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Molecular Biology and Pathogenicity of Mycoplasmas

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"Life is extremely conservative. On whatever level—the individual organism, the species, the biota as a whole—life expends energy such that it preserves its past, even if, paradoxically, various threats force it to innovate."

Lynn Margulis and Dorion Sagan (279a)

INTRODUCTION

The recent sequencing of the entire genomes of *Mycoplasma genitalium* (139) and *M. pneumoniae* (181) has attracted considerable attention among life scientists to the molecular biology of mycoplasmas, the smallest self-replicating organisms. It appears that we are now much closer to the goal of defining, in molecular terms, the entire machinery of a self-replicating cell. Considerable advances were also made toward a better understanding of mycoplasma pathogenesis. Most impressive are the findings concerning the interaction of mycoplasmas with the immune system, macrophage activation, cytokine induction, mycoplasma cell components acting as superantigens, and autoimmune manifestations. Evasion of the host immune system by antigenic variation of mycoplasmal surface components, as well as molecular definition of mycoplasmal adhesins, has also gained much attention recently. The demonstration of the ability of mycoplasmas to enter host cells and the possibility that several human mycoplasmas act as accessory factors in the activation of AIDS played a role in intensifying research on mycoplasma pathogenesis, bringing more researchers into the circle of those interested in this group of organisms. We were thus prompted to try and summarize within the framework of a single comprehensive review the cell biology and pathogenicity of the mycoplasmas, emphasizing when possible the lessons that can be learned from the mycoplasmal genome projects on the minimal complement of genes enabling life.

Mycoplasmas are distinguished phenotypically from other bacteria by their minute size and total lack of a cell wall. Taxonomically, the lack of cell walls is used to separate mycoplasmas from other bacteria in a class named *Mollicutes* (*mollis*, soft; *cutis*, skin, in Latin). The current classification of *Mollicutes* and the properties distinguishing the currently established taxa are presented in Table 1. While the trivial terms "mycoplasmas" or "mollicutes" have been used interchangeably to denote any species included in *Mollicutes*, the trivial names ureaplasmas, entomoplasmas, mesoplasmas, spiroplasmas, acholeplasmas, asteroleplasmas, and anaeroplasmas are routinely used for members of the corresponding genera. The preliminary molecular characterization of the uncultured plant and insect mycoplasma-like organisms (MLOs) has provided strong experimental support for their inclusion in the class *Mollicutes*. Consequently, the trivial term "phytoplasmas" has been proposed to replace the awkward name "mycoplasma-like organisms."

Because of the wide scope of the review and space limitations, emphasis will be put on the more recent findings, published since 1990. The interested reader is referred to a num-

TABLE 1. Major characteristics and taxonomy of the class *Mollicutes*[a]

Classification	Current no. of recognized species	Genome size (kb)	Mol% G+C of genome	Cholesterol requirement	Distinctive properties	Habitat
Order I: *Mycoplasmatales*						
Family I: *Mycoplasmataceae*						
Genus I: *Mycoplasma*	102	580–1,350	23–40	Yes	Optimum growth at 37°C	Humans, animals
Genus II: *Ureaplasma*	6	760–1,170	27–30	Yes	Urea hydrolysis	Humans, animals
Order II: *Entomoplasmatales*						
Family I: *Entomoplasmataceae*						
Genus I: *Entomoplasma*	5	790–1,140	27–29	Yes	Optimum growth at 30°C	Insects, plants
Genus II: *Mesoplasma*	12	870–1,100	27–30	No	Optimum growth at 30°C; 0.04% Tween 80 required in serum-free medium	Insects, plants
Family II: *Spiroplasmataceae*						
Genus I: *Spiroplasma*	33	780–2,220	24–31	Yes	Helical motile filaments; optimum growth at 30–37°C	Insects, plants
Order III *Acholeplasmatales*						
Family I: *Acholeplasmataceae*						
Genus: *Acholeplasma*	13	1,500–1,650	26–36	No	Optimum growth at 30–37°C	Animals, some plants, insects
Order IV: *Anaeroplasmatales*						
Family: *Anaeroplasmataceae*						
Genus I: *Anaeroplasma*	4	1,500–1,600	29–34	Yes	Oxygen-sensitive anaerobes	Bovine/ovine rumen
Genus II: *Asteroleplasma*	1	1,500	40	No	Oxygen-sensitive anaerobes	Bovine/ovine rumen
Undefined taxonomic status						
Phytoplasma	ND[b]	640–1,185	23–29	Not known	Uncultured in vitro	Insects, plants

[a] Updated and modified from reference 361.
[b] The taxonomic status of the uncultured phytoplasmas is not defined (ND) as yet; two *Candidatus* Phytoplasma species have already been published (101, 500).

ber of books on various aspects of mycoplasmology published during the last decade or so (210, 277, 365, 370, 395, 459, 473). A wealth of information can be found in the proceedings of the biannual meetings of the International Organization for Mycoplasmology (the last issues were named *IOM Letters*, volumes 1 through 4), and in a special issue of *Journal of Clinical infectious Diseases* (volume 17, supplement 1, 1993). Reviews covering different aspects of the molecular biology and genetics of mycoplasmas, as well as their general properties and taxonomy, are also available (30, 56, 111, 114, 356, 357, 359–361).

ECOLOGY AND HABITATS

Distribution of Mollicutes

Mycoplasmas are widespread in nature as parasites of humans, mammals, reptiles, fish, arthropods, and plants (361). The list of hosts known to harbor mycoplasmas is continuously increasing, as is the number of established mollicute species, close to 180 at the time this review was written (Table 1). It is widely agreed that the mollicutes that have already been characterized and taxonomically defined constitute only a part, apparently a minor one, of the mollicutes living in nature. It appears as though the main factor for adding an animal or plant to the list of hosts is the willingness of a mycoplasmologist to invest the effort and funds required to isolate and taxonomically characterize the mycoplasmas from the tested host. The larger the number of established mollicute species, the harder it becomes to fulfill the minimal requirements for establishing new species (198). Particularly difficult is the requirement for serological differentiation of the new isolate from the other established species. A large battery of species-specific antisera and seed is needed for this task. The introduction of molecular tools to taxonomy, including the comparison of 16S rRNA and other conserved gene sequences, genomic restriction patterns, etc. (361, 363), has already been found effective in species and strain identification. The fast developments in genome-sequencing methodology are expected to facilitate the trend of basing bacterial taxonomy and species definition on molecular data and phylogeny, decreasing the weight of serology in species and strain identification and classification (see "Taxonomy and phylogeny" below).

The wide occurrence of mycoplasmas has frequently led researchers with little or no expertise in mycoplasmology to suggest that structures resembling mycoplasmas in tissues of oysters, bryozoans, and *Giardia* are mycoplasmas (reference 361 and references therein). While some of the structures may indeed represent mycoplasmas, as long as no culture was available, one could not be certain of their true nature. This statement has to be changed now. The successful application of genomic analysis methodology has led to the identification of the uncultured plant and insect MLOs as bona fide mycoplasmas, showing the way for confirmation of the identity of other MLOs as mollicutes. Thus, the grey lung "virus" disease of mice, caused by an agent that resembles mycoplasmas in thin sections of lung material but resists cultivation, has been identified as a mycoplasma based on its genome size and 16S rRNA sequence analysis. It has been proposed to classify this organism as "*Candidatus* Mycoplasma ravipulmonis", the "*Candidatus*" taxon being reserved to classification of uncultured procaryotes (312). Rather exciting is the recent finding, based on 16S rRNA sequence analysis, that *Haemobartonella* and *Eperythrozoon*, well known uncultured, wall-less pathogenic bacteria, infecting erythrocytes of a wide range of vertebrate hosts, are phylogenetically closely related to *Mollicutes* rather than to rickettsia and should therefore be reclassified as *Mycoplasma* species (313, 375). The inclusion of these hemotropic agents in *Mollicutes* represents an entirely new group of pathogens among the mycoplasmas, widening considerably the scope of mycoplasmology. These findings may encourage those promoting the idea that other mycoplasma-like structures observed in tissues of a variety of animals including humans, are in fact mollicutes. However, the claims by Wirostko's group that uncultured MLOs observed in leukocytes are mollicutes that cause uveitis and possibly other diseases in humans (208) have not been substantiated as yet by any of the molecular tools available for genomic DNA analysis (361). Nevertheless, the fact that in the human host, so extensively searched for mycoplasmas for decades, a new mycoplasma, *M. penetrans*, has recently been discovered (260) encourages the continued search for additional mycoplasma species in humans.

Mycoplasmas in Nontypical Hosts and Tissues

Mycoplasmas usually exhibit a rather strict host and tissue specificity, probably reflecting their nutritionally exacting nature and obligate parasitic mode of life. However, there are numerous examples of the presence of mycoplasmas in hosts and tissues different from their normal habitats (361). Of special interest is the host and tissue specificity of the human respiratory pathogen *M. pneumoniae*. While experimental respiratory infections by this mycoplasma can be induced in hamsters, the development of lung disease is different from in humans, and intratracheal inoculation of the hamsters is essential for successful infection. Only chimpanzees can be infected by droplet infection like humans, and this infection produces a respiratory disease remarkably similar to the naturally occurring pneumonia in humans (21, 138).

The primary habitats of human and animal mycoplasmas are the mucous surfaces of the respiratory and urogenital tracts, the eyes, alimentary canal, mammary glands, and joints. The obligatory anaerobic anaeroplasmas have so far been found in the bovine and ovine rumen only (Table 1). Spiroplasmas and phytoplasmas are widespread in the gut, hemocele, and salivary glands of arthropods. The spiroplasmas and phytoplasmas may be introduced via sap-sucking insects to the phloem tissues of plants, causing disease (473).

Mycoplasmas usually exhibit organ and tissue specificity. Thus, *M. pneumoniae* is found preferentially in the respiratory tract and *M. genitalium* is found primarily in the urogenital tract, but exceptions are possible, since *M. genitalium* has been isolated from the respiratory tract and *M. pneumoniae* has been isolated from the genital tract (163). These two mycoplasmas are genetically closely related, and their entire genomes have been sequenced (see "Genome-sequencing projects" below). Hence, it would be of interest to try and genetically define the factors responsible for tissue specificity of these mycoplasmas.

Accompanying the increasing numbers of patients suffering from various types of immunodeficiencies associated with hypogammaglobulinemia, AIDS, and treatment with immunosuppressive medication in patients undergoing organ transplantation, more and more reports appear on the isolation of mycoplasmas from organs different from their usual habitats. Thus, mycoplasmas and ureaplasmas belonging to the normal urogenital flora have been isolated from the blood of patients suffering from AIDS or treated with immunosuppressive drugs. Hypogammaglobulinemic and immunocompromised patients become susceptible to infections by the urogenital mycoplasmas *M. hominis* and *U. urealyticum* spreading into organs such as the respiratory tract and joints (152, 290) and causing dis-

ease in these organs. In some cases, the infecting mycoplasmas could not be cultivated, so that their identification was based on PCR amplification of their 16S rRNA genes (249, 363).

Cell cultures infected by mycoplasmas constitute an artificial unnatural habitat. The serious problems created by the persistent and elusive infections of cell cultures are reflected in the voluminous literature on this subject (22, 286). Reports from various countries show that 10 to 87% of cell cultures are infected by mycoplasmas (22, 53, 286). The percentage of infected cell cultures depends to a large extent on the population of cell cultures assayed, on the control practices used, and on the efficiency of the assay procedures applied. The mycoplasma species infecting cell cultures have remained essentially the same over the years, with *M. hyorhinis*, *M. orale*, *M. arginini*, and *A. laidlawii* being the dominant contaminants. The increasing percentage of cell cultures infected by *M. fermentans* (53), a mycoplasma incriminated as a cofactor in AIDS (see "Virulence factors" below), is of particular interest in terms of the possible origin of contamination. Another problem concerns the natural habitat of *M. pirum*, a mycoplasma initially detected only in cell cultures. The recent isolation of this mycoplasma from the blood of AIDS patients has been taken to indicate that humans are the natural hosts (164).

Surface versus Intracellular Location

The intracellular location of mollicutes in insect tissues is well established. While human and animal mycoplasmas were shown to be taken up by polymorphonuclear leukocytes and macrophages (281), the question whether mycoplasmas can enter epithelial cells has not been easy to resolve, and for a long time this question was answered in the negative. The stimulus to reexamine this issue came from the studies by Lo (259) showing the intracellular location of *M. fermentans* incognitus in a variety of nonphagocytic cells in AIDS patients. This finding was strengthened by the discovery of a new human mycoplasma capable of entry into a variety of human cells in vivo and in vitro, named accordingly *M. penetrans* (261).

The mechanism of cell entry by mycoplasmas is still unclear. While mycoplasmas such as *M. penetrans* and *M. genitalium* appear to enter the cells through their specialized tip structure (206, 261), other mycoplasmas shown to internalize, such as *M. fermentans* and *M. hominis*, have no tip structures (447). Following contact of *M. genitalium* with human lung fibroblasts, the plasma membrane of the cells appeared to be forced inward to form a cup or a depression. The membrane pockets resembled clathrin-coated pits, suggesting that the mycoplasma might adhere to and enter the cells by a site-directed, receptor-mediated event resembling cell entry by chlamydias (289).

M. penetrans presents, perhaps, the strongest case for the ability of a mycoplasma to actively penetrate into a variety of different types of mammalian cells, many with minimal phagocytic ability (261). Internalization has a definite anatomical orientation, led by the unique structural component, the tip structure. The entry of the pathogen into a host cell is initiated by the binding of the pathogen onto the host cell surface followed by a dramatic rearrangement of microtubule and microfilament proteins (391). Ultrastructural studies of human larynx carcinoma cells infected with *M. penetrans* revealed, as early as 2 h postinfection, that the organisms were invaginated in the cell membrane or internalized in the cytoplasm, free or inside vesicles. Adherence triggered a signal that promoted cytoskeletal changes, namely, aggregation of tubulin and α-actinin and condensation of phosphorylated proteins. Other cytoskeletal components, such as talin, tropomyosin, and vinculin did not appear to accumulate at the site of mycoplasma clustering, suggesting that *M. penetrans* selectively uses signals to induce specific cytoskeletal rearrangements (156). Along these lines, Andreev et al. (11) reported that the actin polymerization inhibitor cytochalasin D markedly inhibited the internalization of *M. penetrans* in HeLa cells whereas the tyrosine phosphorylase inhibitors staurosporin and genistein had only a slight effect. Invasion of enteropathogenic *Escherichia coli* depends on tyrosine phosphorylation of a 90-kDa HeLa cell protein. This was not found to be the case for *M. penetrans*, although tyrosine phosphorylation of another HeLa cell protein of 140 kDa could probably be associated with the internalization of *M. penetrans* (11).

The percentage of the mycoplasma cell population internalized is difficult to determine. Under electron microscopy, *M. genitalium* was demonstrated intracellularly in about 10% of Vero cells infected in vitro (206), indicating significant variability in the capacity of the cells to internalize mycoplasmas. It should be pointed out that electron microscopic observations may lead to conflicting interpretations. It can be argued that the mycoplasmas that appear to be located intracellularly in vacuoles are actually at the bottom of crypts formed by invagination of the cell membrane. To overcome this difficulty, during the preparation of thin sections Taylor-Robinson et al. (447) applied ruthenium red to stain the mucopolysaccharide surface components of both the HeLa cells and the mycoplasmas infecting them. The intracellular location of some mycoplasmas could be confirmed by exclusion of ruthenium red from their membrane surface. Another approach, based on confocal microscopy and flow cytometry of fluorochrome-labeled mycoplasmas, revealed that *M. penetrans*, *M. pneumoniae*, and *M. genitalium* entered the intracellular spaces and were located throughout the cytoplasm and perinuclear regions of cultured human cells (28). The mycoplasmas could be cultivated from the cytoplasmic and nuclear fractions 96 h after infection and persisted intracellularly for at least 7 days. Whether the mycoplasmas replicate intracellularly remains to be resolved. The finding by Baseman et al. (28) of *M. pneumoniae* mutants capable of cytadherence but defective in their capacity to invade cells is of particular interest, since it suggests that mycoplasma cytadherence and invasion are active but separable processes.

As might be expected, invasion of mycoplasmas into the host cell cytoplasm may affect cell function and integrity. Thus, Mernaugh et al. (289) showed by electron microscopy the complete lysis of human lung fibroblasts 96 h after infection by *M. genitalium*, accompanied by large numbers of mycoplasmas in the milieu. Lo et al. (261) also stated that extensive invasion of cells by *M. penetrans* eventually results in cell disruption and necrosis. The presence in the host cell cytoplasm of mycoplasmas, some of which may not even be enclosed within a vacuole, may expose the cytoplasm and the nucleus to mycoplasmal hydrolytic enzymes, such as proteases, nucleases, and phospholipases. The potent endonuclease of *M. penetrans* was suggested to cause chromosomal damage (36).

It should be emphasized that intracellular location, if even for a short period, may protect the mycoplasmas against the effects of the host immune system and antibiotics and may account to some extent for the difficulty of eradicating mycoplasmas from infected cell cultures. Thus, intracellular residence, which sequesters mycoplasmas, promotes the establishment of latent or chronic infection states and circumvents mycoplasmacidal immune mechanisms and selective drug therapies (30).

MORPHOLOGY AND ULTRASTRUCTURE

Motility and Cytoskeletal Elements

Since mycoplasma cells are bounded by a plastic cell membrane only, their dominating shape is a sphere. However, many mollicutes exhibit a variety of morphological entities, including pear-shaped cells, flask-shaped cells with terminal tip structures, filaments of various lengths, and helical filaments. The ability to maintain such shapes in the absence of a rigid cell wall has long indicated the presence of a cytoskeleton in mycoplasmas (356). Some of the flask-shaped *Mycoplasma* species are capable of gliding on solid surfaces. The mechanism of this peculiar gliding motility is still unknown (224). Thus, although *M. pneumoniae* is known to be motile and to exhibit chemotactic behaviour, gliding motility genes could not be identified, since it is not yet known which genes have to be looked for. Furthermore, none of the components of the chemotactic signal pathway, the Che proteins, which are well conserved among bacteria, or any other "two-component signal transduction systems" could be identified in the sequenced *M. pneumoniae* genome (181).

Spiroplasma species are unique in having helical morphology, rotary motility, and chemotaxis. Upon cell lysis by deoxcholate, spiroplasmas release fibrils that are 4 nm in diameter. These fibrils, thought to function as a cytoskeletal element involved in the helical shape and motility of the organisms, consist of a major protein of 59 kDa. The gene for this protein (*fib*) has been cloned, facilitating the characterization of the structural features of the fibril protein (476). It appears that the protein possesses four α-helices; axial projection of the three most prominent ones shows that the hydrophobic and hydrophilic amino acids are located on the opposite sides of the helices. It is therefore possible that the fibril protein attaches to the plasma membrane with the hydrophobic side while the hydrophilic side faces the cytoplasm (476). In light of the finding that reversible phosphorylation controls the assembly of intermediate filaments in eucaryotes, Platt et al. (339) investigated the interrelationship of protein phosphorylation and *Spiroplasma melliferum* fibrils. Their results showed the phosphorylation of a protein resembling the fibril protein in molecular mass, but it was not definitely identified as such. Some information on a gene involved in *S. citri* motility has recently been obtained through transposon mutagenesis (57, 203) (see "Gene transfer" below). An *S. citri* nonmotile mutant was generated by Tn*4001* transposon mutagenesis. The transposon was shown to be inserted into a gene, *scm1*, encoding a putative polypeptide with no significant homology to any known protein. The *scm1* gene was recovered from the wild motile strain and inserted into an *S. citri* cloning vector. Transfection of the nonmotile mutant with this recombinant plasmid restored motility, indicating that the *scm1* gene product is indeed involved in *S. citri* motility.

Williamson et al. (476) have suggested that an actin-like protein found in spiroplasmas may be linked to the fibrils and associated with motility. However, they admit that there is really no evidence for such an association. It should be pointed out that the presence of actin-related proteins in procaryotes in general, and in mollicutes in particular, has long been suspected but was never proven (356). While some workers have claimed to identify actin or actin-related proteins in mollicutes (161), others have failed (77). The complete genomic analyses of *M. genitalum* and *M. pneumoniae* (139, 181) also failed to identify actin-related gene(s), leaving this issue unresolved. In fact, it now appears that the interest in this subject, strong in the 1970s (356), has dwindled. In general, bacterial interconnecting protein networks appear to be important in preserving the integrity of wall-less cells, also keeping an asymmetric distribution of membrane proteins. However, thus far, significant sequence homologies between eucaryotic cytoskeletal proteins and any bacterial protein have not been found (345), although Wasinger et al. (469), applying the proteome technology (see "Genome sequencing and the minimal cell concept" below), have suggested the presence of a tubulin-like protein in *M. genitalium*.

An important group of pathogenic mycoplasmas, including *M. pneumoniae* and *M. genitalium*, have a flask- or clublike cell shape with a protruding tip or bleb structure. These mycoplasmas attach to eucaryotic cells via the tip structure, serving as an attachment organelle (see "Adhesion to host cells" below). Scanning and transmission electron microscopy of *M. pneumoniae* cells grown on grids treated with 1% Triton X-100 revealed a rodlike tip structure and a network of filamentous strands (288). Proteins of this so-called Triton shell are apparently cytoskeleton-forming or cytoskeleton-associated proteins. The cytoskeleton-like structure is thought to function in modulating cell shape and to participate in cell division, gliding motility, and the proper localization of adhesins.

The genomic analysis of *M. pneumoniae* has enabled the identification and molecular characterization of major protein building blocks of the cytoskeleton of this mycoplasma. Some of these proteins function as surface-exposed adhesins, including proteins P1 and P30, while others, named accessory proteins (designated HMW1, HMW2, and HMW3 and A, B, and C) collectively maintain the proper distribution and/or disposition of the adhesins in the mycoplasma membrane (see "Adhesion to host cells" below). Additional proteins, named P65 and P200 (345, 346) share characteristic structural features with HMW1 and HMW3, suggesting their function as elements of the *M. pneumoniae* cytoskeleton, consistent with their presumed scaffolding role (236). HMW2 (about 215 kDa) is predicted to assume a coiled-coil conformation, similar to that of the filamentous portion of the myosin heavy chain, reflecting a likely structural role for this cytoskeletal mycoplasmal protein (237).

An interesting property of these cytoskeletal proteins (such as HMW1, HMW3, and P65) is their abnormal (slower than expected from their molecular weight) migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which is apparently due to proline-rich regions in their molecular structure (penta- and hexapeptide repeats forming a proline-rich acidic domain [345]). Many eucaryotic membrane-associated cytoskeletal proteins (such as spectrin) are characterized by similar internal repeats that function in the binding of accessory proteins of importance in the construction and maintenance of a cytoskeletal network. It should also be mentioned that membrane lipoproteins, common in mycoplasmas, exhibit abnormal electrophoretic migration due to the presence of the lipid moiety (478).

Another interesting feature of the *M. pneumoniae* cytoskeletal proteins concerns their phosphorylation. Protein phosphorylation is a widespread mechanism for regulating intracellular signaling. Through the action of kinases, phosphotransferases, and phosphatases, this posttranslational protein modification results in various reversible phosphorylation states, modulating cellular events by interconverting between active and inactive protein forms (218). Dirksen et al. (106) showed the HMW1 and HMW2 cytadherence-associated proteins of *M. pneumoniae* to be phosphorylated at threonine and serine residues in an ATP-dependent manner, implying that a classical protein kinase phosphatase system is functioning. Whether the phosphorylation state of these cytoskeletal proteins plays a role in regulating the dynamics of cytoskeletal

interactions is a moot point, since the phosphorylation of HMW1 and HMW2 was generally poor. Rather, phosphorylation was associated at very high level with three unidentified proteins of 54, 57, and 60 kDa. Only when the Triton-soluble fraction was removed could phosphorylation be detected in the Triton-insoluble fraction (238).

Cell Division

The mode of reproduction of mycoplasmas is essentially not different from that of other procaryotes dividing by binary fission. For typical binary fission to occur, cytoplasmic division must be fully synchronized with genome replication, and in mycoplasmas the cytoplasmic division may lag behind genome replication, resulting in the formation of multinucleate filaments. The transformation of mycoplasma filaments into chains of cocci followed by microcinematography revealed the appearance of constrictions in the cell membrane at about equal distances along the entire length of the filament, a process that takes a few minutes. At the time these observations were discussed (356), one had no idea about the mechanism of this process. Recent genetic data throw some light on the genes and proteins involved in maintaining the peculiar cell shapes and the cell division process in mycoplasmas.

Since the mechanics of cell septation in conventional eubacterial species are believed to be mediated by cell wall constituents, there is no clear understanding of what coordinates that process in wall-less bacteria. Our knowledge of the genes and their products associated with cell division of walled bacteria, particularly of *E. coli*, is quite extensive. The recent sequencing of several mycoplasma genomes (55, 139, 181) has provided some information on mycoplasmal genes homologous to cell division genes of walled-covered bacteria. The comparative genomics data reveal the lack in mycoplasmas of a significant number of genes belonging to this category, findings which may be relevant in the consideration of the relative importance of the different genes in the procaryotic cell division process.

The most important finding, perhaps, is that of the *ftsZ* gene in mycoplasmas (55, 139, 181, 468). In eubacteria, the FtsZ protein is a polymer-forming, GTP-hydrolyzing protein with tubulin-like elements; it is localized to the site of septation and forms a constricting ring (the Z ring) between the dividing cells. The finding of *ftsZ* in the mollicutes indicates that it is a highly conserved and ubiquitous gene (found also in archeons and chloroplasts) fulfilling a key role in procaryote cell division. Of the additional genes associated with cell division in eubacteria (*ftsA*, *ftsH*, *ftsI*, *ftsQ*, *ftsW*, and *ftsY*) *ftsH* was identified in *M. pneumoniae* (181) and *M. capricolum* (55), and *ftsY* was identified in *M. genitalium* (139) and *M. pneumoniae* (181). More recently, the *ftsY* gene of *M. mycoides* subsp. *mycoides* was cloned and characterized and its role in protein targeting by complexing with the signal-recognition particle was investigated (269). FtsW has been suggested to initiate Z-ring formation, probably serving as a membrane target recognized by FtsZ. The fact that it is absent from the mycoplasmas argues against its being absolutely essential for the localization of FtsZ to the division site. FtsA, FtsI, and FtsQ are believed to act in the division process after the formation of the Z ring; their absence from the mycoplasmas also suggests that they are not absolutely essential for cell division (468). Applying the proteome approach (see "Genome sequencing and the minimal cell concept" below), Wasinger et al. (469) proposed the presence of a tubulin-like protein in *M. genitalium*. It has been suggested that the FtsZ protein of procaryotes is a tubulin homolog; however, the tubular structures formed by FtsZ in vitro are substantially different from microtubules, and sequence similarity between FtsZ and tubulin is barely significant (169).

An integral part of cell division concerns chromosome replication and partition into daughter cells. The mechanism of chromosome partitioning has not yet been examined in any mollicute species. The identification of *parC* and *parE* homologs in *M. genitalium* suggests that topoisomerase IV (a topoisomerase capable of relaxing superhelical DNA and converting knotted DNA to a simple ring in vitro) has been evolutionarily conserved because of its essential role in cell division (20). In conclusion, it appears that on the whole we are still rather far from clearly understanding the factors coordinating the cell division process in mollicutes, since genetic information has only just started to accumulate and has not been entirely analyzed and evaluated.

IN VITRO CULTURE

Definition of Nutritional Requirements

A major impediment to mycoplasma research and laboratory diagnosis of mycoplasma infections has been the difficulty of their in vitro cultivation. There appears to be a consensus that only a minority of the mycoplasmas existing in nature have been cultivated so far. For example, despite many efforts for over 30 years, none of the phytoplasmas infecting insects and plants has been cultivated in vitro in an axenic culture (229). Some of the cultivable mycoplasmas also grow very poorly and slowly on the best mycoplasma media available (363). The recent mycoplasma genome projects have provided definitive genetic explanations of the above-mentioned difficulties by demonstrating the remarkable scarcity in mycoplasmas of genes involved in biosynthetic pathways. For example, both *M. genitalium* and *M. pneumoniae* lack all the genes involved in amino acid synthesis (139, 181), making them totally dependent on the exogenous supply of the complete spectrum of amino acids.

To overcome the assimilative deficiencies of the mycoplasmas, complex media are used for their cultivation. The media are usually based on beef heart infusion, peptone, yeast extract, and serum with various supplements (359). The use of these complex undefined growth media has interfered with the molecular definition of mycoplasmal metabolic pathways, genetic analysis, preparation of mycoplasmal antigens free of serum components, etc. Continual efforts to replace the serum component, with the aim of reaching a defined growth medium, have been made. Serum has been shown to provide, among other nutrients, fatty acids and cholesterol (required for membrane synthesis) in an assimilable nontoxic form. Efforts to replace the serum component with albumin, fatty acids, and cholesterol solubilized in Tween 80 or with liposomes made of phospholipids and cholesterol supplemented with serum albumin to neutralize free fatty acid toxicity (356) have been partially successful. In an attempt to replace the albumin component, cyclodextrins have been proposed as carriers of cholesterol and fatty acids (166). However, cyclodextrin derivatives may have too strong a lipid binding capacity, rendering the essential lipid unavailable to the mycoplasma and consequently inhibiting growth. It is difficult, therefore, to find a cyclodextrin derivative that combines a proper lipid binding capacity with nontoxicity.

The definition of lipid requirements, particularly for cholesterol, has served as an important taxonomic criterion distinguishing the sterol-nonrequiring mollicutes, particularly the *Acholeplasma* species, from the sterol-requiring ones. Testing for sterol requirement has been based on the use of a serum-

free medium containing serum albumin, fatty acids, and various concentrations of cholesterol solubilized in Tween 80, yielding a final Tween concentration of 0.01% (356). The finding (385) that Tween 80 at a final concentration of 0.04% is essential for the growth of some of the sterol-nonrequiring mycoplasmas (later named *Mesoplasma* species [Table 1]) is still an enigma. It seems unlikely that requirement for this relatively high Tween 80 concentration can be explained by the provision of a required fatty acid component, since experiments to test this issue by adding a variety of fatty acids have failed.

One thing is certain: although the numerous nutritional requirements of mollicutes dictate the need for complex growth media, the notion that the richer the medium the better may be wrong. It appears, at least in some cases, that the lack of growth of a mycoplasma in a rich medium is not the result of the lack of a specific nutrient but, rather, is due to the presence of a component(s) toxic to the mycoplasma. Some *M. hyorhinis* strains, common contaminants of cell cultures, have been known to resist cultivation on conventional mycoplasma media, leading us to consider them particularly fastidious strains. This concept proved to be wrong, since these strains could grow well in a minimal serum-free medium, leading Gardella and Del Guidice (150) to suggest that these "noncultivable" strains are not particularly fastidious but are more sensitive to inhibitors found in the complex media, mostly as components of peptone and yeast extract. Hence, the terms "noncultivable" and "fastidious" should be considered relative terms, which take their meaning only in the context of a specific culture system where growth promoters and possibly inhibitors are present.

The possibility that the presence of inhibitory substances in complex mycoplasma media, rather than the lack of essential nutrients, is the reason for the continuous failure to cultivate phytoplasmas is unresolved. The notion that the failure to cultivate phytoplasmas may be associated with a need for anaerobic conditions has not been substantiated. Experiments with phytoplasma-infected *Oenothera* leaf tip cultures, growing under conditions of different levels of O_2 and CO_2 mixed in air, showed no consistent correlation between atmospheric conditions and phytoplasma abundance. However, the phytoplasmas grew better in photosynthesis-defective mutants of *Oenothera*, indicating that the pathogen is more successful in nonphotosynthetic tissue, with no generated O_2 (410). Generally, mollicutes differ markedly in their atmospheric requirements; while most mollicutes are facultative anaerobes and usually favor an anaerobic or low-redox, enhanced CO_2 atmosphere on primary isolation, the ruminal *Anaeroplasma* and *Asteroleplasma* species are strict anaerobes, very sensitive to oxygen (Table 1). Some mycoplasmas, such as *M. hyorhinis*, require an aerobic atmosphere (150).

Cocultivation with Cell Cultures

A novel approach to improve the chances of in vitro cultivation of fastidious mollicutes is based on coculture with eucaryotic cell lines (cell-assisted growth). In this way, uncultivable spiroplasmas, such as the Colorado potato beetle spiroplasma, were first successfully cocultivated in insect cell lines. Subsequently (232), primary cultures of this spiroplasma could be obtained on cell-free media under a low-redox, enhanced CO_2 atmosphere and at a pH lower than 7.0 (for most mollicutes, the initial pH is adjusted to a slightly alkaline value), conditions which imitate those obtained in the insect cell cocultures (232).

A similar cell-assisted cocultivation, using Vero cell cultures, enabled several strains of the highly fastidious human pathogen *M. genitalium* to be cultured from clinical specimens (207). Also, in this case, the mycoplasmas grown in the cell culture (as was indicated by PCR monitoring) could be subsequently subcultured in a cell-free medium (207). The above method, as complex as it is, requiring "heroic" efforts, is certainly inadequate for routine cultivation of fastidious mycoplasmas, leaving the door open for the application of molecular techniques, such as PCR, for detection and identification of fastidious or so far uncultivable mycoplasmas (363) (see also "Taxonomy and phylogeny" below).

GENOME SIZE AND BASE COMPOSITION

Genome Size

The application of pulsed-field gel electrophoresis to mollicute genome size determinations (314, 348) has provided a much more accurate and labor-saving procedure than the previously used renaturation kinetics method and has resulted in a wealth of genome size data (57, 71, 314). The data show a continuum of genome sizes among mollicutes, ranging from less than 600 to over 2,200 kb, with overlapping values between mollicute genera. Thus, the genome sizes of *Mycoplasma* species range from 580 kb for *M. genitalium* to 1,380 kb for *M. mycoides* subsp. *mycoides* LC, while for the helical *Spiroplasma* species the genome size ranges from 780 kb for *S. platyhelix* to 2,220 kb for *S. ixodetis* (57, 71) (Table 1). Clearly, genome size can no longer be taken as the definitive taxonomic criterion to distinguish higher taxa in *Mollicutes* (359). Yet, as a general rule, *Acholeplasma* and *Spiroplasma* species, considered phylogenetically to be "early" mollicutes, have larger genome sizes than *Mycoplasma* and *Ureaplasma* species, considered to be phylogenetically more "recent" mollicutes (see "Taxonomy and phylogeny" below). This is in agreement with the notion that *Mollicutes* have evolved by degenerative or reductive evolution, accompanied by significant losses of genomic sequences (479).

Genome sizes are variable not only within the same genus but even among strains of the same species (71, 191, 241, 382). One of the reasons for this variability is the frequent occurrence in mollicute genomes of repetitive elements, consisting of segments of protein genes, differing in size and number, or insertion sequence (IS) elements (see "Chromosomal rearrangements" below). Intraspecies variability of genome size may also be caused by integration into the chromosome of viral sequences, as was found in *S. citri*, where these sequences may account for up to 150 kb, 1/12 of the entire genome (32, 56, 481, 482). On the whole, in light of the highly dynamic nature of the mycoplasmal genome (see "Chromosomal rearrangements" below), one should be surprised not by intraspecies genomic size variations but, rather, by the relative conservation of genome size observed in mollicute species.

Is there any correlation between genome size and the ability of a mycoplasma to grow in vitro? Although, a priori, a larger genome and consequently a larger number of genes would be expected to endow the organism with better adaptability to in vitro cultivation, the available data do not support this expectation. Thus, *S. ixodetis*, the spiroplasma with the largest genome, is much harder to cultivate than spiroplasmas with much smaller genomes (460). As to the uncultured phytoplasmas, the limited genome size data available suggest that though they may be included among the mollicutes with the smaller genomes (311) such as the western X-disease phytoplasma with a 670-kb genome (127), animal mycoplasmas with even smaller genomes have been easily cultivated in vitro. The conclusion,

therefore, is that there is no simple relationship between genome size and cultivability of mollicutes (see "In vitro culture," above, for a possible explanation). Interestingly, the obligate intracellular bacteria *Chlamydia trachomatis*, *Rickettsia prowazekii*, and *Chlamydia burnetii* have genomes of 1,000, 1,100, and 1,600 kb, respectively (10, 273), larger than the genomes of many of the self-replicating mollicutes, again strengthening the conclusion that there is no simple correlation between genome size and growth in axenic culture.

Genomic Base Composition

The mycoplasma genome has a characteristically low G+C content. With very few exceptions, the G+C content of mycoplasma genomes is within the range of 24 to 33 mol% (appendix in reference 459). The G+C distribution along the genome is uneven. Thus, while the average G+C content of the *M. genitalium* genome is 32 mol%, the G+C content of its rRNA genes is 44 mol% and that of its tRNA genes is 52 mol% (139). The *M. pneumoniae* adhesin genes, P1 and ORF6, and their repetitive sequences, exhibit a G+C content as high as 56 mol%, while on the other extreme the origin of replication of this mycoplasma has a G+C content of only 26 mol%, compared to 40 mol% of the entire *M. pneumoniae* genome (181, 182). Consequently, many of the mycoplasmal intergenic regions have a higher A+T content than do the coding regions, reaching values as high as 80 to 90 mol% (114, 305, 485). The variable G+C content of coding regions within the mycoplasmal genome has phylogenetic relevance, indicating the highly conserved nature of the rRNA and tRNA genes and the possible exogenous origin of the adhesin genes (see "Adhesion to host cells" below).

As in other procaryotes, some of the adenine and cytosine residues in mycoplasmal genomes may be methylated (114, 275, 357). In many mycoplasmas the adenine residues at the GATC site are methylated, while in others the cytosine residues are methylated. Of special interest is the exclusive methylation of the genomic cytosine residues of *S. monobiae* (MQ-1) when they are located 5′ to guanine (CpG), a methylation trait considered unique to eucaryotes (374). The type of base methylated, the extent of methylation, and methylation sequence specificity have been suggested as markers in mollicute taxonomy (361).

Extrachromosomal Elements

Among the mollicutes, the spiroplasmas and acholeplasmas are the most frequently infected by a variety of viruses (phages), whereas very few viruses are known to infect *Mycoplasma* species (499). The characteristics of mollicute viruses have been described and discussed in great detail in previous reviews (271, 357, 372). The mollicute phage DNA genomes range in size from 4 to 40 kb and may be either circular or linear and single or double stranded (114). Of the very few phages infecting *Mycoplasma* species, the most recent phage discovered is the lysogenic phage MAV1, infecting *M. arthritidis* (465). The finding that this phage is associated with highly arthritogenic strains and that experimental infection of low-virulence *M. arthritidis* strains with this phage significantly increases their arthritogenicity suggests that MAV1 carries a virulence enhancing factor (see "Virulence factors" below).

Plasmids were detected in *S. citri* and more recently in *M. mycoides* subsp. *mycoides* (221, 222) and in phytoplasmas (229). The potential of the mollicute phages and plasmids to serve as cloning and shuttle vectors has been the main focus of interest in these elements (see "Gene transfer" below).

GENOME SEQUENCING AND THE MINIMAL CELL CONCEPT

One of the first and most intriguing issues supported by the National Aeronautics and Space Administration was the search for extraterrestrial forms of life. The assumption that these living forms, if they exist, may be extremely simple, led Harold Morowitz to look for the simplest self-replicating cells existing on our planet. A short search aided by Mark Tourtellotte pointed at the mycoplasmas as the smallest and simplest self-replicating organisms. To attract attention to these organisms, in 1962 Morowitz and Tourtellotte published an article in *Scientific American* on mycoplasmas as the smallest living cells (298) and organized, with the support of the National Aeronautics and Space Administration, the first meeting on the molecular biology of mycoplasmas. We were then enchanted by the "crazy" idea of assembling a living cell from its components. It should be recalled that these were the 1960s, a period of revolutionary ideas including the trip to the Moon. The mycoplasmas, being built of the minimum set of organelles (a plasma membrane, ribosomes, and a circular double-stranded DNA molecule, the typical prokaryotic genome), were naturally selected by us as the best candidates for cell reassembly. Starting with detergent solubilization and reassembly of the cell membrane appeared to be the most logical step to initiate this venture. To make a long story short, we succeeded in reconstituting vesicular membranous structures from the solubilized components (369), but the resulting membranes differed in molecular organization from the native membranes. Nevertheless, our study had an impact, giving a considerable push to the field of membrane reconstitution (355). However, the failure to reconstitute a functional plasma membrane led us to abandon the idea of reconstituting a living cell in the laboratory.

Harold Morowitz did not entirely give up this idea. In 1984, 20 years after our pioneering membrane reconstitution experiments, Morowitz was invited to deliver a keynote lecture at the International Congress of Mycoplasmology in Jerusalem. This lecture has had a major impact on mycoplasma research, culminating in the significant achievements to be discussed below. This time, Morowitz proposed another, more practical and down-to-earth approach (297). His major idea was to define in molecular terms the entire machinery of a mycoplasma cell, in order to prove the dogma of the completeness of molecular biology, that is, that the "logic of life" is finite, relatively simple, and subject to full exploration. He proposed the launching of an international effort to accomplish this ambitious goal. The project appeared to require an enormous amount of work and generous funding, but even in 1984, it did not appear to involve conceptual and methodological difficulties. The proposed plan consisted of physical and functional mapping of a *Mycoplasma* genome including its complete sequencing, determining the open reading frames (ORFs), reading out the encoded amino acids, defining the genes and their products, and in this way achieving a complete molecular description of the cell machinery (297).

Genome-Sequencing Projects

Although the proposal by Morowitz was met with enthusiasm, a collaborative international effort was not established. Instead, several laboratories initiated independent projects focusing on sequencing and genetic mapping of different mycoplasma genomes. Since the late 1980s, there has been a continuous flow of papers on the construction of physical maps of mollicute genomes, followed by the sequencing of genomic fragments and trials to identify the genes they carry (references

TABLE 2. Gross properties of bacterial genomes[a]

Property	*H. influenzae*	*M. pneumoniae*	*M. genitalium*	*E. coli*
No. of bp	1,830,137	816,394	580,070	4,639,221
G+C content (mol%)	38	40	32	50
No. of putative coding sequences (ORFs)	1,703	677	479	4,288
No. of ORFs tentatively identified[b]	1,408	603	468	2,659
No. of ORFs with no functional prediction or database match	295	74	11	1,629

[a] Based on data from Fleischmann et al. (130), Himmelreich et al. (181, 182), Fraser et al. (139), and Blattner et al. (52).
[b] The numbers of tentatively identified ORFs are subject to the reservations expressed in "Proteome Approach" below.

56, 360, and 364 and references therein). The first large-scale studies directed at sequencing entire mycoplasma genomes were initiated at about 1990. The Harvard Genome Lab chose to sequence the genome of *M. capricolum*. The selection of this mycoplasma was not optimal, as it carries a relatively large genome, of over 1,000 kb (294). By 1995, when the project terminated, only 214 kb of the *M. capricolum* genome had been definitely sequenced and genetically analyzed in collaboration with the Heidelberg European Molecular Biology Laboratory (55). Although this study was apparently the first to provide extensive genetic data based on sequences of a significant part of a mycoplasma genome, generalizations based on these partial data could have led to erroneous conclusions.

Richard Herrmann's laboratory at the University of Heidelberg chose to sequence the 800-kb genome of *M. pneumoniae*. Despite being small and limited in resources, this laboratory succeeded, in about 3 years of hard work, in fully sequencing and genetically characterizing the entire *M. pneumoniae* genome (181). They first constructed a cosmid library and then, by applying the sequence strategies and methods described by Hilbert et al. (180), sequenced both DNA strands in a directed fashion by primer walking, limiting random (shotgun) sequencing to a minimum.

However, Herrmann's group was not the first to publish the complete sequence of a mycoplasma genome. The first report was published in October 1995 by a large team from The Institute for Genomic Research (TIGR), Gaithersburg, Md., collaborating with teams from Johns Hopkins University and from the University of North Carolina at Chapel Hill (139). They selected apparently the most adequate mycoplasma for whole-genome sequencing: *M. genitalium*. This mycoplasma carries the smallest genome known so far for a self-replicating organism, a genome of 580 kb only. The foundations of the project were laid down by the Chapel Hill team, employing the conventional chromosome-walking strategy on a set of ordered cosmids. This led to the partial sequencing and genetic characterization of the *M. genitalium* genome (264, 334). However, the breakthrough occurred when the *M. genitalium* genome sequencing was moved to Gaithersburg in January 1995. The TIGR team sequenced the entire mycoplasma genome in less than 6 months. This remarkable achievement was made possible by application of the whole-genome shotgun sequencing strategy, based on the random fragmentation of genomic DNA to small segments of about 2 kb followed by their cloning and sequencing. The reassembly of the thousands of the sequenced overlapping fragments in the right order has been made possible by the TIGR Assembler software program. The predicted coding regions of the genome were defined with the GeneMark software. Role assignments were made, where possible, for each of the predicted coding regions by database matches to all proteins in the public archives (130, 138a, 139). The remarkable power and cost-effectiveness of the new genome sequencing technology mean that complete genome sequences of over 40 of major bacterial pathogens as well as archeons could be available by the year 2000 (96, 192). Included in these are several mollicutes, such as *U. urealyticum* (158), *M. gallisepticum* (192), and *M. mycoides* subsp. *mycoides* (192).

Comparative Genomics

The complete sequence of the *M. genitalium* genome was reported only a few months after publication by the TIGR and Johns Hopkins teams of the first complete sequence of a bacterial genome, that of *Haemophilus influenzae* (130). The voluminous amount of genetic data provided by the genome projects has opened the way for "comparative genomics," by which the total genomic complements of organisms could be compared. This provides an opportunity to explore the functional content of genomes and evolutionary relationships between them at a new qualitative level, signifying the onset of a new era in biology. We intend to discuss in this section the cell biology of *M. genitalium* and its close relative *M. pneumoniae*, comparing it to that of *H. influenzae* and other eubacteria. The number of coding regions, the ORFs for proteins, in the *M. genitalium* genome is only 479, compared to 677 in *M. pneumoniae*, 1,703 in *H. influenzae*, and 4,288 in *E. coli* K-12 (Table 2).

Assimilative processes. Examination of the mycoplasmal genomic data to find where economization in genes took place may help us define the genes which are really essential for a minimal cell. Most striking are the results concerning genes of biosynthetic pathways. During their evolution, the two mycoplasmas *M. pneumoniae* and *M. genitalium* have apparently lost all the genes involved in amino acid biosynthesis and thus require the full spectrum of the essential amino acids from the host or from the artificial culture medium (Fig. 1). The mycoplasmas have also lost most of the genes involved in cofactor biosynthesis, so that to cultivate them in vitro, the medium has to be supplemented with essentially all the vitamins (Fig. 1). Very significant savings in genetic information have resulted through the loss of the cell wall during mycoplasma evolution. Compared with the 105 genes involved in the synthesis of the cell envelope (comprising the outer membrane and the cytoplasmic membrane) in the gram-negative *H. influenzae*, only 54 genes were found to be directly associated with the synthesis of the *M. pneumoniae* cytoplasmic membrane proteins, mostly lipoproteins and only 30 were found in *M. genitalium* (Fig. 1). Nevertheless, it should be mentioned in this context that in *M. pneumoniae* 275 predicted gene products carry transmembrane segments, in line with SDS-PAGE analysis showing that the membrane fraction of this mycoplasma contains about 50% of the cell proteins (181). Yet, each gene saving has its price. Being limited by a cell membrane only, the mycoplasmas are osmotically much more sensitive than the walled bacteria (356). Adaptation to a parasitic mode of life has provided the osmotically sensitive mycoplasmas with a rather osmotically

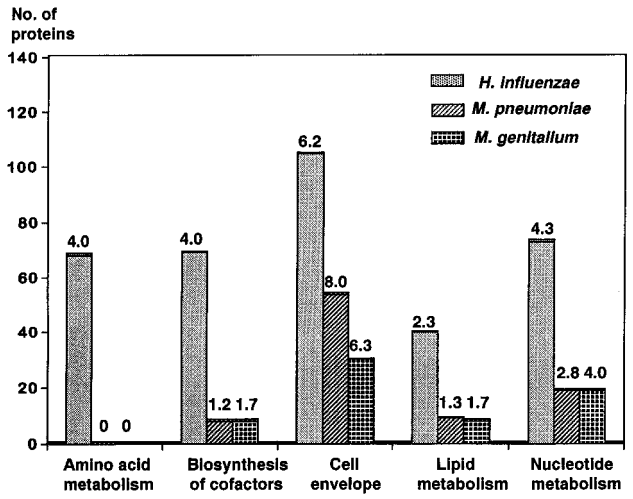

FIG. 1. Biosynthetic pathways genes in the genomes of *H. influenzae*, *M. pneumoniae*, and *M. genitalium*. Numbers above the bars indicate the percentages of the total putatively identified genes. Based on data from Fleischmann et al. (130), Fraser et al. (139), and Himmelreich et al. (181, 182).

constant milieu. *M. genitalium* is a parasite of the human urogenital tract, and its transmission by sexual contact ensures its minimal exposure to the external, osmotically variable, environment.

As can be seen in Fig. 1, significant gene saving is also pronounced in lipid metabolism. Most mycoplasmas cannot synthesize any fatty acid and therefore depend on the host for their supply (see "Cell membrane" below). Although mycoplasmas generally synthesize their own membrane phospholipids and glycolipids from the exogenously provided fatty acids, some mycoplasmas incorporate preformed host phospholipids into their membrane. There is also a price for this gene saving. Being deficient in the ability to regulate membrane fluidity by preferential fatty acid biosynthesis, the mycoplasmas overcome this difficulty by incorporating large quantities of exogenous cholesterol into their membrane, and, as our laboratory showed in the 1970s, cholesterol serves as a very effective buffer of membrane fluidity (356).

Early studies in our laboratory on mycoplasma nutrition revealed the requirement for the nucleic acid precursors, purines and pyrimidines. These may be provided by RNA and DNA that have been degraded by the potent mycoplasmal nucleases (292, 356). The present genetic data (Fig. 1) provide an explanation for this observation by revealing the scarcity of genes and enzymes responsible for purine and pyrimidine synthesis in the mycoplasmas, compared to *H. influenzae*. The genes for the salvage pathways utilizing purines and pyrimidines for the synthesis of ribonucleotides and their conversion to deoxyribonucleotides have been detected in the mycoplasmas (139, 181, 449).

Cellular processes. The number of genes involved in cellular processes, such as the *fts* genes associated with cell division, heat shock proteins, and genes for chaperones functioning in protein secretion, is definitely smaller in the mycoplasmas than in *H. influenzae* (Fig. 2). The protein secretion system in *M. pneumoniae* is much less complex than in *E. coli*. The channel-forming proteins SecG, SecF, SecE, and SecD and the cytosolic receptor protein SecB were not identified in the mycoplasma. Also missing is the signal peptidase Spase I (181). The simplified protein export system might reflect the fact that the mycoplasma cell is bounded by a cytoplasmic membrane only. The problem concerns the refolding of the proteins which are exported in an unfolded configuration. Refolding is catalyzed by chaperones which have to function on the cell surface. This might impose a problem in mollicutes that lack a periplasmic space, a space which prevents the proteins from diffusing away. A way to solve this problem is by anchoring the proteins to the membrane surface via long acyl chains, and, indeed, *M. pneumoniae*, *M. genitalium*, and mycoplasmas in general are rich in cell surface lipoproteins (181) (see "Cell membrane" and "Antigenic variation" below).

The difference between the mycoplasmas and *H. influenzae* is even more pronounced in the category of regulatory functions (Fig. 2). Many of the regulatory system genes found in other bacteria, such as the two-component signal transduction systems consisting of a sensor and response regulator, were not detected in the two mycoplasmas but were present in *H. influenzae*. The absence of identifiable transcription factors from *M. genitalium* is most striking. While *E. coli* has 55 known transcriptional activators and 58 repressors, *M. genitalium* contains only a single transcription elongation factor apart from *nusA* and *nusG* (329). How regulation of transcription takes place with such a small number of factors is a moot point. Although mycoplasmas produce hydrogen peroxide, *M. pneumoniae* and *M. genitalium* lack the genes dealing with oxidative stress, such as those encoding catalase, peroxidase, and superoxide dismutase (139, 181). It has been suggested that the thioredoxin reductase system, identified in the mycoplasmas, may protect them from reactive oxygen compounds (37). What is the price paid for this drastic saving in genes? Are the mycoplasmas less adaptable to environmental changes? What are the mechanisms for maintaining cellular homeostasis in mycoplasmas? Answering these questions and unraveling the subtleties of the interconnected regulation mechanisms in mycoplasmas is expected to be a demanding enterprise.

Energy metabolism and transport. The genomes of the two mycoplasmas are deficient in genes coding for components of intermediary and energy metabolism (Fig. 2). Thus, the two mycoplasmas depend mostly on glycolysis as a means of synthesizing ATP. Genes that encode the components of the pyruvate dehydrogenase complex, phosphotransacetylase, and ace-

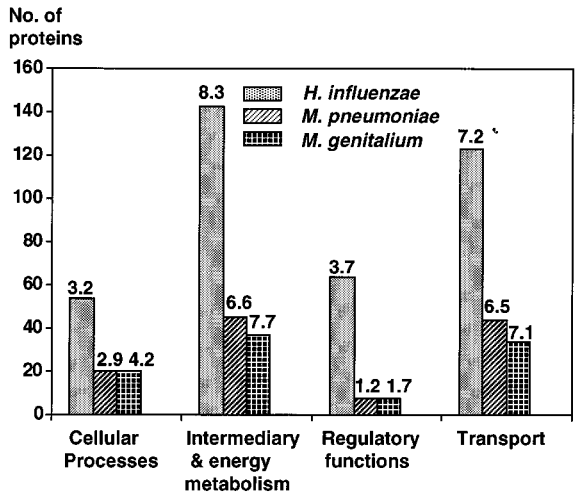

FIG. 2. Cellular processes, metabolic pathways, and regulatory functions genes in the genomes of *H. influenzae*, *M. pneumoniae*, and *M. genitalium*. Numbers above the bars indicate the percentages of total putatively identified genes. Based on data from Fleischmann et al. (130), Fraser et al. (139), and Himmelreich et al. (181, 182).

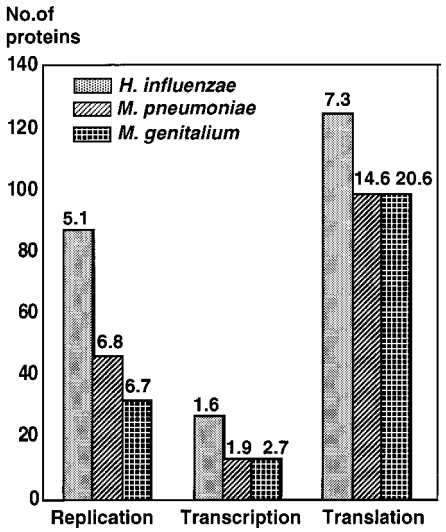

FIG. 3. DNA replication, transcription, and translation genes in the genomes of *H. influenzae*, *M. pneumoniae*, and *M. genitalium*. Numbers above the bars indicate the percentages of total putatively identified genes. Based on data from Fleischmann et al. (130), Fraser et al. (139), and Himmelreich et al. (181, 182).

tate kinase were also detected, as well as a deficient pentose phosphate pathway (139, 181). Most striking is the lack of many energy-yielding systems from the mycoplasmas. No tricarboxylic acid cycle, and no quinones and cytochromes were found in any of the mycoplasmas (see "Metabolism and Transport" below). The electron transport system in mycoplasmas is flavin terminated. Thus, ATP is produced in mycoplasmas by substrate-level phosphorylation, a less efficient mechanism than oxidative phosphorylation. However, it appears that the parasitic mycoplasmas grow well in vivo despite the truncated and inefficient ATP-yielding systems at their disposal. A major reason probably is the relatively small investment of ATP needed for the limited biosynthetic pathways characterizing mycoplasmas.

Being dependent on the exogenous supply of many nutrients would predict that mycoplasmas need many transport systems. As can be seen in Fig. 2, the percentage of genes devoted to transport in *M. pneumoniae* and *M. genitalium* is not higher than in *H. influenzae*, while their absolute number is much smaller. The small number of transport proteins in the two mycoplasmas is even more striking compared to the 281 transport and binding proteins annotated in *E. coli* K-12 (52) and almost 400 in *B. subtilis* (238a). Possible reasons for the small number of transport systems in the mycoplasmas may be the presence of only one permeability barrier in the wall-less mycoplasmas, compared to two in the gram-negative *H. influenzae* and *E. coli*. The apparent low substrate specificity of some of the mycoplasmal transport systems, such as those for amino acids (181), may also contribute to the significant gene saving observed in this category.

Replication, transcription, and translation. Moving on to the comparative genomics of genes involved in DNA replication, transcription, and translation, the picture is somewhat different (Fig. 3). The essential role of these basic processes in cell biology, leads us to expect that saving of genes in these categories will be more restricted as compared to metabolic processes. Cells are capable of importing and utilizing exogenous metabolites but not functional proteins; therefore, they have to rely on their own gene products to provide housekeeping functions. Figure 3 shows that while the absolute number of genes involved in DNA replication in the mycoplasmas is still much smaller than in *Haemophilus*, the percentage of genes devoted to DNA replication and degradation in *M. genitalium* and *M. pneumoniae* is higher than in *H. influenzae*, pointing to the essential biological role of these genes, so that evolutionary deletion of genes in this category had to be limited.

Obviously, it is of interest to define the genes missing from the mycoplasmas, since these are apparently of secondary importance. For example, the estimated numbers of genes functioning in DNA repair in *E. coli* and *H. influenzae* are estimated to be about 100 and approximately 30, respectively (52, 139, 181). The numbers are definitely smaller in *M. genitalium* and *M. pneumoniae*: only 13 of the genes known to be involved in excision repair, recombination, and SOS repair of DNA were found by Himmelreich et al. (181) in *M. pneumoniae*. We may assume that the genes detected in the mycoplasmas, including the uracil DNA glycosylase and the exinuclease ABC genes and *recA*, must represent the genes really essential for DNA repair.

As for transcription, the percentage of genes devoted to transcription is higher in the mycoplasmas than in *Haemophilus* (Fig. 3). Included in this category are the genes for the RNA polymerase subunits, which are basically similar in mycoplasmas and the other eubacteria (see "Transcription and translation" below). Included in this category are the genes for RNA helicases. The two mycoplasmas encode only a single sigma factor, compared to at least 6 in *E. coli* (139) and 18 in *B. subtilis* (238a). The *M. genitalium* genome carries only 2 RNase genes, compared to 10 in *H. influenzae* (139, 181). It should be emphasized again that even with this considerable saving in genes, the percentage of genes devoted to transcription in the mycoplasma genomes is considerably higher than in *Haemophilus* (Fig. 3).

The translation and protein synthesis machinery constitutes apparently the most highly conserved system in eubacteria, indicating that it represents a very efficient and successful evolutionary development. The highly conserved nature of the genes involved in translation, ribosome, and tRNA synthesis is reflected in their G+C content. Thus, while the overall G+C content of the *M. genitalium* genome is 32 mol%, the G+C content of the mycoplasmal rRNA genes is 44 mol% and that of the mycoplasmal tRNA genes is 52 mol% (139). The degree of homology of the mycoplasmal ribosomal proteins to those of other eubacteria is also very high. Thus, the composition of mycoplasmal translation components closely resembles that of the components of other bacteria.

A marked difference can nevertheless be observed in the copy number of the rRNA and tRNA genes. While *E. coli* and *H. influenzae* genomes carry seven and six copies of rRNA operons, respectively, *M. genitalium* and *M. pneumoniae* have only one copy (Table 3). We could show long ago that savings in the number of rRNA genes is a general property of mycoplasmas, since the maximum copy number of rRNA genes in these organisms is only 2, except for *Mesoplasma lactucae*, in which it is 3 (5, 56). The number of tRNA genes in mycoplasmas is also kept to a minimum, with very few, if any, gene duplicates. Thus, the *M. genitalium* and *M. pneumoniae* genomes carry only 33 genes (139, 181) and *M. capricolum* carries 30 genes (304), compared to 54 in *H. influenzae* and 86 in *E. coli* (52, 130, 139). Accordingly, the number of anticodons in the mycoplasma is not much greater than in mitochondria and is close to the essential minimum for translation of all the amino acid codons by wobbling (364). Nevertheless, of the 20 standard amino acyl-tRNA synthetases, the glutaminyl-tRNA synthetase was the only one not detected in *M. genitalium* and *M. pneumoniae* (139, 181). Thus, the mycoplasmas possess the

TABLE 3. Properties distinguishing mollicutes from other eubacteria[a]

Property	Mollicutes	Other eubacteria
Cell wall	Absent	Present
Plasma membrane	Cholesterol present in most species	Cholesterol absent
Genome size	580–2,220 kb	1,050–>10,000 kb
G+C content of genome	23–40 mol%	25–75 mol%
No. of rRNA operons	1 or 2[b]	1–10
5S rRNA length	104–113 nt[c]	>114 nt
No. of tRNA genes	30 (*M. capricolum*), 33 (*M. pneumoniae*)	84 (*B. subtilis*), 86 (*E. coli*)
UGA codon usage	Tryptophan codon in *Mycoplasma*, *Ureaplasma*, *Spiroplasma*, *Mesoplasma*	Stop codon
RNA polymerase	Rifampin resistant	Rifampin sensitive

[a] Adapted from reference 359.
[b] Three rRNA operons in *Mesoplasma lactucae* (56).
[c] nt, nucleotides.

minimum machinery needed for protein synthesis. As to the price to be paid for these drastic savings: protein synthesis and consequently cell replication in mycoplasmas are much slower than in *E. coli*. Thus, the velocity of the DNA replication fork was about 10 times slower in *M. capricolum* than in *E. coli* (411a). However, mycoplasmas appear to grow well in the protective and constant environment of their host and a low replication rate cannot really be considered a deficiency of a parasite that can lose only by killing its host. In fact, mycoplasma infections are usually rather mild and chronic.

Parasitism-associated genes. As emphasized above, the considerable economization in genes depends primarily on the obligate parasitic mode of life of mycoplasmas. However, there is no "free lunch," and some genomic price has to be paid for parasitism; that is, mycoplasma cells must possess surface components enabling their attachment to the host cells. In some cases, including *M. genitalium* and *M. pneumoniae*, the mycoplasmas developed special attachment organelles (see "Morphology and ultrastructure" above). Obviously, a significant number of genes is involved in the construction of such an organelle (342a). Some of these genes have already been identified and characterized, but some have not and are apparently included in the parts of the mycoplasmal coding regions or genes that are unidentified as yet. Of those characterized, the genes for the major adhesins of *M. pneumoniae* and *M. genitalium*, P1 and MgPa, are well known. The two adhesin proteins resemble each other in structure, with both being large integral membrane proteins having regions exposed on the mycoplasma cell surface that attach to receptor sites on the host epithelial cell (see "Adhesion to host cells" below). Somewhat unexpected was the finding of repetitive elements composed of short segments of the MgPa and P1 operon distributed over the genome. These repetitive sequences, together with the intact MgPa and P1 operons, constitute 4.7% of the total *M. genitalium* genome and about 8% of the *M. pneumoniae* genome (139, 181, 182). Repetitive elements have also been found in other mollicute genomes (55) (see "Genome size and base composition" above). These observations appear to contradict our expectation from a minimal genome. However, recent evidence provides a rationale for the presence of repeats in the minimal genome. The repetitive elements are conducive to homologous recombination and genomic rearrangements and may play a role in induction of antigenic variation of the mycoplasmal cell surface and in this way help the parasite to evade the host immune response (332) (see "Chromosomal rearrangements" and "Antigenic variation" below). But again, by genetic manipulations, a minimum number of genes are usually needed to produce a great variety of epitopes on the mycoplasma cell surface, in line with the theme of gene saving (114, 477, 485).

Comparison of the *M. genitalium* and *M. pneumoniae* genomes. Having at hand the complete sequences of the genomes of the two mycoplasmas enabled the comparison of the gene content and mode of organization of the two genomes (182). The most important points revealed were as follows.

(i) All 479 ORFs of the smaller *M. genitalium* genome are contained in the *M. pneumoniae* genome.

(ii) The additional 209 proposed ORFs observed in the *M. pneumoniae* genome include 110 ORFs not detected in *M. genitalium*. Among these *M. pneumoniae*-specific genes, Himmelreich et al. (182) could tentatively identify those encoding an hsd-type restriction-modification system, an NADP-dependent alcohol dehydrogenase, and two additional phosphotransferase (PTS) transport systems for carbohydrates (one probably for mannitol), as well as the three genes of the arginine dihydrolase pathway. In addition, 23 ORFs containing a copy of either one of the three repetitive DNA sequences RepMP2/3, RepMP4, and RepMP5 were found in *M. pneumoniae* but not in *M. genitalium*, although the DNA sequences were present. Himmrelreich et al. (182) could not determine the functions of the proteins encoded by these genes, although at least some of them were expressed. Very interesting is the finding of the 76 ORFs in *M. pneumoniae* representing amplifications of genes found only as single copies in *M. genitalium*. Most prominent among these are genes for cell surface lipoproteins, more prevalent in *M. pneumoniae* than in *M. genitalium*.

(iii) Another very interesting finding concerns the organization of the two genomes. The genomes of the two mycoplasmas could be subdivided into six segments. The order of orthologous genes was well conserved within each of the individual segments, but the arrangement of the six segments differed. Himmelreich et al. (182) suggest that the different order of the genomic segments in the two genomes is the result of translocation of entire segments via homologous recombination between the repetitive DNA sequences located at the border of each of the segments. It remains to be determined how all these data are related to the different properties of these mycoplasmas. Are the great difficulties of in vitro cultivation of *M. genitalium* a reflection of the smaller number of genes? What are the reasons for the different location of these mycoplasmas in humans (*M. pneumoniae* is a respiratory pathogen, while *M. genitalium* is a pathogen of the human urogenital tract)?

Gene density. The sequencing of entire bacterial genomes and definition of the coding regions provide us with an answer to an important question, i.e., how much of the bacterial ge-

nome is occupied by intergenic noncoding regions, including control elements, promoters, and terminators. The answer is about 12% in the *M. genitalium* and *M. pneumoniae* genomes (139, 181, 182), as in *E. coli* (52), and 15% in the *H. influenzae* genome (130). The intergenic sequences in *Helicobacter pylori* were estimated to occupy only 6% of the genome (455). *M. genitalium* appears to make heavy use of operon systems, potentially reducing the number of regulatory elements required for controlling the transcription of genes (333) and thus increasing the gene density (422). Does gene overlapping occur in mollicutes? There are some examples of overlapping where the promoter for one coding sequence is placed within the C-terminal coding sequence of the upstream gene, but such overlaps appear to be few and short and hence do not appear to have a significant effect on genome compaction in mollicutes (55, 94, 333). On the whole, the marked reduction in mycoplasma genome size did not result in any significant increase in gene density. Neither could a decrease in gene size be found. In fact, the average gene size is 1,040 bp in *M. genitalium* and 1,011 bp in *M. pneumoniae*, compared with 900 bp in *H. influenzae* (139, 181, 182).

Another related point concerns gene duplication and DNA repeats. For many cellular functions of *E. coli*, there are two or more genes, as if the genetic program of this bacterium calls for backup systems (52). It appears that despite the genome size limitation, the occurrence of duplicate or even multiple gene copies has been recorded in mycoplasmas, such as duplicate lipoprotein genes in *M. pneumoniae* (182) and, more striking, the large number of pMGA genes in *M. gallisepticum*, occupying up to 16% of the genome (27) (see "Antigenic variation" below). Thus, physiological needs crucial for survival in the host, such as antigenic variability, may overrule the principle of gene saving.

Proteome Approach

Although over 90% of the ORFs in the *M. genitalium* and *M. pneumoniae* genomes have been definitely or tentatively identified (Table 2), there much still remains to be done to identify the unclassified coding regions that have no database match, prove the DNA-based predictions experimentally, and assign functions to proposed ORFs with hitherto unknown functions. As could be expected, the easy access to the entire genomic sequence data on the World Wide Web facilitated reevaluation of the original *M. genitalium* ORF annotations, so that some of the original functional annotations of the genes were corrected and new functional predictions for a number of gene products were added (66, 329). A major difficulty in gene identification by computer database matching arises from the fact that the degree of identity required (percent homology) is not precisely defined and that this approach is restricted to the genes or proteins that have been detected in other species and are available in data banks.

The construction of a functional genetic map on the basis of sequence data may be hampered by the occurrence of silent genes or cryptic operons with recognizable genes that cannot be expressed because of lack of functional promoters, because of mutations, or because of insertion of genetic material that prevents normal expression, as well as by regulation and suppression of gene expression. Since the genomes of many organisms are expected to be fully sequenced within the next few years, the ability to ascribe function to a given gene product will become increasingly significant (see "Metabolism and Transport" below). One approach to tackle this problem involves proteome analysis; "proteome" is defined as the expressed total protein complement of a genome. The method of proteome analysis is based on two-dimensional gel electrophoresis, electroblotting and silver staining procedures, amino acid composition analysis of the peptide spots combined with peptide mass fingerprinting, N-terminal protein microsequencing, and computer database matching. Automated procedures are being developed so that the proteome approach may be more expeditious in terms of speed, economy of effort, and cost (192, 469).

Mycoplasmas are particularly suitable for proteome analysis, since a high percentage of their proteins can be visualized by two-dimensional PAGE (2D-PAGE), analyzed, and identified. Moreover, the highly conserved nature of a large part of the mycoplasmal proteins facilitates their identification (96). Thus, of the 58 *M. pneumoniae* proteins analyzed by this method, 49 could be putatively identified (470). The major limitations of the proteome approach are as follows. (i) Not all cell proteins are expressed at a given time. (ii) The 2D-PAGE is effective only for small organisms, such as mycoplasmas. (iii) The detection threshold of 2D-PAGE is a limiting factor, since protein molecules with a low copy number may not be detected. (iv) Proteins with an isoelectric point higher than 10 cannot be separated well in the first dimension of immobilized pH gradients. (iv) At present, proteome analysis is useful only for identifying proteins for which the DNA sequence of their genes is available.

The *M. genitalium* genomic sequences have recently been used to assign computerized three-dimensional folds to cell proteins. Of the total 468 ORFs, 22% could be assigned a known protein fold with high confidence. Of the remaining 78% of sequences, 18% belong to membrane proteins (128). This approach is expected to bring us closer to structural and functional identification of proteins predicted on the basis of genomic sequences. Nevertheless, as much as all the above approaches may be helpful, it must be emphasized that the question of gene product function can be answered definitely only by an enzyme assay, gene "knockout" mutagenesis, structural analyses, etc.

Minimal Cell Concept

M. genitalium has the smallest genome known at present. Does its gene complement represent the minimal self-sufficient gene set? Mushegian and Koonin (303) approached this intriguing issue by applying comparative genomics to *M. genitalium* and *H. influenzae*, organisms belonging to two ancient bacterial lineages, i.e., gram-positive and gram-negative bacteria, which separated from their common ancestor at least 1.5 billion years ago. They assumed that genes that are conserved in both bacteria are essential for cellular function. It is this category of genes that is most likely to approximate the minimal gene set. Mushegian and Koonin (303) identified 240 genes associated with similar amino acid sequences and functions in both organisms. Adding to this set 22 genes needed to fill gaps in essential metabolic pathways not encoded by similar sequences in the two organisms and subtracting 6 genes which are functionally redundant and parasite specific resulted in 256 genes, regarded by Mushegian and Koonin as the approximation of the minimal gene set necessary for a "modern-type" cell.

While *M. genitalium* is the cellular form with the smallest number of genes (Table 2), its genome still carries almost double the number of genes included in the minimal gene set of Mushegian and Koonin. Clearly, some of the genes in addition to the minimal gene set encode proteins implicated in parasite-host interactions. Maniloff (273) estimated that at most 10% of the *M. genitalium* genes are host specific (e.g.,

cytadherence and surface antigen genes), a value which may be an underestimate, when the specific genes involved in construction of the cytoskeleton are added. However, even when these specific genes are subtracted, the *M. genitalium* gene set is still larger than the minimal gene set of Mushegian and Koonin, leading Maniloff to suggest that, "This probably reflects the difference between a small (minimal) tinkered-together gene set, produced by a couple of billions years of evolution, and a small (minimal) engineered gene set produced by a computer." It seems that unless the smallest genomes of mycoplasmas are still evolving and undergoing genome reductions, 600- to 800-kb genomes appear to be the lower limit for mycoplasmas and presumably for any other self-replicating cells (273).

The idea that a hypothetical cell with the minimal gene set will be a simplified derivative of *M. genitalium* has led Himmelreich et al. (182) to propose an experimental approach to define and construct a minimal cell. Accordingly, *M. genitalium* will be genetically manipulated by inactivation or deletion of genes, testing for the effects of these manipulations on survival and replication under defined conditions. Clearly, the minimal set of essential genes depends on the growth conditions, e.g., whether the minimal cell is growing in vitro in a serum-enriched medium or in the urogenital tract of the host, where adhesion and antigen variation genes are expected to play an essential role. Thus, when defining a minimal cell, one must also define the environmental conditions for growth of this cell.

The finding that *M. genitalium* and *M. pneumoniae*, while differing significantly in genome size, carry a similar number of genes involved in DNA replication and the same number of transcription and translation genes (Fig. 3) led Himmelreich et al. (182) to suggest that the minimal gene set for these essential functional categories has already been established. Strengthening this notion are the recent results derived from the sequencing of the *E. coli* genome, showing that the number of proteins involved in translation in this relatively large genome is similar to that found in *H. influenzae* and in the two mycoplasmas (52). In other functional categories, such as cell envelope, cytoskeletal proteins, energy metabolism, and transport, more flexibility seems possible, since environmental conditions might strongly influence the number of genes and functions required to be supplied by the minimal cell itself.

In conclusion, the sequencing of entire bacterial genomes has been the fastest and most cost-effective way to acquire huge amounts of new information. Knowledge of the complete sequence of the *M. genitalium* genome has in less than 6 months contributed more information on the cell biology of this organism than many years of hard work involving conventional methods. We are now much closer to achieving the goal of completely deciphering, in molecular terms, the machinery of a self-replicating cell, and we already have an overall, although somewhat blurred, picture of a minimal cell after investing, perhaps, less effort than was predicted by Morowitz in 1984 (297).

DNA REPLICATION AND REPAIR

Origin of Replication

The organization of genes at the origin of replication (*oriC*) of mollicute genomes has attracted much attention, since the eubacterial *oriC* genes and their order (*rnpA-rmpH-dnaA-dnaN-recF-gyrB*) are highly conserved. This order has been only partially retained in the mollicutes tested. The lack of *recF* is common to all the mycoplasmas tested so far (19, 139, 180, 293, 483). The DNA gyrase subunits A and B (*gyrA* and *gyrB*) are linked at the origin of replication of gram-positive bacteria, while in gram-negative bacteria *gyrA* is usually not associated with this region. Interestingly, the tandem arrangement of the two gyrase genes has been found at the origin of replication of *M. genitalium* (19), *M. pneumoniae* (180), *M. gallisepticum* (136), and *S. citri* (483) but not *M. capricolum* or *M. hominis*, where the *gyrB* and *gyrA* genes were located separately outside the origin of replication region (242, 400).

The data of the *M. genitalium* and *M. pneumoniae* genome sequencing projects, led Hilbert et al. (180) to conclude that the genes located in the *dnaA* region of the two mycoplasmas are unusual, since a number of them have not been found in the corresponding *oriC* region of other bacteria and the conserved genes *gyrB*, *dnaA*, and *dnaN* are in a different order or orientation with respect to each other. Hilbert et al. (180) also failed to detect in *M. pneumoniae* the dnaA box regions adjacent to *dnaA*, characterized by repeats of the nonamer TTAT CCACA, in line with earlier findings by Fujita et al. (144). Since the dnaA boxes are essential for binding of the DnaA protein initiating DNA replication, the boxes are expected to exist, but their sequence apparently differs from the consensus to different degrees depending on the mollicute species (412). The overall conclusion of these studies is that the conserved order of genes (*rnpA-rpmH-dnaA-dnaN-recF-gyrB*) is not a prerequisite for a functional origin of replication (180).

DNA Polymerases

The nature of the mycoplasmal DNA polymerases has presented an enigma for many years. Early studies involving classical biochemical procedures (reviewed in references 284 and 357) detected a single DNA polymerase in *M. orale*, *M. hyorhinis*, *M. mycoides* subsp. *mycoides*, and *U. urealyticum*, whereas *Spiroplasma* species and *A. laidlawaii* were shown to possess three different DNA polymerases, resembling *E. coli* and *B. subtilis* in this respect. Most intriguing was the apparent lack of the 3′→5′ proofreading exonuclease activity from the *M. orale* and *M. hyorhinis* polymerases, a finding in support of a high mutation rates in mollicutes (479). The recent *M. genitalium* and *M. pneumoniae* genome sequencing projects, as well as the study of Barnes et al. (24) on *M. pulmonis* DNA polymerases, have clarified this issue considerably. It appears that the mycoplasmas carry the central enzyme for DNA replication—the DNA polymerase III holoenzyme (Pol III), which has the essential characteristics of the eubacterial gram-positive Pol III. Although the genes responsible for building the major subunits of the holoenzyme, such as the α (*dnaE* or *polC*) and β (*dnaN*) subunits, were detected, several other genes responsible for other subunits were missing from the *M. genitalium* and *M. pneumoniae* genomic analysis (139, 181), indicating a simplified DNA replication complex in the mycoplasmas compared to that of *E. coli* or *H. influenzae*. However, most importantly, the mycoplasmal Pol III α subunit (about 1,400 amino acids) included the motif for 3′→5′ exonuclease activity typical of Pol C of gram-positive bacteria (24, 181, 238a), providing an answer to the question of the way DNA proofreading is done in mycoplasmas.

Barnes et al. (24) presented experimental evidence for a second enzyme with DNA polymerase activity in *M. pulmonis*. This correlates with sequence data for *M. genitalium* and *M. pneumoniae* indicating that at least these three *Mycoplasma* species carry two DNA polymerase genes, one coding for the larger protein (about 166 kDa) with a 3′→5′ exonuclease activity, with high sequence similarity to the *B. subtilis* Pol III, and the other coding for a Pol III homolog (about 100 kDa) that more closely resembles the *polC* gene from *E. coli*, lacking

a 3′→5′ exonuclease domain. This smaller enzyme may correspond to the DNA polymerases lacking exonuclease activity, isolated earlier from *M. orale* and *M. hyorhinis* (284, 357).

It should be emphasized that while the major components of the DNA polymerase machinery are present in *M. genitalium* and *M. pneumoniae*, their genetic maps reveal the absence of many genes, including some major ones, involved in DNA replication, e.g., initiation, elongation, and termination of replication (Fig. 3). The failure of sequence analysis to detect the primer removal protein RNase H and a protein for the termination of replication is most puzzling and may just reflect the weakness of basing gene identification on sequence matching only (181). As for DNA polymerase I (Pol A), an enzyme important in DNA repair, only a truncated *polA* gene was detected in *M. genitalium* (139) and *M. pneumoniae* (181), encoding only the 5′→3′ exonuclease domain of the much larger Pol A of *E. coli* and *B. subtilis*, which also have a 5′→3′ polymerase-specific domain. The mycoplasmal 5′→3′ exonuclease activity could be responsible for the removal of the RNA primers in the lagging strand of the replication fork, resembling the activity of Pol I in *E. coli* (114).

Topoisomerases catalyze the interconversions of topological isomers of DNA molecules, fulfilling an essential role in DNA replication, transcription, recombination, and repair. The mycoplasmal DNA topoisomerases characterized so far are the DNA gyrase and topoisomerase IV, both belonging to type II topoisomerases. Both mycoplasmal enzymes resemble their eubacterial counterparts in having a tetrameric structure composed of two subunits: A and B in the case of DNA gyrase, and the products of *parC* and *parE* genes in the case of topoisomerase IV (20, 242). The DNA gyrase introduces negative supercoils into a relaxed closed-circular DNA molecule in a reaction requiring ATP. In the absence of ATP, the gyrase can relax negatively supercoiled DNA (242). Topoisomerase IV resembles DNA gyrase in its effect on DNA but lacks the ATP-independent relaxation activity (187). Resembling other eubacterial topos, the mycoplasmal enzymes are inhibited by novobiocin and quinolones (33, 187). However, the topoisomerase IV of *M. fermentans* and *M. pirum* was also inhibited by several eucaryotic topo IV antagonists, not known to inhibit eubacterial topoisomerases (187).

DNA Repair

Injury to DNA is minimized by damage containment systems that recognize the occurrence of a change and then rectify it. The bacterial repair systems are likely to be as complex as the replication apparatus itself, an indication of their importance for the survival of a cell. The measured rate of mutation of an organism reflects a balance between the number of damaging events occurring in the DNA and the numbers that have been corrected.

As mentioned above in "Genome sequencing and the minimal cell concept," the number of genes involved in DNA repair in *M. genitalium* and *M. pneumoniae* is considerably smaller than in *E. coli*, *B. subtilis*, or even in *H. influenzae* (52, 130, 139, 181). Thus, *M. pneumoniae* codes only for 13 genes involved in excision repair, recombination, and SOS repair. Obviously, comparative genetic analysis of the mycoplasmal DNA repair systems with those of other eubacteria may indicate the relative importance of specific genes, assuming that those retained in the mycoplasmas are the more essential ones. The failure to identify the dUTPase gene in *M. genitalium* and *M. pneumoniae* (139, 181) and to detect dUTPase activity in mollicutes (342) should be considered on evaluation of the efficiency of the DNA repair system in these organisms. This enzymatic activity is ubiquitous in nature, and it has been claimed that DNA repair processes may be impaired in its absence. Hence, this deficiency may contribute to the high mutation rates and the rapid or tachytalic evolution characterizing the mollicutes (272, 342).

Mismatch and excision repair. Excision repair of regions containing missing bases (apurinic or apyrimidinic sites) is likely to occur by a pathway involving endonuclease IV (*nfo*), Pol A, and ligase, found in *M. genitalium* (139). The *ung* gene, coding for uracil-DNA glycosylase, functioning in the removal of uracil residues from DNA arising by spontaneous deamination of cytosine residues, is present in *M. genitalium* (139) and *M. pneumoniae* (181). This enzyme was purified from *Mesoplasma lactucae*, but its specific activity was low. No activity of this enzyme could be detected in *Mesoplasma entomophilum* (341), *M. gallisepticum*, *M. capricolum*, and *U. urealyticum* (475). While this failure may simply be due to insufficient sensitivity of the assay procedure, the decreased capacity of the mycoplasmas to remove uracil residues from DNA would favor the gradual replacement of G · C base pairs with A · T base pairs, leading to the low G+C content of mollicute genomes (475).

The three genes coding for the ABC excinucleases are present in *M. genitalium*, *M. pneumoniae*, and *M. capricolum* (55, 139, 181); these nucleases, together with Pol I, helicase II, and ligase, should provide the mechanism for repair of UV damage, such as cross-linking. While dark reactivation of UV damage (excision repair) operates in the above mycoplasmas, photoreactivation may be missing, because the photolyase (*phr*) gene could not be identified in *M. genitalium* or *M. pneumoniae* (139, 181). Earlier studies testing for light and dark repair of UV damage revealed the presence of both in *A. laidlawii* and only dark repair in *M. buccale*. The two systems were apparently absent or acting at very low efficiency in *M. gallisepticum* and *S. citri*, corresponding to the high sensitivity of these mycoplasmas to UV irradiation (reference 239 and references therein). The mismatch repair system (*mutS*, *mutL*, and *mutH* genes) has not been identified in *M. pneumoniae* or *M. genitalium* (181, 182). It appears likely that the absence of these genes is also associated with an increased mutation rate in these organisms (181).

RecA and the SOS response. The RecA protein plays a central role in recombination repair, homologous recombination, and initiation of the SOS response. The *recA* gene has been found essentially in all the mollicutes analyzed so far, including *A. laidlawii* (115), *M. pulmonis*, *M. mycoides* subsp. *mycoides* (223), *M. capricolum* (55), *M. genitalium* (139), and *M. pneumoniae* (181). Surprisingly, *S. citri* R8A2 was found to lack a significant portion of the N terminal of RecA (279) affecting its function, in line with the high sensitivity of the organism to UV irradiation (239). Although *recA* is generally mutable in bacteria, including *A. laidlawii*, where the mutant became deficient in DNA repair activity (115), trials to introduce mutations into *M. pulmonis recA* have failed, suggesting that RecA is critical for cell survival of this mycoplasma (223).

RecA functions in exchanging strands between DNA molecules. In this way, it may fill the gap that could be formed due to jumping of the polymerase system over a pyrimidine dimer in one of the strands. RecA transfers the proper section retrieved by replication of the intact strand. RecA also has other functions; thus, following UV irradiation it activates proteolytic activities, leading to induction of the expression of many genes. LexA is a small (22-kDa) protein acting as a repressor of many genes, including those for repair functions. Activation of RecA leads to proteolytic cleavage of LexA and thus to induction of all these genes. The SOS response occurs within

minutes of the damaging treatment. In the absence of activated RecA, the LexA protein rapidly accumulates, turning off the SOS genes. Surprisingly, the *lexA* gene could not be detected in *M. genitalium* (139) or *M. pneumoniae* (181). Thus, it is not clear how the SOS response is regulated in these mycoplasmas in the absence of LexA. The *M. pneumoniae* genomic analysis has shown that genes for *recB*, *recC*, *recD*, *recG*, and *ruvC*, involved in recombination, are missing, as well as the genes *recN*, *recO*, *recQ*, and *recR* involved in SOS repair in *E. coli* (181). It appears, nevertheless, that a rudimentary stock of enzymes has been conserved in *M. pneumoniae* to permit homologous recombination (RecA, Ssb, PolA, GyrA, GyrB, RuvA, and RuvB), excision repair, and a kind of truncated SOS repair. Apparently, although much less sophisticated than the homologous systems of *E. coli*, the mycoplasmal systems enable the performance of the basic processes of DNA replication and repair under the protective environment of the host.

TRANSCRIPTION AND TRANSLATION

RNA Polymerase

The DNA-dependent RNA polymerase of mollicutes resembles that of other eubacteria in subunit structure. Thus, the core RNA polymerase of *M. genitalium* (139) and *M. pneumoniae* (181) is encoded by the conserved genes *rpoA* (α subunit), *rpoB* (β subunit), and *rpoC* (β′ subunit). One of the peculiar properties of mollicutes is their resistance to the rifamycin and streptolydigin antibiotics (56, 357) (Table 3). Interestingly, the phylogenetically related *Clostridium ramosum*, *C. innocuum*, and *C. acidiurici* are also resistant to rifamycin, suggesting that this property has been acquired from their common ancestor with mollicutes (331). Since rifamycins act by binding to the β subunit of the eubacterial RNA polymerase, it was suspected long ago that rifamycin resistance is associated with some structural change in the β subunit of the mollicute RNA polymerase. The recent cloning and sequencing of the *rpoB* gene from *S. citri* (153, 245) and *M. gallisepticum* (425) suggest that minor differences in amino acid sequences in the β-subunit Rif region, the region responsible for rifamycin binding, may confer rifamycin resistance. Gaurivaud et al. (153), by constructing a chimeric *E. coli rpoB* with the Rif region from *S. citri rpoB*, pinpointed rifamycin resistance to a single amino acid substitution in the Rif region of *S. citri*.

The single sigma factor found in *M. genitalium* and *M. pneumoniae* shows high homology to sigma factor SigA (vegetative) from *B. subtilis* (139, 181). Modulation of promoter selectivity of RNA polymerase by replacement of the sigma subunits is an efficient way of altering the global pattern of gene expression in response to changes in environmental conditions. Thus, the *E. coli* genome carries at least 6 sigma factors and that of *B. subtilis* carries at least 18 (238a). The presence of only one sigma factor in the mycoplasmas suggests that the response to external stimuli in these organisms is not controlled by the level of expression of alternative sigma factors. Mycoplasmas have conserved the heat shock response, and heat shock proteins, resembling the eubacterial DnaK (HSP70) and GroEL proteins, have been identified in several mollicutes (100). The *M. genitalium* and *M. pneumoniae* genomes carry genes for seven heat shock proteins (139, 181). In gram-negative bacteria, heat shock responses are regulated by alternative sigma factors that interact with the core RNA polymerase to alter promoter selectivity. Himmelreich et al. (181) suggest that the heat shock response in *M. pneumoniae*, at least in some cases, is regulated by the interaction of a proposed repressor (*hrcA* homolog) with the CIRCE-like element preceding the heat shock genes, resembling gram-negative bacteria in this respect (174a). However, this is not true for all heat shock genes, since the CIRCE element was not found in front of all the heat shock genes in *M. pneumoniae*.

Transcription Signals

The conclusion reached long ago (357) that mycoplasmal transcription signals resemble the classical eubacterial ones has been strengthened considerably by the voluminous gene sequencing data obtained in the 1990s. The mycoplasmal −10 region (Pribnow box) and to a lesser extent the −35 region resemble the eubacterial promoter consensus sequences recognized by the RNA polymerase holoenzyme (for examples, see references 19, 139, 181, and 440). A consensus Shine-Dalgarno (SD) sequence could also be identified upstream of the initiation codon (56, 114), although in many cases the SD sequence could not be identified, indicating that signals other than the SD sequence may function as a ribosomal binding site for some mollicute genes (180, 181, 262).

Termination of transcription in mollicutes appears to be independent of the termination factor Rho, because its gene could not be detected in the mycoplasmal genomes analyzed thus far. Typical terminator sequences composed of short interrupted palindromic regions, forming a stem-loop structure, followed by a run of U residues, also serve as transcription stops in mollicutes (56, 157). While in most cases transcription signals were identified by their similarity to consensus sequences, the identity of promoters and terminators of mycoplasmal genes has also been experimentally established in a significant number of cases. Of the Nus transcription factors, NusA, which increases the efficiency of termination, and NusG, which is involved in antitermination in other bacteria, were found in *M. genitalium* and *M. pneumoniae* (139, 181).

As to the direction of transcription, in both the *M. genitalium* and *M. pneumoniae* genomes, a remarkable uniformity in this parameter has been observed. Only about 15% of the proposed ORFs are transcribed against the general direction of transcription (182).

Ribosomes and rRNAs

As mentioned under "Genome sequencing and the minimal cell concept" above, the translation machinery of mycoplasmas is rather comprehensive. In *M. pneumoniae* and *M. genitalium*, it is made up of 99 proposed genes, constituting about 15 to 20% of the ORFs of the genome (Fig. 3). It is apparently the most highly conserved system in eubacteria, facilitating considerably the cloning and identification of its genes. The mycoplasmal ribosomes are typically procaryotic in size, shape, and composition (357). The *M. pneumoniae* ribosomal protein genes are organized in operons, retaining some of the gene order found in the *E. coli* and *B. subtilis* genomes (180). Of the 50 ribosomal proteins found in *E. coli*, only the gene for protein S1 could not be detected in *M. genitalium* and *M. pneumoniae*; in this respect, these species resemble a number of gram-positive bacteria, including *Bacillus* (139, 181). The highly conserved nature of the ribosomal protein genes has also found its use in establishing mollicute phylogeny (258, 456) (see "Taxonomy and phylogeny" below).

The rRNA genes are perhaps the best-characterized mollicute genes. Sequences of these highly conserved genes, particularly those of 16S rRNA, serve as most important phylogenetic, taxonomic, and diagnostic tools (see "Taxonomy and phylogeny" below). The organization of the mollicute rRNA genes generally follows the characteristic eubacterial order, 16S-23S-5S, functioning as an operon (157, 357). Some excep-

tions to the above classical rRNA gene organization have been reported. The 5S rRNA gene is separated from the 16S-23S genes in *M. hyopneumoniae* (445a) and *M. flocculare* (429). *M. gallisepticum* S6 carries two rRNA operons, one with the complete set of 16S-23S-5S genes and the other with the 16S rRNA genes separated from the 23S-5S rRNA genes (162). The existence in *M. gallisepticum* A5969, but not in S6, of a third truncated 16S rRNA pseudogene was reported, but it is unclear whether this truncated gene is expressed (424). Another peculiar arrangement of rRNA genes was found in the *M. fermentans* incognitus genome, which carries two rRNA gene sets. In this case the 16S and 23S rRNA genes of the two gene sets are closely linked, since two clusters were arranged in the unusual tail-to-tail orientation while both the 5S rRNA genes were positioned separately (191).

Mollicute genomes carry only one or two rRNA gene sets (Table 3), whereas *Clostridium ramosum* and *C. innocuum*, phylogenetically closest to mollicutes, have four and five sets of rRNA genes, respectively (56). Interestingly, each of the 28 phytoplasmas examined by Schneider and Seemuller (409), representing five primary taxonomic clusters, carried two rRNA gene sets, resembling in this respect their phylogenetic relatives, the acholeplasmas (see "Taxonomy and phylogeny" below). Another feature of interest is the presence of tRNA genes in the intergenic region between the 16S and 23S rRNA genes. While all the seven rRNA operons of *E. coli* carry two tRNA genes in this region, no tRNA genes could be found in this region in mollicutes (357), except for phytoplasmas, where a single $tRNA^{Ile}$ was located in this region (229, 426, 500), and *A. laidlawii*, where the spacer region in one of its two rRNA operons carries the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes (306).

tRNAs

The number of tRNA genes in mollicutes is kept to a minimum, with very few gene duplicates (Table 3). It is the smallest number among all known genetic systems, except for mitochondria (10, 139, 181, 304). The recent sequencing of the *E. coli* K-12 genome revealed 6 new tRNA genes, bringing the total number of tRNA genes in this strain to 86 (52). This number is almost three times higher than the number of tRNA genes in the mycoplasmas tested so far (Table 3). Accordingly, the number of anticodons in *M. capricolum* is only 28 (8) and that in *M. pneumoniae* is only 32 (422), not much higher than the number in yeast mitochondria (24 anticodons) and close to the theoretical essential minimum of 23 anticodons needed to translate all 61 amino acids codons according to the wobble rules (8, 10, 56). Thus, the limited coding space of the mycoplasmal genome has led to a paucity of tRNA genes achieved by the use of a simplified decoding method (422). The mycoplasmal tRNAs contain significantly fewer modified nucleosides than do other bacterial tRNAs, economizing in genes for enzymes involved in tRNA posttranscriptional nucleoside modification. Thus, only 13 types of modified nucleosides were found in the total tRNAs of *M. capricolum*, compared to 23 types in *E. coli* (8, 56).

RNase P is the ubiquitous RNA-processing endonuclease that removes the leader sequences from precursor tRNAs, forming the mature 5′ end of tRNAs. RNase P is a ribonucleoprotein whose RNA component is catalytically active in vitro; i.e., it is a ribozyme. It has been identified and characterized in several mollicutes (170, 181, 418, 440). Sequencing the conserved RNA moiety of the mycoplasmal RNase P appears to have some phylogenetic implications. As could be expected, the sequence basically resembled the type B RNase P of gram-positive bacteria, although the RNase P of *M. flocculare*, *M. hyopneumoniae* (440), and *M. fermentans* (418) showed some divergence from the *Bacillus* type while the sequences of the *A. laidlawii* and *M. genitalium* RNase P resembled the *Bacillus* type more closely (170).

Stable RNAs, other than rRNAs and tRNAs, were also found in mollicutes. Thus, a gene coding for the 10Sa RNA, which functions as tRNA and mRNA and is implicated in *trans*-translation (305a), has been detected in *M. pneumoniae* (181) and in *M. genitalium*, as have genes for 4.5S, 6S, and M1 stable RNA species (66). The *M. capricolum* genome carries three additional small stable RNAs not detected in *M. genitalium* and *M. pneumoniae* (463a).

Codon Usage

Most mollicutes have genomes with a very low G+C content (Table 1), the outcome of an AT-biased directional mutation pressure (A-T pressure) during their evolution (305). This has resulted in codon usage favoring synonymous codons with A and T, in particular in the wobble (3′) position. Codon bias in the AT-rich mycoplasmas is not limited to the third nucleotide position but is evident also in the first and second positions (305). Mycoplasmas have fewer GGN, CCN, GCN, and CGN codons. As a consequence, the mycoplasmal proteins have fewer Gly, Pro, Ala, and Arg residues than their *E. coli* counterparts. In conserved proteins, mycoplasmas often have Lys residues (codons AAA and AAG) at amino acid positions that contain Arg residues (codons AGA, AGG, and CGN) in other bacteria (114). A codon rich in G and C, such as CGG (arginine) does not appear in the coding frames of *M. capricolum*, leading Oba et al. (324) to suggest that CGG is a nonsense or unassigned codon in this mycoplasma. Nevertheless, the CGG codon is used for arginine in *M. hyopneumoniae*, an organism with a higher-G+C genome (145). As expected, in *M. pneumoniae*, with a genome of 40 mol% G+C, all the codons are being represented and used, although to a different degree (181).

Another apparent outcome of codon reassignment under strong A-T pressure is the reassignment in most mollicutes of UGA from a stop codon to a tryptophan codon, a feature found in mitochondria (328). The use of UGA as a tryptophan codon is so unusual that its presence may indicate the mycoplasmal origin of an element located in a mycoplasmal chromosome, such as IS-like elements (116). As can be seen in Table 3, not all mollicutes share this property. The phylogenetically early acholeplasmas and phytoplasmas (see "Taxonomy and phylogeny" below) use the conventional UGG codon for tryptophan (456), carrying $tRNA^{Trp}$ (CCA) and retaining UGA as a stop codon. The phylogenetically more recent spiroplasmas, mycoplasmas, ureaplasmas, and mesoplasmas have evolved the new tryptophan codon, UGA, and a new tRNA to read UGA as tryptophan. The spiroplasmas, as well as *M. capricolum* and *M. gallisepticum*, use both UGG and UGA and have the corresponding two tRNAs: the ancestral $tRNA^{Trp}$ (CCA) and $tRNA^{Trp}$ (UCA) (81, 196). Early claims (196) that *M. pneumoniae* and *M. genitalium* did lose $tRNA^{Trp}$ (CCA) and kept only $tRNA^{Trp}$ (UCA) proved to be wrong, since the genomes of these mycoplasmas were found to carry both tRNA genes (139, 422).

As a consequence of the use of UGA as a tryptophan codon, the peptide chain release factor 2 (RF2), which recognizes the stop codons UGA and UAA, has become obsolete and was deleted, but release factor 1 (RF1), which recognizes the stop codons UAG and UAA, was retained. That this really happened was proved experimentally in *M. capricolum* (193) and genetically by showing that the genomes of *M. capricolum*, *M.*

genitalium, and *M. pneumoniae* carry only RF1 (139, 181, 194). Thus, mollicutes that use UGA as a tryptophan codon use only UAA and UAG as termination codons, giving preference to UAA in the mollicutes with very low G+C genomes, like *M. capricolum* (193).

Another consequence of the use of UGA as a tryptophan codon is the difficulty in expressing cloned mollicute genes in *E. coli*. Since *E. coli* regards UGA as a stop codon, translation of a mycoplasmal mRNA in *E. coli* will stop where originally there should be tryptophan, so that mycoplasmal proteins expressed in *E. coli* may be truncated. One way to overcome this difficulty is to use an *E. coli* opal suppressor strain for expression (316, 374, 427), although the efficiency of expression falls considerably with increasing numbers of UGAs in the mycoplasmal mRNA (344).

In mitochondria, family codon boxes are each read by a single tRNA having an unmodified uridine at the first position of the anticodon, the wobble position (10). Similarly, *M. capricolum* and *M. mycoides* carry only a single isoacceptor tRNA for six of the eight family boxes (8, 195) and *M. pneumoniae* carries a single tRNA for each of the Ala, Leu, Pro, and Val family boxes (422). However, analysis of the anticodon set and codon usage revealed some features distinguishing *M. pneumoniae* from the low-G+C mollicutes: (i) there is no obvious preference for AT-rich synonymous codons, (ii) CGG codons are assigned for arginine and are translated by $tRNA^{Arg}$ (UCG), and (iii) CNN or GNN anticodons are encountered in the Ser, Thr, Arg, and Gly family boxes. Thus, in the case of *M. pneumoniae*, which has a genome of 40 mol% G+C, the codon-anticodon recognition pattern has followed the genomic economization strategy but without the influence of A-T pressure (422).

Amino Acyl-tRNA Synthetases and Elongation Factors

As a rule, there is one activating enzyme for each amino acid. Of the 20 standard amino acyl-tRNA synthetases, glutaminyl-tRNA synthetase was the only one not detected in *M. genitalium* (139) or *M. pneumoniae* (181). This deficiency is shared with *B. subtilis* and other gram-positive bacteria, where the organisms charge the $tRNA^{Glu}$ first with glutamate, which is subsequently converted to glutamine by an amidotransferase, an enzyme not identified as yet in the two mycoplasmas.

The elongation factor genes, *fus*, *efp*, *tsf*, and *tuf*, were identified in the *M. genitalium* and *M. pneumoniae* genomes (139, 181). Of these, the *tuf* gene, encoding elongation factor EF-Tu, which mediates attachment of amino acyl-tRNAs to the ribosome, has attracted most attention. It was one of the first mollicute genes to be cloned, sequenced, and expressed in *E. coli* (240, 265, 486). Being a highly conserved gene, it was applied as an effective genetic probe in taxonomy and phylogeny of mollicutes (see "Taxonomy and phylogeny" below). Gram-negative bacteria usually have two copies of the *tuf* gene, whereas most gram-positive bacteria and mycoplasmas carry a single copy, another finding supporting the phylogenetic relatedness of mollicutes to gram-positive bacteria (408, 486).

GENE TRANSFER

The lack of a cell wall in mollicutes would be expected to facilitate the introduction of exogenous DNA into the cells. In fact, the exchange of chromosomal DNA during direct contact of mycoplasma cells (25) and conjugative transposition of transposon Tn*916* from *Streptococcus (Enterococcus) faecalis* to *M. hominis*, by a spontaneous mating process (380), probably involves transient fusion of the cell membranes at the zone of contact. However, DNA transfer efficiency in these cases was rather low. Increased transformation and transfection efficiencies have been achieved either in the presence of polyethylene glycol (PEG) or by application of the electroporation procedure. Other procedures, such as liposome-mediated transformation, in which the DNA element to be transformed is encapsulated in liposomes (399), were less effective with spiroplasmas (151). It appears that the method of choice depends on the mollicute species and on the particular DNA to be transferred (114).

Genetic studies with mollicutes have also been hampered by the paucity of selectable markers (111). Most studies on gene transfer in mollicutes have used as selectable markers the *tetM* tetracycline resistance determinant found on Tn*916*, or the gentamicin-resistance determinant of Tn*4001*. The *tetM* gene is preferable as a marker, since mollicutes in general are sensitive to tetracycline while not all mollicutes are sensitive to gentamicin (114).

Cloning and Shuttle Vectors

Gene function can be ascertained only by mutational analysis. The recent deluge of information on putatively identified mycoplasmal genes, based on sequence analysis (see "Genome sequencing and the minimal cell concept" above), has emphasized the need for complementation of these predictions by mutational analysis. Having efficient transformation procedures at hand, major efforts have been directed at developing cloning and shuttle vectors for mollicutes. Transfection experiments with the replicative form of the *S. citri* virus SpV1, carrying as an insert a segment of the gene coding for the P1 adhesin of *M. pneumoniae* (G-fragment), resulted in the expression of the fragment in the spiroplasma. In this case, the presence of seven UGA codons in the G-fragment insert did not interfere with expression, since *S. citri* can read UGA as tryptophan (278). However, the SpV1-RF/*S. citri* cloning system was deficient because it suffered from the rapid loss of the cloned DNA insert. The loss of the insert was suggested to be the result of illegitimate and homologous recombination (279). Thus, for one such clone, deletion did occur via a double-crossing-over exchange between the circular free viral RF and SpV1 viral sequences present in the *S. citri* host chromosome. Surprisingly, the *recA* gene, which is usually required for homologous recombination, was found to be truncated in the *S. citri* R8A2 host. Another possible explanation suggested by Marais et al. (279) was based on illegitimate recombination of short direct repeats flanking the G-fragment. During replication, these short repeats could be targets for a copy choice process, leading to the loss of the sequence between the direct repeats.

Another type of a cloning vector for *S. citri* was subsequently constructed by combining the *oriC* of *S. citri* with *tetM*. The artificial recombinant plasmid, named pOT1, was introduced by electroporation into *S. citri* cells, in which it replicated and was stably maintained as a free extrachromosomal element (373). This plasmid could be used as a vector to introduce into *S. citri* the G-fragment of the *M. pneumoniae* P1 gene or the spiralin gene of *S. phoeniceum*. Both inserts were transcribed and the spiralin gene was also translated in the *S. citri* host. These recombinant plasmids, in contrast to the SpV1 RF vector, were stably maintained in the spiroplasmal transformants for at least 40 generations. The addition of the ColE1-derived *E. coli* replicon to pOT1 enabled the plasmid to replicate both in *S. citri* and in *E. coli*, fulfilling the requirement of a shuttle vector. However, after a few passages in *S. citri*, this plasmid

integrated into the spiroplasma chromosome, affecting its use as a shuttle vector (373).

Foreign genes could be expressed in *A. laidlawii*, a mollicute that uses the conventional UGG codon for tryptophan. A recombinant *Lactobacillus lactis* plasmid pNZ18, carrying the α-amylase gene of *Bacillus licheniformis*, replicated stably in *A. laidlawii*, and the amylase gene was expressed in the mycoplasma (205). The *S. citri* spiralin gene inserted into pNZ18 was also expressed in *A. laidlawii*. However, while the spiralin in the spiroplasma is a membrane lipoprotein, the spiralin expressed in the acholeplasma did not undergo postranslational modification by fatty acids, remaining as a soluble protein in the cytoplasm (205).

The *M. mycoides* subsp. *mycoides* plasmids pADP201 and pKMK1 are the only characterized plasmids known in the genus *Mycoplasma* (114). Naturally, they were considered as candidates for construction of cloning and shuttle vectors in *Mycoplasma* species. A construct of pKMK1 with an *E. coli* replicon and *tetM*, named p2D4, was shuttled from *E. coli* to *M. mycoides* and back to *E. coli*, with no discernible deletions or rearrangements occurring in the plasmid. Another construct, pIKΔ, without the *E. coli* replicon, could be transformed effectively into *M. mycoides*. The incorporation of the erythromycin resistance determinant (*erm*) into both plasmids contributed erythromycin resistance to the mycoplasma. Resistance was stably maintained, even in the absence of erythromycin, indicating that these plasmids have the potential of serving as useful mycoplasmal cloning vectors. The above vectors may serve as vehicles for delivering exogenous genes into mycoplasmas, or for generating mutations of endogenous genes via recombination with cloned gene fragments (221). The deficiency of these vectors is that they are low-copy-number vectors that have been shown to replicate only in *M. mycoides* and *M. capricolum*, and the stability of inserts in these vectors has yet to be examined (114). In summary, it appears that although several potential cloning and shuttle vectors have already been developed for mycoplasmas, each of them suffers from some deficiency, so that the search for new and effective vectors should be continued.

Transposition

Successful transformation of *M. pulmonis* and *A. laidlawii* to antibiotic resistance was first achieved by a plasmid, pAM120, harboring the streptococcal transposon Tn*916*, which contains the tetracycline resistance determinant *tetM* (113). The transposon was excised from the plasmid and integrated into the mycoplasmal chromosome at random sites, rendering the mycoplasmas resistant to tetracycline. Since then, a variety of mollicute species have been shown to be transformed to tetracycline resistance by Tn*916* or Tn*1545* or to gentamicin resistance by the staphylococcal conjugative transposon Tn*4001*, which contains a gentamicin resistance determinant (68, 102, 133, 213, 464). All these transformations were aided by PEG or electroporation, and the transposons were excised from the plasmids and integrated into the mycoplasma chromosomes at random sites (133, 464). In some cases, the integrated transposons were also stably maintained in the absence of the selective antibiotic (marker) and could transpose to other sites on the chromosome at relatively high frequency (133, 220). The integrated transposon could be accompanied by an additional element. Thus, on integration of Tn*1545* into the *U. urealyticum* chromosome, the integrated fragment contained *int*-Tn, the gene encoding the integrase protein required for the movement of Tn*1545* (102).

Transposon mutagenesis is generated by insertion of transposons into genes, causing their inactivation. The genes carrying the inserted transposon are mapped and identified. The next step is to prove the predicted function of the inactivated gene by complementation with the same gene from the wild type. Transposon mutagenesis has already been applied, rather effectively, to generate mutants of *M. pneumoniae* and *M. genitalium* deficient in cytadherence (175, 237, 371) and of *S. citri* mutants deficient in motility and pathogenicity to plants (57, 131, 203). Characterization of these mutants has already contributed significantly to the identification of genes and their protein products, associated with the mycoplasmal cytoskeleton and cytadherence (see "Adhesion to host cells" below), spiroplasmal motility (see "Morphology and ultrastructure" above), and pathogenicity (see "Virulence factors" below). There is no doubt that transposon mutagenesis will continue to be a most useful tool in mollicute genomic studies.

Restriction and Modification

Restriction and modification of DNA allow a bacterium to distinguish between its own DNA and any foreign DNA which lacks the characteristic host modification pattern. This difference renders an invading foreign DNA susceptible to attack by restriction enzymes that recognize the absence of methyl groups at the appropriate sites. Hence, these systems may pose a serious barrier to gene transfer. Restriction-modification (R-M) systems in mollicutes have been previously reviewed (114, 275). Generally, the restriction-modification systems characterized in mollicutes in the past were defined as type II R-M systems, in which the endonuclease is separate from the methylase (275). More recent findings indicate that this generalization may not be valid any more. Thus, the predicted amino acid sequences encoded by the *M. pulmonis hsd1* locus are very similar to the *E. coli* Hsd proteins that comprise the three subunits of type I R-M systems (116, 423). This finding was somewhat unexpected since type I R-M systems have until recently been found only in gram-negative bacteria and the finding of a type I R-M system in a mycoplasma is the first example for a type I R-M system in an organism related to gram-positive bacteria (116). Strong support for the presence of a type I (*hsd*-type) R-M system in other mollicutes has been derived from the sequence analysis of the *M. pneumoniae* genome, revealing the genes for the three characteristic subunits (*hsdM*, *hsdR*, and *hsdS*) of the type I R-M system (181, 182). Interestingly, *M. genitalium*, the close relative of *M. pneumoniae*, apparently does not have the type I R-M system, since it lacks genes analogous to *hsdM* and *hsdR*, although the presence of a gene related to *hsdS* was reported, a puzzling finding (139). Similarly, an incomplete ORF in a sequenced genomic fragment of *S. citri* showed significant homology to *hsdS*, but no other component of the type I R-M system was identified (245), leaving this issue open.

An interesting finding by Dybvig and Yu (116) concerns the regulation of *M. pulmonis* R-M properties by DNA inversion. The first DNA inversion system to be described involved the *hsd1* element. *Mycoplasma* virus P1 is an *M. pulmonis*-specific phage that contains double-stranded DNA (499). Inversion of *hsd1* changes the R-M properties of *M. pulmonis*, as evidenced by changes in the plating efficiency of P1 on *M. pulmonis* lawns. Another DNA inversion system in *M. pulmonis* is *hsd2*, which is highly homologous to *hsd1* (116). It should be emphasized that this is the first description of a phage-variable R-M system. It has been suggested (114, 116) that the above *hsd* loci may be important regulatory elements. Thus, changes in DNA methylation resulting from inversion of the loci may regulate the expression of some genes. In addition, nuclease activity in-

duced by inversion of the *hsd* loci may lead to double-strand breaks in the mycoplasmal chromosome that initiate DNA rearrangements associated with repair pathways.

As mentioned above, R-M systems may present effective barriers to gene transfer. An appealing approach to overcoming such a difficulty has been described by Voelker and Dybvig (464), who studied conjugal transfer of Tn*916* from an enterococcal donor to *M. arthritidis*. The *M. arthritidis* strains carried an *Alu*I-like restriction enzyme that would digest the foreign DNA that carries the unmethylated AGCT recognition site. Since Tn*916* carries 68 unmethylated AGCT sequences, it failed to transfer into these strains. The difficulty was overcome by modifying in vitro the DNA of the plasmid carrying the Tn*916* by using the appropriate *Alu*I site-specific methylase.

CHROMOSOMAL REARRANGEMENTS

The mollicute chromosome is a genetically dynamic structure that undergoes frequent rearrangements, insertions, deletions, and inversions of genes or entire genomic segments. The mollicute genome may carry repetitive elements of various types, such as IS-like sequences, integrated viral or plasmid genomic segments, or repetitive elements of endogenous origin, consisting of segments of mycoplasmal genes. These are the elements which facilitate, through homologous recombination, chromosomal rearrangements, as well as loss of genomic material by deletion of intervening sequences during recombination, a process presumably taking place during the reductive evolution of mollicutes. The various types and mechanisms of chromosomal gene rearrangements in mycoplasmas have been thoroughly reviewed by Dybvig and Voelker (114). Chromosomal rearrangements that play a crucial role in antigenic variation are discussed in "Antigenic variation" (below).

Repetitive Genomic Sequences

Structural gene fragments. Large portions of the coding regions of the *M. pneumoniae* P1 operon exist as multiple extragenic copies over the chromosome, named RepMP2/3 and RepMP4, originating in the P1 gene, and RepMP5, originating in the ORF6 gene of the P1 operon. Relics of these sequences detected in *M. genitalium*, named MgPa repeats, revealed strong sequence similarities to the repetitive DNA sequences from *M. pneumoniae* (182). RepMP1 is a repetitive DNA sequence not related to the P1 operon and not identified in *M. genitalium*. RepMP1 sequences are translated, since several proteins were identified in *M. pneumoniae* with an antibody directed against the conserved part of RepMP1 (182). Forsyth and Geary (135) found that the 300-bp RepMP1 element forms a core flanked by short repeated sequences of 56, 71, and 80 nucleotides arranged in various combinations, forming a mosaic pattern. These variable sequences do not exist independently of the core RepMP1 element. Whether these newly discovered elements have anything to do with recombination is a moot point. There is no indication that any of the repetitive elements of the mosaic exist as mobile elements.

Perhaps the most striking example of chromosomal rearrangements on a large scale, associated with the above repetitive sequences, is that disclosed on comparison of the *M. genitalium* and *M. pneumoniae* genomes (182). The genome of both mycoplasmas can be subdivided into six genomic segments. Within these segments the order of genes is conserved, although additional genes were interspaced in the larger *M. pneumoniae* genome, but the order of the six genomic segments was different in the two genomes. Since each of the six genomic segments was bordered by one or more of the repetitive sequences, Himmelreich et al. (182) concluded that reorganization of the genomes took place by translocation of entire segments via homologous recombination between the repetitive DNA sequences, employing the mycoplasmal RecA.

It has been shown that at a lower genomic level, that of the P1 gene itself, clinical isolates of *M. pneumoniae* exhibit some nucleotide sequence variation in the P1 gene and that variable regions in the gene correspond to regions of some of the repetitive elements, suggesting that the P1 gene may vary due to recombination between repetitive regions (436). Accordingly, the *M. pneumoniae* strains were divided into two groups, I and II (436). That this division is also associated with sequence divergence in the ORF6 gene was indicated by Ruland et al. (396a), showing nucleotide sequence variation in the RepMP5 copies residing in the ORF6 gene of the prototype strains of the two groups.

Integrated viral sequences. Sequences of the spiroplasmal virus SpV1 were detected all over the chromosome of every *S. citri* strain examined (32). The results suggest that insertion of the viral sequences occurred by linearization of the circular viral genome between two inverted 31-bp repeat sequences. Integration of the viral sequences into the spiroplasmal chromosome possibly involved a virus-encoded transposase, but no experimental evidence for such a mechanism was reported. The biological meaning of the integrated viral sequences in the cell biology of *S. citri* is not known, although it could be hypothesized that they may play a role in genomic rearrangements in the spiroplasma. Site-specific integration of mycoplasma virus L2 into the host *A. laidlawii* chromosome probably takes place via a site-specific recombinase encoded by the virus during the lysogenation process (276).

Insertion-like elements. Repetition of mobile genetic elements, such as IS elements and transposons, distributed throughout the genome, provides a fertile source of DNA homologies. The fully sequenced *E. coli* K-12 genome (52) has revealed a number of autonomously transposable elements that are implicated in the generation of many spontaneous mutants, not only by insertional inactivation but also by deletions, duplications, and inversions. The presence of such elements in mollicutes may provide an attractive substrate for recombinational deletion of large genomic regions during mycoplasma evolution. Repetitive elements may also contribute to the genetic plasticity associated with the high-frequency antigenic variation common in mollicutes (see "Antigenic variation" below).

Repetitive elements resembling the large IS*3* family of bacterial IS elements appear to be rather prevalent in mollicutes. However, of these, only IS*1138* was shown to actively transpose in *M. pulmonis* (44) while all the other elements, which failed to show transposition within the mycoplasma chromosome, are usually referred to as IS-like elements. IS-like elements have so far been described in *M. hyorhinis* and *M. hyopneumoniae* (173, 493), *M. fermentans* (190), and *M. mycoides* subsp. *mycoides* (140). Interestingly, the high A+T content and the presence of UGA codons in the ORFs of mycoplasmal IS-like elements indicate that the acquisition of the elements by the mycoplasmas was not a recent event (44, 140, 493).

The *M. hyopneumoniae* and *M. hyorhinis* IS-like elements, designated IS*1221*, were the first elements of this kind identified in mollicutes (125) and have been well characterized (493). IS*1221* is present in approximately 16 copies in chromosomes of several *M. hyorhinis* strains and in two copies in *M. hyopneumoniae* J. It is a 1,513-bp element with highly conserved 28-bp imperfect terminal inverted repeats and three distinctive internal inverted repeats. Although the IS*1221* nucleotide sequence reveals two partially overlapping ORFs, as in other members of

the IS*3* family, expected to encode and regulate the production of transposases, in vitro protein expression experiments suggested that IS*1221* produces a truncated product, resulting in low-level production of the full-length transposase. This may account for the failure to show transposition of IS*1221* by Southern hybridization against genomic DNA from many individual *M. hyorhinis* isolates (493). A totally different and much larger repetitive DNA element, with two long direct terminal repeats at each terminus, was found in *M. hyopneumoniae*. It is a unique element, different in structure from bacterial transposons and IS elements, and its biological significance remains unknown (173). Whether this element represents a repetitive sequence of a structural gene, like the P1 repetitive sequences, remains to be seen.

As mentioned above, the *M. pulmonis* IS*1138* is the only mycoplasmal IS element shown to transpose in the *M. pulmonis* chromosome at high frequency. The predicted amino acids encoded by the major IS*1138* ORF have significant similarity to the transposases of the IS*3* family, suggesting that IS*1138* has a potential for development into a cloning vector and/or mutagenesis vehicle in *M. pulmonis*. It should be emphasized that IS*1138* is species specific to *M. pulmonis* but is ubiquitous among the strains of this species (44).

CELL MEMBRANE

Lacking a cell wall and intracytoplasmic membranes, the mollicutes have only one type of membrane, the plasma membrane. The ease with which this membrane can be isolated and the ability to introduce controlled alterations in its composition have made mycoplasma membranes effective tools in membrane research (356, 362). A multiauthored treatise devoted to various aspects of mycoplasma membrane research was published in 1993 (395).

Membrane Proteins

Proteins constitute over two-thirds of the mycoplasma membrane mass, with the rest being membrane lipids. Membrane lipoproteins have attracted much attention in recent years, since their abundance in mycoplasma membranes is most remarkable in contrast to the limited number of lipoproteins in membranes of other eubacteria. Furthermore, membrane lipoproteins are among the most dominant antigens in mollicutes, and a majority of the mycoplasma cell surface antigens known to undergo antigenic and/or size variation are lipoproteins (see "Antigenic variation" below). Cultivation of mollicutes in the presence of labeled palmitate or myristate labels a significant number of membrane proteins (75, 126, 132, 323, 453).

Based on characteristic lipoprotein-specific features, Himmelreich et al. (181) enumerated 46 lipoprotein genes in the *M. pneumoniae* genome, while 21 putative lipoprotein genes were found in the *M. genitalium* genome (139, 181). The unusually large number of lipoproteins in mollicutes may be attributed to the absence of a periplasmic space in the wall-less mollicutes. Mollicutes possess typical eubacterial signal peptides that direct the newly synthesized proteins into a secretory pathway for transport across the cell membrane (487). For surface-exposed membrane proteins which have to function on the outside of the cell, acylation of the proteins with long-chain fatty acids is an effective way to anchor the proteins to the cell surface.

The classification of *M. pneumoniae* ORFs as genes coding for lipoproteins is based on detection of a signal peptide sequence preceding a cysteine residue (181). The cysteine is modified by the transfer of a diacylglycerol moiety from glycerophospholipid to the cysteine sulfhydryl group. The precursor form with the modified cysteine, the prolipoprotein, is cleaved in front of the modified cysteine by a specific signal peptidase II, so that cysteine becomes the first amino acid of the processed protein. It is still unclear whether all of the lipoprotein genes are expressed, since in vivo labeling of *M. pneumoniae* with [^{14}C]palmitic acid followed by SDS-PAGE analysis revealed between 20 and 25 labeled proteins only, compared with the 46 predicted genes (181, 182). This discrepancy could be due to regulated expression of the lipoprotein genes or to weak labeling with palmitic acid, in case some other fatty acids are required for acylation (181, 182).

The processing of a prolipoprotein to a mature lipoprotein in *E. coli* requires the enzymes prolipoprotein diacylglycerol transferase, prolipoprotein signal peptidase, and apolipoprotein transacylase. Himmelreich et al. (181) could identify in the *M. pneumoniae* genome only the transferase, which catalyzes the thioester linkage between diacylglycerol (DAG) and cysteine; the transacylase responsible for the acylation of the amino group of the cysteine could not be identified in *M. pneumoniae* or in *M. genitalium*. Therefore, it is still an open question whether in these mycoplasmas a third fatty acid is linked to the cysteine residue by an amide bond, as was found for the lipoproteins of *E. coli* (181, 182). In fact, some of the *A. laidlawii* lipoproteins carry only one acyl chain and others carry two acyl chains, both attached to the apoprotein by ester bonds (323). Release of all labeled palmitate by alkaline hydroxylamine treatment of *M. hyorhinis* and *M. fermentans* lipoproteins suggested linkage through the more labile thioester or *o*-ester bonds (59, 300). The cleavage of signal peptides in bacterial prolipoproteins, an essential step enabling the attachment of the amide-linked fatty acid to the N-terminal cysteine, is inhibited by globomycin, a cyclic lipopeptide specifically inhibiting signal peptidase II. High globomycin concentrations did not affect the extent of acylation of most *A. laidlawii* acylated proteins, suggesting the absence of amide-linked acyl chains in this mollicute (323). On the other hand, globomycin inhibited the growth of *Spiroplasma melliferum* (40). Evidence for amide-linked fatty acids has been reported for membrane lipoproteins of *M. gallisepticum* (204a) and *M. mycoides* subsp. *mycoides* (204b), suggesting that the acylation mechanism of membrane proteins, at least in some mycoplasmas, resembles that depicted for lipoproteins of gram-negative bacteria (204a).

Spiralin, a major membrane protein of spiroplasmas, has been extensively studied. Its gene was among the first mycoplasmal genes to be cloned, sequenced, and expressed in *E. coli* (76). Based on secondary-structure predictions, a 20-residue amphipathic α-helix localized within the last one-third of the amino acid sequence has been proposed as the putative transmembrane segment of the protein (76). It was suggested that several spiralin molecules assemble in the membrane to form a homo-oligomer with the hydrophobic faces of the α-helices facing the lipid bilayer and the hydrophilic domains turning toward the inside, defining a channel-like space or pore, possibly endowed with transport properties (56). This concept of spiralin disposition in the membrane now has to be reconsidered in light of recent findings that spiralin is acylated (54, 132). Accordingly, spiralin does not span the lipid bilayer but is anchored to the outer face of the bilayer through its acyl chains. The α-helix mentioned above is related to globular protein helices and contains a B-cell epitope, so that it does not act in the capacity of a membrane-spanning domain (61). Mycoplasmal membrane proteins associated with surface antigenic variation, adhesion to host cells, and transport are discussed in the relevant sections.

Membrane Lipids

Virtually all mycoplasma lipids are located in the cell membrane and, as in other biological membranes, consist of phospholipids, glycolipids, and neutral lipids. A detailed description of the composition, distribution, and biosynthesis of mycoplasmal membrane lipids can be found in previous reviews (342, 356, 395). As mentioned in "Genome sequencing and the minimal cell concept" (above), the mycoplasmas are partially or totally incapable of fatty acid synthesis and depend on the host or the culture medium for their supply. In addition, most mycoplasmas require cholesterol for growth, a unique requirement among procaryotes. The fatty acid residues of membrane phospholipids and glycolipids, as well as cholesterol, constitute a major portion of the hydrophobic core of the membrane. The dependence of mycoplasmas on the exogenous supply of fatty acids and cholesterol has been one of their greatest advantages as models for membrane studies. The ability to introduce controlled alterations in the mycoplasma membrane lipid composition simply by controlling the composition and content of fatty acids and sterols in the growth medium has been used most effectively in elucidating the molecular organization and function of the lipids in mycoplasma membranes (reviewed in references 285, 362, and 376). The present review updates the data on mycoplasma lipid composition, emphasizing the more recent studies on lipid synthesis, and its relationship to regulation of the physical state of the membrane and its effect on cell growth.

Almost all recent studies concerning mycoplasma lipid biosynthesis and its regulation were carried out on *A. laidlawii*, an organism which has been used for a long time as a model organism, particularly in studies dealing with the physical state of the membrane (356, 362). It is the organism used by Wieslander, Rilfors, Lindblom and their associates at Umeå University to show that altering the polar head group structure of membrane phospho- and glycolipids and changing the acyl chain structure are the two strategies used by the organisms to adapt their membrane lipid composition to various environmental and physiological conditions (9). A common method by which procaryotes respond to varying growth temperatures is to regulate the degree of unsaturation of the acyl chains of the lipids. *A. laidlawii* cannot synthesize unsaturated fatty acids, and it synthesizes saturated fatty acids in very limited amounts, when the organism is grown in a thoroughly lipid-depleted medium. Thus, at least one exogenous fatty acid must be supplied in the growth medium for the cells to grow.

A. laidlawii exploits a different mechanism to cope with changes in environmental conditions, namely, altering the proportion of lipids with different polar head groups. It has been hypothesized and experimentally supported by the Umeå group (for a review, see reference 376) that *A. laidlawii* A-EF22 regulates its membrane lipid composition to maintain a proper balance between lipids forming a lamellar crystalline phase and lipids forming reversed nonlamellar phases. The first acholeplasmal lipid shown to form a nonlamellar phase was monoglucosyl diacylglycerol (MGlcDAG). The other major glycolipid, diglucosyl diacylglycerol (DGlcDAG), resembles the acholeplasmal phospholipids in forming solely the lamellar phase at all temperatures and with all acyl chain compositions. It was then found that the acholeplasma is capable of varying the proportion of MGlcDAG and DGlcDAG in response to the prevailing growth conditions (376). More recently, two additional glycolipids have been identified in *A. laidlawii* A-EF22 that have the ability to induce the formation of reversed nonlamellar phases. The lipids are monoacyldiglucosyldiacylglycerol (MADGlcDAG) and monoacylmonoglucosyldiacylglycerol (MAMGlcDAG). Thus, the regulation of the balance between lamella- and non-lamella-forming lipids now appears more complex and sophisticated than before, since four non-lamella-forming lipids (including DAG synthesized by the acholeplasma) act in concert to maintain the right balance between the lamellar and nonlamellar lipid components of the membrane in response to varying growth conditions (9).

The acholeplasma membrane lipid composition is also strongly affected by the acyl chain length and degree of unsaturation. *A. laidlawii* can grow with exogenously supplied fatty acids varying considerably in chain length (474). The metabolic responses to changes in the acyl chain length could be readily explained as a regulatory mechanism based on the established phase equilibria of the individual lipids in the *A. laidlawii* membrane. MGlcDAG was the dominating lipid with short acyl chains, but the fraction of this lipid decreased with increasing average acyl chain length, correlating with the decreasing lamellar to nonlamellar phase transition temperatures for this lipid. The fractions of DGlcDAG and phosphatidylglycerol (PG), forming lamellar phases only, increased with increasing average acyl chain length. A weaker correlation was observed between the relative amount of the lipid and the extent of chain unsaturation; however, the fractions of DGlcDAG and PG increased significantly with increasing degree of chain unsaturation. Moreover, the synthesis of the non-bilayer-forming lipids, MGlcDAG and DAG, was strongly stimulated by a high degree of chain saturation. Concomitantly, the phase equilibria of MGlcDAC are shifted towards the lamellar phase at the growth temperature. The fraction of the three potential non-bilayer-forming lipids could vary between 10 and 80% of the total membrane lipids as a function of acyl chain composition (474). Interestingly, the size of the *A. laidlawii* cells was found to change in a systematic manner and correlated quantitatively with the packing properties of the lipids. The cell diameter increased with increases in saturation and acyl chain length, a finding confirming our earlier observation published in 1966 (366).

It has been long anticipated that the physical properties of the mycoplasma membrane lipid bilayer are governed by the concerted actions of the lipid-synthesizing enzymes. Studies to elucidate the biosynthetic pathways of the acholeplasma membrane polar lipids have been initiated only recently. It appears that MGlcDAG is synthesized by glucosylation of DAG as follows: DAG + UDP-Glc→MGlcDAG + UDP. This reaction is catalyzed by a membrane-bound enzyme 1,2-diacylglycerol-3-glucosyltransferase (MGlcDAG synthase), purified and characterized by Karlsson et al. (214). MGlcDAG is the first glucolipid in the glucolipids biosynthetic pathway, and is further glucosylated by another glucosyltransferase to give DGlcDAG (98). Acylated variants of the two major glucolipids are also synthesized under some conditions. The DAG is synthesized from phosphatidic acid by the enzyme phosphatidic acid phosphatase. Phosphatidic acid is also the precursor of the separate and competing pathway leading to the major anionic PG. In *A. laidlawii*, these lipid syntheses are regulated in response to external and internal factors influencing the physical properties of the lipid bilayer, to maintain phase equilibria close to a potential bilayer/nonbilayer transition, a nearly constant radius of spontaneous curvature, and a constant anionic surface charge for the membrane lipid bilayer (98, 214). Regulation of the packing properties is accomplished mainly by altering the relative proportions of MGlcDAG and DGlcDAG, forming essentially nonlamellar and lamellar phases, respectively. In vivo, conditions promoting nonlamellar properties of the bilayer, like higher growth temperature or unsaturated acyl chains, result in an increase in DGlcDAG and a decrease in

MGlcDAG (474). In vitro, the activity of the two glucosyltransferases is critically dependent upon the lipid environment. The best enzyme activator appears to be PG, a major endogenous anionic phospholipid of *A. laidlawii* (98). This dependency of the enzyme is most probably involved in keeping the rate balance between the PG and glucolipid pathways, maintaining a constant surface charge density of the membrane. The lamellar/nonlamellar balance is more likely to be regulated by the consecutive synthesis of DGlcDAG, since the activity of this step is strongly influenced by the presence of molecules increasing the bilayer chain order, like certain sterols (215).

In conclusion, the extensive studies of the Umeå laboratory show that several basic features of the membrane homeostasis mechanism in *A. laidlawii*, including the maintenance of phase equilibria, spontaneous curvature, and surface charge density of the membrane lipid bilayer, are dependent upon the physical properties of the bilayer and are sensed by the two consecutively acting glucosyltransferases synthesizing the major lipids MGlcDAG and DGlcDAG. While the fine details of the molecular mechanisms regulating the synthesis of the two glucolipids have to be worked out, the experiments with the purified MGlcDAG synthase suggest that enzyme regulation may depend on the enzyme proteins themselves and may not require a complex multiprotein regulatory mechanism (98, 214).

While essentially all recent work on mycoplasmal membrane lipid biosynthesis described above dealt with *A. laidlawii* lipids, the *M. genitalium* and *M. pneumoniae* genome sequencing projects have provided some genetic information on the enzymes involved in the biosynthesis of phospholipids and glycolipids in these mycoplasmas. The results were somewhat disappointing, since of the about 10 genes predicted for the biosynthesis of the various *M. pneumoniae* phospholipids and glycolipids reported by Plackett et al. (338), only 3 could be identified by homology search (181). It is possible that the other genes belong to the category of mycoplasmal specific genes unidentified as yet. As expected, no genes involved in fatty acid and cholesterol biosynthesis could be detected in the genomes of these two mycoplasmas.

Membrane Fusion

Due to the lack of a cell wall, the mycoplasma cell membrane is exposed to the external environment. This can be expected to facilitate direct contact of the mycoplasma membrane with that of its eucaryotic host, creating a condition which, in principle, could lead to fusion of the two membranes, enabling the transfer or exchange of membrane components (356, 358, 368). The entry of *M. penetrans* into cells and the intracellular location of some mycoplasmas in nonphagocytic cells (see "Ecology and habitats" above) may also be taken to support membrane fusion. While solid evidence for the occurrence of these events, as part of the interaction of mycoplasmas with their host cells, is still lacking, the topic of fusion has continued to attract attention, particularly as related to the possible potential of membrane lipids to act as fusogenic agents. Cholesterol, a major lipid component of the cholesterol-requiring mollicutes, is the first membrane lipid to be associated with fusogenic properties. A positive correlation was found between the cholesterol content of the mycoplasmal membrane and fusion of Sendai virus with mycoplasma cells (79) and for the formation of intergeneric hybrids of various *Mycoplasma* and *Spiroplasma* species (444). Fusion was much less pronounced with *A. laidlawii* cells, whose membrane contains relatively small amounts of cholesterol. The requirement of cholesterol for fusion could also be demonstrated with *M. capricolum* cells containing variable amounts of cholesterol in their membrane, or with small unilamellar lipid vesicles in which the cholesterol content was manipulated (445). In all these cases, fusion was induced by PEG 8000 and the extent of fusion was monitored by following the dequenching of the fluorescent label octadecylrhodamine B chloride, incorporated into donor cell membranes after their incubation with recipient cells. An increase in the fluorescence observed is interpreted as a result of dilution of the probe in the unlabeled recipient membrane, due to the mixing of the lipids of the donor and recipient membranes. Why does cholesterol promote fusion? Tarshis et al. (445) argue that the increase in membrane viscosity caused by the high cholesterol content of the membrane cannot explain the fusogenic activity of cholesterol, since increasing the membrane viscosity by raising the saturated-to-unsaturated-fatty-acid ratio of membrane lipids decreased rather than increased fusion activity. They suggest that a high cholesterol level promotes fusion by decreasing the density of membrane surface charges of the membrane acidic phospholipids PG and di-PG. If this thesis is correct, why cannot Mg^{2+}, required for fusion apparently by neutralizing the repulsive forces between the negatively-charged head groups of the phospholipids, replace cholesterol? On the whole, one would expect membrane fusion to be a complex process, depending upon or influenced by many other factors as well as the type of membrane lipids. Thus, membrane proteins do certainly play a role in fusion. Treating the recipient cells with 0.1% glutaraldehyde or with proteolytic enzymes inhibited fusion completely (444), but the nature of membrane protein participation in the fusion process is still unknown.

A unique glycoglycerolipid containing phosphocholine, recently identified in *M. fermentans* (38, 104, 282, 489), exhibits fusogenic properties in addition to serving as an important surface immunogen, inducing the secretion of tumor necrosis factor alpha (TNF-α) by human monocytes. This complex lipid was found to markedly enhance the fusion of small unilamellar lipid vesicles with $CD4^+$ (MOLT 3) lymphocytes. Consequently, it has been proposed that this lipid serves as a natural fusogen in the membrane of the AIDS-associated *M. fermentans* cells, facilitating their fusion with host cells (104). In fact, *M. fermentans* incognitus was found to be capable of fusing with T-cell lines, as well as with peripheral blood lymphocytes. It has been suggested that the possible role of *M. fermentans* in the pathogenesis of AIDS may be attributed, at least in part, to the delivery of mycoplasmal cell components into the lymphocytes upon mycoplasma-lymphocyte fusion (137).

While the study of mollicute fusion with eucaryotic host cells may be relevant to the understanding of mycoplasma-host cell interactions and mycoplasma pathogenicity (see "Damage to host cell membrane" below), fusion of mycoplasmas with lipid vesicles entrapping DNA may be applied to the introduction of foreign DNA into the cells and in this capacity may serve as another method for mollicute transformation (see "Gene transfer" above). Whether this approach is more effective than the conventional ones (e.g., electroporation) is still questionable (151). Even more questionable is the value of the proposal to produce cell hybrids of different mollicute species by fusion (444). Even when polyploid heterozygotes with chimeric properties are produced by intergenic mycoplasma fusion, they may simply represent bizarre artifacts. It can be predicted that such hybrid cells, carrying entire genomes of the parental organisms, will not be able to grow and replicate, since the complex species-specific regulatory systems in the hybrid could be expected to be incompatible.

METABOLISM AND TRANSPORT

The extensive studies on the metabolism of mollicutes have been recently reviewed by Pollack et al. (342), summarizing the information in the form of large metabolic maps, linking over 130 enzymatic activities detected in the cytoplasmic fraction of the mollicutes tested so far. Metabolic activities serve as valuable phenotypic markers in *Mollicutes* taxonomy and should be taken into account when considering the phylogeny of this large group of organisms (see "Taxonomy and phylogeny" below).

Energy-Yielding Pathways

The small genome of mollicutes precludes their possession of an extensive range of metabolic activities present in other bacterial groups. Demonstrated metabolic activities appear to be associated primarily with energy generation rather than with the provision of substrates for synthetic pathways. As mentioned above (see "Genome sequencing and the minimal cell concept"), all the mollicutes examined so far have truncated respiratory systems. They lack a complete tricarboxylic acid cycle and have no quinones and cytochromes, ruling out oxidative phosphorylation as an ATP-generating mechanism (references 291, 342, and 356 and references therein). Thus, the demonstrated energy-yielding pathways of mollicutes produce low ATP yields and relatively large quantities of metabolic end products, in some cases depleting host tissues of the specific substrate metabolized.

Based on their ability to metabolize carbohydrates, the mollicutes are divided into fermentative and nonfermentative organisms. Members of the fermentative group produce acids from carbohydrates, decreasing the pH of the growth medium. The sequencing projects of the *M. genitalium* and the *M. pneumoniae* genomes (139, 181) showed that these mycoplasmas carried all the enzymes of the Embden-Meyerhof-Parnas pathway, while the second pathway for metabolizing glucose, the pentose phosphate pathway, is incomplete. Pyruvate generated by glycolysis can be further metabolized either to lactate by lactate dehydrogenase or to acetyl coenzyme A (acetyl-CoA) by the pyruvate dehydrogenase pathway (139, 181).

Most of the nonfermentative mollicutes and some fermentative species possess the arginine dihydrolase pathway. Arginine hydrolysis by this pathway results in the production of ornithine, ATP, CO_2, and ammonia, raising the pH of the culture medium (356). The pathway uses three enzymes: arginine deiminase, ornithine carbamoyl transferase, and carbamate kinase (396). The degradation of arginine is coupled to equimolar generation of ATP by substrate-level phosphorylation. The arginine dihydrolase pathway is found in a variety of phylogenetic bacterial groups, e.g., *Pseudomonas*, *Bacillus*, and lactic acid bacteria (396). The role of this pathway as a sole energy-generating source in nonfermentative mollicutes has been frequently questioned (181, 182, 356). The demonstration of an arginine-ornithine antiport system in *Spiroplasma melliferum* requiring no ATP for arginine import into the cells supports an energetic advantage in arginine utilization (417), but the question whether arginine degradation can serve as a sole or even a major energy-generating mechanism remains unanswered.

The arginine dihydrolase pathway can be found also in some fermentative *Spiroplasma* and *Mycoplasma* species (see Appendix in reference 459). In this case, when both glucose and arginine are present in the medium, the acids produced by glycolysis would mask the alkalinization caused by the ammonia liberated on arginine degradation, hampering the test for arginine utilization by the simple pH change assay. However, as was shown in a ^{13}C nuclear magnetic resonance spectroscopy study, the accumulation of lactate and the breakdown of arginine by *M. fermentans* were observed in the simultaneous presence of both glucose and arginine, suggesting that glucose utilization has little or no effect on deimination of arginine to citrulline (325). Furthermore, proteome analysis of *S. melliferum* showed that the arginine dihydrolase enzyme proteins were also constitutively expressed in the presence of glucose (96). Finding all the genes of the arginine dihydrolase pathway in the *M. pneumoniae* genome (181, 182) was unexpected, since this mycoplasma was believed to have no arginine dihydrolase activity, as tested by the conventional methods (see Appendix in reference 459). It is possible that these genes represent an example of an "orphan" pathway, whose coding elements are present, or even expressed, but at levels of activity too low to be metabolically useful and detectable (340). Pollack (340) suggests that of the three genes constituting the arginine dihydrolase pathway, only the arginine deiminase gene has been definitely annotated, explaining the failure to demonstrate this pathway in *M. pneumoniae*. If so, the arginine deiminase gene falls in the category of "remnant" or "abandoned" genes (340).

Some mycoplasmas, such as *M. agalactiae*, *M. bovigenitalium*, and *M. bovis* (see Appendix in reference 459), metabolize neither sugars nor arginine but are capable of oxidation of organic acids (lactate, pyruvate) to acetate and CO_2 (291, 446). The contribution of the oxidation of these substrates to the energy provision of the above mycoplasmas should be worked out. Pyruvate metabolism genes were identified in some mycoplasmas, being clustered in *M. capricolum* (496) but scattered in the *M. genitalium* genome (139). The clustering of the genes in *M. capricolum* constitutes a unique arrangement of pyruvate metabolism genes, probably facilitating the regulation of gene function. However, the function of the gene cluster as a polycistronic operon has not been experimentally shown (496). Another potential energy-yielding mechanism in mollicutes is based on ATP generation from acetyl phosphate and ADP by acetate kinase, coupled with acetyl phosphate formation from acetyl-CoA by phosphate acetyl transferase; both enzymes are commonly found in fermentative and nonfermentative mollicutes. Acetyl-CoA can be produced by oxidative phosphorylation of pyruvate by mycoplasmas (356). The weight that should be given to this pathway in the energy metabolism of mollicutes has not been critically evaluated.

Energy metabolism of ureaplasmas presents a special case. Neither glycolysis nor arginine dihydrolase-ATP- or acetate kinase-ATP-generating pathways could be detected in these organisms. Ureaplasmas are unique among the mollicutes in possessing a very potent urease. Although protein and gene analysis of the ureaplasmal urease complex has shown that it resembles other procaryotic ureases in subunit structure and composition, the specific activity of the ureaplasmal urease is much higher and was estimated to exceed that of jack bean urease by about 100-fold (50, 316, 428, 452). More importantly, ureaplasmas appear to be unique among procaryotes in requiring urea for growth (356). Thus, the specific urease inhibitor flurofamide inhibits ureaplasma growth (50). The dependence of ureaplasmas on urea for growth has led to the hypothesis that intracellular urea hydrolysis and the resulting intracellular accumulation of ammonia/ammonium ions is coupled to ATP synthesis through a chemiosmotic type of mechanism (356). Experimental support for the generation of a transmembrane potential, with resultant ATP synthesis through the ureaplasmal F_OF_1-type ATPase, first provided by Romano et al. (383) was more recently extended and confirmed by Smith et al. (428). At an external pH of 6.0, the pH optimum for ureaplasma growth in vitro, urea hydrolysis generated an ammonia

chemical potential equivalent to almost 80 mV and, simultaneously, an increase in proton electrochemical potential (Δp) of about 24 mV with resultant de novo synthesis of ATP (428). When the external pH of the growth medium reaches about 8.1, due to ammonia released from the cells, the intracellular pH rises to 8.6 and urease activity ceases (as shown by the pH activity profile of the ureaplasmal urease). ATP generation is blocked at this alkaline pH, leading to the abrupt and steep decline of growth characterizing the ureaplasmal growth curve (356, 428). Inhibition of the urease by flurofamide abolished both the chemical potential and the increase of Δp, such that ATP synthesis decreased to ~5% of the normally obtained levels (428). Lansoprazole, another specific urease inhibitor, also inhibited ATP synthesis and ureaplasma growth (320).

It is worth mentioning at this point that the pH of the urogenital tract is usually on the acidic side of neutrality, corresponding to the pH values optimal for ureaplasma growth, maximum increase in Δp, maximum ammonia chemical potential, maximum urease activity, and maximum ATP generation.

Mycoplasmal ATPase. Resembling other eubacteria, mollicutes possess an F-type ATPase (356). The number and order of genes of the *atp* operon coding for the F_OF_1-ATPase of *M. gallisepticum* (351), *M. genitalium* (139), and *M. pneumoniae* (180) are identical to those of *E. coli* and *B. subtilis*. However, the b subunit (atpF) of the mycoplasmal ATPase was found to carry the characteristic features of a lipoprotein. This feature, not known for any other eubacterial ATPase, may explain the well-known inability to detach the mycoplasmal ATPase activity from the membrane by varying the osmolarity of the medium or by treating the membrane with EDTA (356).

Annotated Genes Correlated with Metabolic Activities

Annotation of genes associated with mycoplasma metabolism, an outcome of the recent mycoplasma genome projects, has yielded several unexpected results, raising key questions concerning the correlation of gene annotation based on ORF sequences and actual enzymatic activities demonstrated experimentally. Comparison of the genomic data of *M. genitalium* with its metabolic activities has revealed that several predicted enzymatic activities based on ORF sequences could not be experimentally detected while other enzymatic activities demonstrated experimentally could not be assigned to any primary gene sequence (340). The failure to demonstrate an enzymatic activity that was putatively assigned to a gene sequence may be due to an inactivating mutation, inappropriate or insensitive experimental technique, or simply the lack of expression of this gene at the time of cell harvest. The lack of correlation of genetic and metabolic data may also be explained by incorrect gene assignment or may occur because the assigned sequence was not translated or because posttranslational modification rendered the translated protein inactive. Pollack (340) concludes that appraisal of metabolic capabilities based only upon putative gene assignments might be erroneous, emphasizing the need for genomic sequencing analysis to be correlated with enzymatic data.

Pollack (340) goes further by distinguishing several categories of enzymatic activities. "Orphan enzymes" are activities for which the corresponding genes were not identified. In *M. genitalium* these include malate dehydrogenase, phosphoenolpyruvate carboxylase, aspartate aminotransferase, and 5′-nucleotidase. The failure to detect, in both *M. pneumoniae* and *M. genitalium*, the nucleoside diphosphate kinase (*ndk*) gene, encoding the key enzyme responsible for conversion of nucleotide diphosphates to nucleotide triphosphates (181, 182), is another striking example. There is little doubt that such a gene must be present; can its structure be so different from those of the homologous genes of other procaryotes? On the other hand, the gene for 6-phosphogluconate dehydrogenase was tentatively identified in the *M. genitalium* genome, but no such activity could be detected, leading Pollack (340) to classify such genes as "abandoned" or "remnant" genes, that in the past may have played a metabolic role but had not yet been eliminated from the genome. Another interesting case is that of malate dehydrogenase (MDH). This activity is found in *M. genitalium* and in many other mollicutes. However, no ORF could be found for MDH in the *M. genitalium* and *M. pneumoniae* genomes (139, 181). Cordwell et al. (95) hypothesized that the product of the gene putatively assigned to lactate dehydrogenase (LDH) in *M. genitalium* and *M. hyopneumoniae* fulfills both LDH and MDH functions, basing this on the finding of a common unique motif: arginine-proline-glutamine in the sequence of the active site of the mycoplasmal LDH, a motif that reduces the specificity of the active site, allowing the binding of both pyruvate and oxalacetate. In contrast, *S. melliferum*, with a genome about three times as large as that of *M. genitalium*, was shown to possess two unrelated gene products for MDH and LDH (96). While multienzyme proteins may represent one of the solutions for gene economization in organisms with minimal genomes, its contribution to genomic economization in mycoplasmas appears very marginal, since examples for multienzyme proteins are also known for bacteria with no apparent constraint in genome size (340).

Transport

The expectation to find in *M. genitalium* and *M. pneumoniae* a proportionally large number of genes involved in transport of the many essential nutrients required for growth has not been substantiated (139, 181) (see "Genome sequencing and the minimal cell concept" above). This has led to the notion that at least some of the transport systems, such as those for amino acids, might not be very specific, an assumption which still requires experimental verification.

Three types of transport systems were found in *M. genitalium* and *M. pneumoniae* (139, 181). (i) The first is ABC transporter systems, consisting of two ATP binding domains, two membrane spanning- and one substrate-binding domain. These different domains are frequently present on separate polypeptides. (ii) The second type consists of PTS systems for transporting sugars, resembling homologous systems of gram-positive bacteria. (iii) The third is facilitated diffusion by transmembrane proteins functioning as specific carriers (not defined so far). Surprisingly, a transport system for the essential nucleic acids precursors, purines and pyrimidines, or for nucleoside 5′-monophosphates was not identified in *M. pneumoniae* (181).

ABC systems. The ABC transporter systems are involved in import or export of a large variety of substrates, including sugars, peptides, proteins, and toxins. In fact, the ABC transporters were the most frequent class of proteins found in *B. subtilis* (238a) and in *E. coli* (52). Generally, bacterial ABC importers have an ATP binding domain and membrane-spanning domains located on separate polypeptides whereas the ABC exporters may carry these components on the same or on different polypeptides (51). The first to show homology of mollicute proteins with ABC transporter systems were Dudler et al. (110) studying *M. hyorhinis*. Putative ABC transporter genes were also reported in *M. hyopneumoniae* (51). The deduced amino acid sequence showed significant homology to the ABC transporter proteins, particularly those of the eucary-

otic multidrug resistance protein family, a finding previously reported for other bacteria (317).

Himmelreich et al. (181) reports the presence in *M. pneumoniae* of an ABC transport system for oligopeptides consisting of two different transmembrane and ATP binding domains. Striking is the absence from the mycoplasma of the substrate binding domain (OppA) present in *B. subtilis*. It remains to be experimentally determined whether the substrate binding protein is dispensable in *M. pneumoniae* and *M. genitalium* or is part of the transmembrane or of the ATP binding proteins. Saurin and Dassa (403) suggest that the apparent absence of binding proteins seems to be a general property of the class *Mollicutes*. However, it is hard to conceive the total absence of substrate binding proteins in mycoplasmas. To deal with this dilemma, Saurin and Dassa (403) put forward the thesis that the mycoplasmal binding proteins diverged so much from the homologous proteins in other organisms that their identification by standard sequence analysis becomes extremely difficult. The high divergence might be the result of extensive sequence changes determining a broader substrate binding specificity, in line with the need to compensate for the loss of specific transporters during genome compaction. In fact, only one amino acid transporter has been identified in *M. genitalium*; no transporters specific for other classes of amino acids were found (403). Himmelreich et al. (181) emphasize that all statements concerning substrate specificities should be taken with caution, because high scores are obtained in similarity searches with many different substrate specificities. Thus, all substrate specificity annotations must be verified experimentally. The recent characterization of an ABC transporter four-gene operon in *M. fermentans* (451) sheds some light on this problem. One of the four gene products, a surface-exposed P78 lipoprotein, presumably serves in substrate binding capacity. Interestingly, this lipoprotein is subject to phase variation (see "Antigenic variation" below), probably conferring alternative substrate specificity and transport function, in line with gene saving in mollicutes.

PTS systems. The highly efficient phosphoenolpyruvate-dependent sugar phosphotransferase transport systems (PTS) were identified in mollicutes long ago, and some of their components were partially characterized by classical biochemical methods (78, 356). The recent molecular genetic approach has enabled a more precise and detailed description of the genes and protein components of these systems. While the *M. genitalium* genome carries the genes for a PTS specific for glucose (139), the closely related *M. pneumoniae* was found to carry two additional PTS systems, one with an apparent specificity for mannitol and another with an unknown specificity (181, 182). Fructose also appears to be imported by the PTS system of *M. pneumoniae*, although Himmelreich et al. (181) failed to detect in the mycoplasma the *fruF* gene which is part of the fructose operon in enteric bacteria.

The PTS glucose transport system of *M. capricolum* revealed a unique arrangement of the *pts* operon (497, 498), later found also to be true for *M. genitalium* (139) and *M. pneumoniae* (181). While in all other bacterial species examined thus far, the *ptsH* (HPr) gene is located immediately upstream to the gene *ptsI*, encoding enzyme I, the mycoplasmal *ptsH* gene is part of another operon. This finding is reminiscent of the unusual location of the *gyrB* gene in the *M. capricolum* genome, far away from its usual location at the origin of replication (293), an apparent outcome of the extensive genomic rearrangements so common in mollicutes (see "Chromosomal rearrangements" above). In sequence, the mycoplasmal HPr more closely resembled the HPrs of gram-positive bacteria than those of gram-negative bacteria and could not serve as a phosphoacceptor for the *E. coli* enzyme I. Nevertheless, the *M. capricolum* enzyme IIA^{Glu} could accept and transfer phosphate from both the *E. coli* and *B. subtilis* PTS components (497, 498). The probable regulator of sugar transport systems, Hpr (Ser) kinase, was found in extracts of *M. capricolum* and *M. genitalium* (495), in this respect resembling typical gram-positive bacteria.

It has been known for a long time (78) that *Acholeplasma* species lack a functional PTS. The reason was recently found by Hoischen et al. (184), who showed that *A. laidlawii* possesses the activities of enzyme I, HPr, HPr (Ser) kinase, and HPr (Ser-P) phosphatase but lacks detectable activities of enzymes II. Thus, the absence of PTS activity from *A. laidlawii* reflects an incomplete PTS, providing another example of a rudimentary or orphan system common in mollicutes (see above). Nonetheless, Hoischen et al. (184) suggested that components of the incomplete PTS of *A. laidlawii* have a potential to function in a unique regulatory capacity associated with the phosphorylation of an as yet an undefined target protein(s).

TAXONOMY AND PHYLOGENY

There is a consensus among bacterial taxonomists that the complete sequences of bacterial genomes will form the basis for phylogeny and, ultimately, taxonomy. However, as long as complete genomic sequences are available for a few bacteria only, current bacterial taxonomy, including that of mollicutes (198), relies on the combination of phenotypic characteristics and phylogenetic data based on partial genomic sequences, mostly those of the conserved rRNA genes. Detailed descriptions and evaluations of current molecular methods in the diagnostics, taxonomy, and phylogeny of mollicutes can be found in reference 361 and in the two volumes of *Molecular and Diagnostic Procedures in Mycoplasmology* (370, 459).

Molecular Tools in Taxonomy and Phylogeny

New definition of the species concept. While the availability of complete genomic sequences is expected to provide a sound basis for establishing phylogenetic relatedness among bacterial species and consequently to enable the construction of taxonomic entities based on phylogeny, the way to achieve this has not yet been worked out, since it presents several key problems. Thus, the current, somewhat arbitrary definition of the basic taxonomic entity, that of a bacterial species, includes all strains with approximately 70% or higher DNA homology and with 5°C or lower ΔT_m (361). There can be little doubt that this definition will have to be modified once comparison of entire genomic sequences replaces the cumbersome and not too accurate determination of genetic homology based on DNA-DNA hybridization tests. However, as long as the number of bacterial genomes sequenced remains small, there is still more than enough time to work out a solution to the above problem. Comparison of the genomic sequences of *M. genitalium* and *M. pneumoniae* (182) has revealed the significant genomic relatedness of these mycoplasmas but also underlines the problems that taxonomists may face when using comparative genomic data to redefine taxonomic entities, including that of a bacterial species.

Another issue concerning the application of complete genomic sequences to taxonomy was raised by Pollack (340). The primary DNA sequence may not be completely determinative with regard to function (see "Metabolism and transport" above). Although function is dependent on the primary sequence, tertiary effects, such as the degree of oligomerization and peptide-peptide interactions, and the relative concentra-

tion of substrates and cofactors also affect the function of the gene product. In addition, the extent of posttranslational modification of metabolic enzymes in procaryotes remains largely undescribed (340, 341). Thus, genotypic characterization of a bacterium may differ from its phenotypic characterization, placing another obstacle in the way of basing taxonomy on a combination of genotypic and phenotypic characteristics.

rRNA sequences. According to 16S rRNA sequences (272, 472), the mollicutes are divided into five phylogenetic units (clades). Acholeplasmas and anaeroplasmas are considered the earliest mollicutes to have evolved by degenerative (reductive) evolution from their gram-positive bacterial ancestors. The spiroplasmas evolved by an early splitting of the acholeplasmal branch, and the mycoplasmas and ureaplasmas are thought to have spiroplasmal ancestors. An important feature of mollicute phylogeny proposed by Woese (479) is the rapid pace of their evolution, in line with the marked genotypic and phenotypic variability characterizing the mollicutes as a group. The great weight given to 16S rDNA sequences in mycoplasma phylogeny, taxonomy, and species identification (335, 361) led the *Mollicutes* Taxonomy Committee (199) to recommend the inclusion of the 16S rDNA sequence in any description of a new mollicute species.

Phytoplasma classification. A most important contribution of 16S rRNA gene sequences to mollicute phylogeny and taxonomy has been the placing of the uncultured phytoplasmas as a distinct monophyletic clade within *Mollicutes*, closely related to the acholeplasmas (258). This conclusion has been supported by the small genome size of phytoplasmas, resembling that of the culturable mollicutes (256, 311), by the finding of only two rRNA operons in the phytoplasmas, and by the presence of a $tRNA^{Ile}$ gene in the spacer region between the 16S and 23S rRNA genes (255, 409, 500), a feature found also in acholeplasmas (306). The deduced amino acid sequences of some of the highly conserved ribosomal protein genes also indicated that the phytoplasmas are more closely related to *A. laidlawii* than to *Mycoplasma* species; like acholeplasmas, they read UGA as a stop codon rather than a tryptophan codon (168, 257, 258, 456). The 16S rRNA gene sequences delineated about 14 distinct subclades within the phytoplasma clade, generally in accord with the phytoplasma strain clusters established by DNA homology, restriction enzyme analyses of 16S rRNA genes, and sequences of the 16S/23S intergenic spacer regions (167, 229, 308, 407, 411, 426). The taxonomic implications of these studies are that the phytoplasmas should be distinguished at the minimal taxonomic level of a genus and that each phytoplasma subclade should represent at least one distinct species (199). However, in the absence of the phenotypic markers used to classify mollicutes, taxonomic affiliations cannot be resolved in the conventional way at present. Thus, a provisional classification of the uncultured phytoplasmas may be introduced by applying the *Candidatus* category proposed for classification of uncultured bacteria (197, 199). So far two *Candidatus* species names for phytoplasmas have been published (101, 500), but more *Candidatus* species descriptions can be expected to appear in the press soon. The phytoplasma case demonstrates well the revolution occurring in bacterial taxonomy, where molecular data suffice to lay the phylogenetic and taxonomic basis for classification of a group of organisms for which very few phenotypic characteristics are available. The molecular approach to phytoplasma classification has recently been applied to uncultured MLOs of vertebrates. Thus, genome size and 16S rRNA analyses of the grey lung agent, a mouse pathogen, have led to the classification of this agent as *Candidatus* Mycoplasma ravipulmonis, based on its phylogenetic relatedness to the hominis group (clade) of mycoplasmas (312). Similarly, the wall-less rickettsia *Eperythrozoon wenyonii*, phylogenetically related to the pneumoniae group of mycoplasmas according to its 16S rRNA sequence, requires reclassification as a *Mycoplasma*, employing the *Candidatus* taxon (313; see "Ecology and habitats" above).

More phylogenetic markers. Although the 16S rRNA sequences have proved to be very effective tools in the phylogeny and taxonomy of mollicutes, it was thought that additional phylogenetic markers are desirable to support the conclusions based on the 16S rRNA data. In fact, such markers have already been applied, including the conserved ribosomal protein genes (168), the elongation factor EF-Tu (*tuf*) gene (211, 408), the heat shock protein gene *hsp70* (121), and the 16S/23SrRNA intergenic sequences (426). Use of these markers has supplemented and complemented the 16S rRNA comparative data. A priori, wobble in the genetic code permits more variations in protein gene sequences, even of highly conserved genes, than is possible in rRNA sequences. Thus, while the ribosomal protein-encoding genes are conserved, they vary in size and primary sequence more than the 16S rRNA genes do. Therefore, the ribosomal protein genes have a greater potential to reveal variations among closely related strains. Gundersen et al. (168) have applied such data to suggest the division to subspecies of some of the phytoplasma subclades representing future *Candidatus* species, a proposal considered premature by the *Mollicutes* Taxonomy Committee (199). Likewise, the intergenic spacer region located between the 16S and 23S rRNA genes, being less subjected to evolutionary constraints than the genes themselves, exhibits greater sequence variation, a property of use in strain differentiation (426).

The highly conserved and ubiquitous elongation factor EF-Tu (encoded by the *tuf* gene) has also proved to be an effective phylogenetic marker. A phylogenetic tree constructed on the basis of the deduced amino acid sequences of this protein placed *M. pneumoniae* closest to *M. genitalium*, while an alternative dendrogram based on 16S rRNA sequences showed *M. genitalium* to be nearest to *M. hominis* (211), a result which disagrees with the recent genome sequencing data, revealing that the 16S rRNA of *M. genitalium* is closest in sequence to that of *M. pneumoniae* (139, 181). The heat shock Hsp70 family of proteins shows, perhaps, the highest degree of sequence conservation and is therefore well suited for examining deep phylogenetic relationships. The recent application of the *hsp70* gene to phylogenetic analysis of mollicutes has considerably strengthened the evidence gained through the 16S rRNA sequence analyses that mollicutes are evolutionary closely related to the gram-positive bacteria (121). The genome-sequencing projects have also provided strong evidence for the phylogenetic relatedness of mollicutes to gram-positive bacteria. Thus, in the majority of cases where *M. genitalium* coding regions matched sequences of both *E. coli* and *Bacillus* species, the better match was to sequences of *Bacillus* (average, 62% similarity) than to sequences of *E. coli* (average, 56% similarity) (139).

Intraspecies genetic heterogeneity. The rather arbitrary and somewhat vague definition of a bacterial species and, even more so, the rather frequent and significant chromosomal rearrangements taking place in mycoplasmas (see "Chromosomal rearrangements" above) are conducive to the most common finding of genotypic heterogeneity of strains within the same species. Intraspecies genotypic heterogeneity, noticeable through serological testing and electrophoretic analysis of cell proteins, has become much easier to distinguish by applying the much more sensitive molecular tools, such as restriction fragment length polymorphism, restriction modification pro-

files, conserved gene sequences, and genomic physical maps (361). These tests have provided epidemiologists with very sensitive tools to distinguish strains of interest in the field (51, 141, 335, 484).

Phenotypic Markers in Taxonomy

Metabolic markers. The present tendency to depend on direct genomic analysis, made available by the dramatic advancements of molecular genetic methods, has put aside further development of classical taxonomic tools based on determination of nutritional requirements and enzymatic activities (361). Even electrophoretic cell protein profiles are now used as taxonomic tools less extensively than they were in the 1970s and early 1980s. The reason is that these profiles reflect the expression of specific genes and are thus liable to change. It is now possible to clone and sequence the protein genes themselves and thus have a more direct comparative measure. However, the recent development of the proteome approach to genomic analysis may revive the use of electrophoretic patterns of cell proteins as a taxonomic and phylogenetic tool (see "Genome sequencing and the minimal cell concept" above).

It should also be pointed out in this context that distinguishing members of the class *Mollicutes* by their metabolic characteristics has been generally of limited phylogenetic and taxonomic usefulness. There are a few metabolic exceptions, including the ability to ferment glucose, the ability to hydrolyze arginine and urea, and dependence on cholesterol for growth and anaerobiosis. Cellular localization of NADH oxidase activity has also been helpful in the classification of mollicutes at and above the genus level. While *Acholeplasma* species resemble other procaryotes in having the NADH oxidase localized in the plasma membrane, this enzymatic activity is located in the cytoplasm of *Mycoplasma*, *Spiroplasma*, and *Ureaplasma* species. The finding of this activity in the cytoplasm of the *Mesoplasma* and *Entomoplasma* species has helped to distinguish these newly described genera from *Acholeplasma* (341, 458).

Serology. At the species level, serologic relatedness has until recently overshadowed all other features used in routine mollicute identification, but the weight given to the determination of molecular properties in mollicute classification and identification is steadily increasing (361). The high rate of surface antigenic variation characterizing mollicutes (see "Antigenic variation" below) may impose some limitations on the use of monoclonal antibodies to surface antigens as tools in mycoplasma identification (390), difficulties usually not encountered when polyclonal antibodies are used (199).

Cholesterol requirement. The weight to be given to cholesterol requirement in mollicute taxonomy provides a good illustration of the conflict that may occur between taxonomy based on phenotypic characteristics and that based on molecular phylogenetic data. Cholesterol requirement has long been considered a major criterion in establishing high taxonomic groupings within the mollicutes (367). Recent findings have weakened the importance of cholesterol requirement in mollicute classification. Thus, the cholesterol-nonrequiring mesoplasmas, considered previously to be acholeplasmas, were shown to possess molecular properties very different from those of the classic acholeplasmas, requiring their taxonomic separation (458). In addition, several *Spiroplasma* species were recently shown to grow in the absence of cholesterol (58, 458). These data suggest that nutritional dependency on exogenous sterols is a trait that has arisen independently several times during the evolution of mollicutes, since it occurs in at least three different phylogenetic groups (456). It thus appears that cholesterol requirement should not be used as a sole definitive criterion in defining higher taxa of the class *Mollicutes* but, rather, should serve as one of a matrix of characters in mollicute classification (58). Consequently, nutritional requirements, although being the most practical traits to assess in routine laboratory tests, may not always be reliable indicators of phylogenetic relationships.

Blending Taxonomy with Phylogeny

While it is generally agreed that basing bacterial taxonomy on phylogeny is both inevitable and advantageous, there is still disagreement among bacterial taxonomists whether the time is ripe for radical changes in bacterial taxonomy (for a detailed discussion, see reference 361). The taxonomic status of the *Mycoplasma mycoides* cluster clearly illustrates the problem. The *Mollicutes* phylogenetic tree based on 16S rDNA sequences has suggested that the *M. mycoides* cluster is phylogenetically related to the genus *Spiroplasma* (472). However, the proposal to reclassify this cluster accordingly has not been adopted by the *Mollicutes* Taxonomy Committee (199), which argues that such a reclassification would create considerable problems in diagnostic veterinary medicine. Likewise, reassignment of all other *Mycoplasma* species to a new genus would have unacceptable consequences in human and veterinary medicine. For the time being, a polyphasic classification based only partly on phylogeny is recommended, striving to fit mollicute taxa into the phylogenetic classification as far as possible (199).

Mollicute evolution. The voluminous data that has already accumulated (described above) has led Maniloff (273, 274) to try and construct a hypothetical scheme for mycoplasma phylogeny. Accordingly, the ancestral mycoplasma arose from the *Streptococcus* phylogenetic branch about 600 million years ago, probably from an organism with a genome of about 2,000 kb. The mycoplasma phylogenetic tree split later into two major branches, about 450 million years ago, probably from an organism with a genome size of 1,700 to 2,000 kb. One branch (the AAA branch) led to the *Asteroleplasma*, *Anaeroplasma*, and *Acholeplasma* branches, and the other (the SEM branch) led to the *Spiroplasma*, *Entomoplasma*, and *Mycoplasma* branches. The phytoplasmas subsequently arose from the *Acholeplasma* branch, and *Ureaplasma* arose from the *Mycoplasma* branch. The *Entomoplasma* branch also contains *Mesoplasma* and the *M. mycoides* sublines. Maniloff suggested that in both major branches genome reductions had occurred independently during their degenerate evolution. Thus, degenerate evolution of the AAA branch after the appearance of flowering plants has led to the phytoplasmas with 600- to 1,200-kb genomes, while phylogeny of the SEM branch produced the *Spiroplasma* branch with 1,000- to 2,000-kb genomes and the *Entomoplasma*, *Mesoplasma*, *Mycoplasma*, and *Ureaplasma* branches with 600- to 1,200-kb genomes. The conversion of UGA from a stop codon to a tryptophan codon occurred apparently during early phylogeny of the SEM branch. Using bacterial rRNA phylogenetic rates to calculate the timing of mycoplasma evolution, Maniloff estimated that the AAA branch arose during the Paleozoic and the SEM branch arose just after the Permian extinction, during the establishment of modern evolutionary flora and fauna (see reference 174 for a discussion of the evolution of the ureaplasmas). Although all of the above should be taken as hypothetical assessments, they emphasize a cardinal point: the mollicutes are relatively late evolutionary products.

VIRULENCE FACTORS

Most mollicutes live as commensals, and in many arthropods they may even be considered symbionts (171). Infections with pathogenic mycoplasmas are rarely of the fulminant type but, rather, follow a chronic course. It could be argued that mycoplasmas are close to the concept of "ideal parasites," usually living in harmony with their host. Extensive descriptions of the diseases caused by mollicutes to humans, animals, plants, and insects can be found in *The Mycoplasmas*, volumes IV (365) and V (473), and in reference 277. A recent, somewhat inciting review discusses the role of mycoplasmas in disease pathogenesis, referring also to the variety of diseases of unknown etiology that have been linked to mycoplasmas (30). These include the possible role of mycoplasmas as cofactors in AIDS pathogenesis, the Gulf War Syndrome, and other diseases of unexplained etiology such as the chronic fatigue syndrome, Crohn's disease, and various arthritides. While the association of mycoplasmas with many of these diseases remains doubtful, the possible role of mycoplasmas in AIDS activation has attracted the most attention during the last decade. The substantial number of studies carried out on the AIDS-associated mycoplasmas, *M. fermentans* and *M. penetrans*, have considerably advanced our knowledge of mycoplasma cell biology and host immune system modulation by mycoplasmas, as evidenced in many sections of this review. However, thus far the proposed role of mycoplasmas in AIDS activation remains controversial and doubtful (30, 49, 62).

The molecular basis of mycoplasma pathogenicity remains largely elusive. The clinical picture of mycoplasma infections in humans and animals is more suggestive of damage due to host immune and inflammatory responses rather than to direct toxic effects by mycoplasmal cell components (see "Interactions with the host immune system" below).

Damage to Host Cell Membranes

Potent toxins have not been associated with mycoplasmas. The mildly toxic by-products of mycoplasma metabolism, such as hydrogen peroxide and superoxide radicals, have been incriminated as causing oxidative damage to host cell membranes (359). The ammonia released from urea hydrolyzed by the potent urease of ureaplasmas may also be considered a virulence factor, probably affecting the tissues immediately adjacent to live ureaplasmas (254). Another pathogenicity factor associated with ureaplasma infection has been proposed by Kim et al. (219). They found a significant decrease in prostaglandin E_2 and F_{2a} production by bovine endometrial cells following infection by the bovine *Ureaplasma diversum*. Prostaglandins are necessary for the implantation and maintenance of pregnancy. The ureaplasmas are able to disturb prostaglandin production probably by their potent phospholipase A_2 activity, as Lamont et al. (246) have shown with amniotic cells exposed to media conditioned by culturing a variety of urogenital bacteria, including ureaplasmas. The ureaplasmal phospholipase could release excessive amounts of arachidonic acid, resulting in substrate inhibition of prostaglandin synthesis, a point which may be relevant to the understanding of the pathogenesis of ureaplasmas in pregnancy (73).

Izutsu et al. (202) reported that *M. orale* infection disrupted the regulation and reduced the numbers of Ca^{2+}-activated K^+ channels and of Cl^- channels in plasma membranes of a human submandibular gland cell line. Such changes in salivary gland cells in vivo could result in diminished salivary gland fluid production, leading to xerostomia. Similarly, if a mycoplasma-associated preferential loss of K^+ channels occurs in tissues, such as ciliated bronchial epithelia (103), the resulting depolarization of the cell membrane could lead to the ciliostasis observed in mycoplasma-infected ciliary cells, a novel idea that deserves further investigation (202).

Clastogenic and Oncogenic Effects

It has been proposed that the intimate contact of the wall-less mycoplasmas with the host cell membrane may result in local, perhaps transient fusion of the two membranes or exchange of membrane components and hence in direct "injection" of the mycoplasma cytoplasmic content, including hydrolytic enzymes, into the host cell cytoplasm (see "Cell membrane" above). Thus, the potent nucleases of mollicutes combined with superoxide radicals may be responsible for clastogenic effects (430). Peripheral blood leukocyte cultures exposed to *M. fermentans* were shown to display a dose-dependent increase in the frequency of sister chromatid exchanges. The addition of antioxidants and free radical scavengers, such as superoxide dismutase and the hydroxyl radical mannitol, had an anticlastogenic effect (430). In fact, reports on chromosomal aberrations, altered morphology, and cell transformation in cell cultures infected by mycoplasmas have appeared rather sporadically since the early 1960s (354). Interest in this subject has been recently rekindled following the claims that *M. penetrans* may be a cofactor in the induction of HIV-associated Kaposi's sarcoma (467). The fundamental question raised is whether mycoplasmas can indeed induce neoplastic growth and cause cancer in infected hosts. Several arguments can be advanced in support of an oncogenic role for mycoplasmas. Mycoplasmas may be the only procaryotes which can "symbiotically" grow in the eucaryotic host and have a close interaction with mammalian cells for long periods. Intimate interactions of the mycoplasmas with the host cell surface may trigger a cascade of signals transduced from the cell membrane to the nuclei, altering the function of many genes. Furthermore, mycoplasmas are known to induce a variety of cytokines, which may effectively mediate a wide range of biological actions on cell proliferation and differentiation (see "Interactions with the host immune system" below). Results of experiments with cultured mouse embryo cells reported by Tsai et al. (457) appear to support an oncogenic potential of the AIDS-associated mycoplasmas *M. fermentans* and *M. penetrans*. However, instead of acute transformation, a multistage process in promotion and progression of malignant cell transformation with long latency was noted. Only after 18 passages (1 week per passage) of persistent infection with mycoplasmas was an irreversible form of transformation, which included the ability to form tumors in mice, achieved. It should be pointed out, however, that the tumors had developed very slowly and not in all mice. The irreversible phase of transformation coincided with permanent karyotypic alterations. Once induced, chromosomal alterations continued to accumulate both in cultured cells and in animals without the continued presence of the transforming mycoplasmas. Hence, the mycoplasma-mediated multistage oncogenesis exhibited many characteristics found in the development of human cancer. It is obviously too early to be able to draw conclusions about whether the findings of Tsai et al. bear any relevance to oncogenesis in humans. The claims of Wang et al. (467) that Kaposi's sarcoma is associated with *M. penetrans* infection were not verified by Grau et al. (165). However, oncogenesis by mycoplasmas is a topic that offers an additional dimension to the pathogenic potential of mycoplasmas and certainly deserves further study.

Another issue that came up recently concerns the possible role of mycoplasma infection on the induction of apoptosis. DNA fragmentation, a common biochemical hallmark of apo-

ptosis, is generally considered to be catalyzed by endogenous endonucleases. In light of the relatively large amounts of nucleases expressed by mycoplasmas (36), Paddenberg et al. (330) tested the effects of *M. hyorhinis* infection of NIH 3T3 cells, checking for parameters characteristic of apoptosis. The infected cells exhibited intranucleosomal DNA degradation into multimers of 200 bp, forming a ladder in agarose gels. Nuclease activities were detected in cell homogenates and culture supernatants. Their mycoplasmal origin was indicated by inhibition of their expression following chloramphenicol treatment of the infected cells. However, to demonstrate the intranucleosomal DNA degradation, the infected cells first had to be treated with cycloheximide, a treatment inducing apoptotic cell death. It has been suggested that the cellular stress caused by cycloheximide enabled the mycoplasmal nucleases to penetrate the cells, leading to the cleavage of the host cell DNA. Thus, mycoplasmal infection by itself does not cause apoptosis, but once the cells have undergone apoptosis, the infecting mycoplasmas may be responsible for the cleavage of the host cell DNA into multimers of 200 bp (330).

Searching for Pathogenicity-Associated Genes

A promising genetic approach, based on transposon Tn*4001* mutagenesis, has recently been applied to studying the pathogenicity factors of *S. citri* (57, 131). One of the Tn*4001* mutants did not multiply in the leafhopper vector and therefore could not be transmitted to the plant. Another mutant multiplied well in the plant but did not induce symptoms. It was found that in this nonpathogenic mutant, Tn*4001* was inserted in the spiroplasmal fructose operon, abolishing the ability of the organism to utilize fructose, probably in this way affecting its pathogenicity. It would be of interest to test if the pathogenicity of the mutant can be restored by complementation with the wild-type genes, like the successful restoration of motility in a Tn*4001 S. citri* mutant by complementation with the wild-type genes (203) (see "Morphology and ultrastructure" above).

The lysogenic bacteriophage MAV1, infecting *M. arthritidis* (465), appears to play an important role in the pathogenicity of this murine mycoplasma. All virulent *M. arthritidis* strains carry MAV1 DNA integrated at various sites of the mycoplasmal chromosome, while avirulent strains lack MAV1. Furthermore, the arthritogenic potential of a low-virulence *M. arthritidis* strain could be considerably enhanced by lysogenization with MAV1 (465). The mechanism by which MAV1 enhances virulence is not known. The fact that several MAV1-negative strains are not completely avirulent suggests that MAV1 integration may activate other virulence factors, such as the superantigen MAM (see "The *M. arthritidis* superantigen MAM" below), or increase the expression of *M. arthritidis* adhesins. However, no experimental data to support these suggestions are available. Additional molecular characterization of MAV1 may help to clarify its mode of action (465).

In summing up this section, the unavoidable conclusion is that despite the various mycoplasmal virulence factors that have been described, there appears to be no clear causal relationship between these factors and mycoplasma pathogenicity, a conclusion justifying the statement that the molecular basis of mycoplasma pathogenicity remains largely elusive.

Adhesion to Host Cells

Most human and animal mycoplasmas adhere tenaciously to the epithelial linings of the respiratory or urogenital tract, rarely invading tissues. Hence, they may be considered surface parasites. Adhesion of mollicutes to host cells is a prerequisite for colonization and for infection. The loss of adhesion capacity by mutation results in a loss of infectivity, and reversion to the cytadhering phenotype is accompanied by regaining infectivity and virulence. The best-defined mycoplasmal adhesins—membrane components responsible for adhesion—are those of *M. pneumoniae* and *M. genitalium*. Detailed information on the genes and the molecular properties of the encoded adhesin proteins of these mycoplasmas (P1, MgPa, and P30) can be found in several recent reviews (29, 236, 368). The topography of the adhesin molecules embedded in the mycoplasma membrane, as predicted by computer analysis of amino acid sequences, has been supplemented by epitope mapping with monoclonal antibodies acting on adhesin molecules in situ or on synthetic oligopeptides corresponding to defined segments of the adhesin molecules. In this way, the presumed three-dimensional conformation of the adhesins in the membrane and of the regions in the adhesin molecules acting directly in adhesion could be proposed (326, 368).

Although the above-mentioned adhesins play the major role in cytadhesion, the process appears to be multifactorial, also involving a number of accessory membrane proteins. These accessory proteins act in concert with cytoskeletal elements to facilitate the lateral movement and concentration of the adhesin molecules at the attachment tip organelle, an organelle characterizing these and several other cytadhering mollicutes. Much of the recent effort, forming part of the *M. pneumoniae* genome project, has been directed to the molecular definition of the attachment organelle components, forming part of the mollicute cytoskeleton (Triton shell). Some of the genes and the encoded proteins of the *M. pneumoniae* Triton shell (30, 40, and 90 kDa; P65 and P200; HMW1 and HMW3) have been identified and characterized. Interestingly, they are proline rich and exhibit repeat sequences and other motifs characteristic of eukaryotic cytoskeletal proteins. Efforts are being made to localize these proteins in the membrane, looking for the interactions of the proteins with each other to form the cytoskeletal network (171a, 250, 251, 342a, 346).

As stated above, most attention has been given to the adhesins and cytoskeletal elements of *M. pneumoniae* and *M. genitalium*. The recent isolation of *M. pirum* from the blood of AIDS patients has focused attention on this mycoplasma, which is also characterized by a tip organelle. A gene encoding a protein showing about 26% amino acid homology to the *M. pneumoniae* P1 adhesin and exhibiting a proline-rich region has been cloned from *M. pirum* (450). The question whether this protein acts as an adhesin is still open. Similarly, a 150-kDa adhesin-like protein has been identified in *M. gallisepticum*; it exhibits 28.7 and 26.3% homology at the amino acid level to the P1 and MgPa cytadhesins, respectively (216). Collectively, these findings suggest that there may be a conserved family of mycoplasmal cytadhesins that are used to colonize widely divergent hosts. The receptors on host cell membranes responsible for mycoplasma attachment, identified so far, are mostly sialoglycoconjugates and sulfated glycolipids (reviewed in references 358, 368, and 492).

Lipid-modified membrane proteins, probably acting as adhesins, have also been characterized in another human mycoplasma, *M. hominis* (176, 177). The notion that the same lipid-modified proteins responsible for the antigenic variation phenomenon also act as adhesins has recently gained experimental support. By "fine-tuning" of the specificities of variant receptors or adhesion factors throughout the cell population, there is better chance that a given variant will be successful in finding the preferred receptors on the mosaic of different tissues displayed by the host. It may also provide the pathogen, during the course of infection, with the flexibility to reach and adapt to different niches where distinctive receptors may be

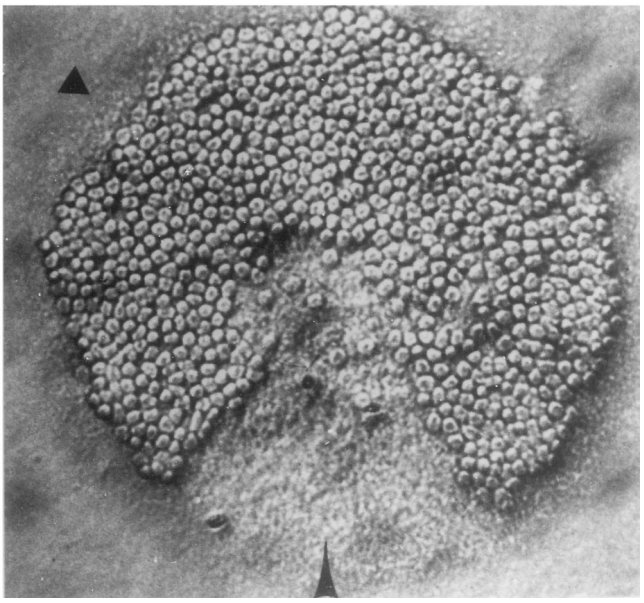

FIG. 4. Selective hemadsorption of human erythrocytes to a distinct sector of a *M. gallisepticum* colony (16).

required for colonization. A novel approach may allow the identification of phase-variable proteins which may be involved in mycoplasma adherence. The approach is based on linkage between the ability of the mycoplasma cells to attach to erythrocytes (hemadsorption), a common tool in mycoplasma adherence studies (358), and the expression of variable surface antigens. A well-established test for determining whether a particular surface antigen undergoes high-frequency phenotypic switching is based on the colony immunoblot technique. Immunostaining with monoclonal or polyclonal antibodies allows the identification of colonies exhibiting variation in expression of surface proteins (388). One of the most conspicuous ways in which this heterogeneity takes shape in in vitro studies is colony sectoring (388). A sector is defined as an immunologically distinct region in which a change in protein expression has occurred within a single colony. A typical feature of cells recovered from the sectorial region is their ability to generate colonies predominantly with the sectorial phenotype. Within this population, colonies exhibiting the nonsectorial phenotype can also be found, indicating high-frequency switching of the corresponding protein. Attachment of erythrocytes via a variable surface membrane protein(s) could be identified by the selective adherence of the erythrocytes to organisms within a single mycoplasma colony, thus generating a typical sector. Experiments conducted with *M. gallisepticum* (Fig. 4) (16) or with *M. synoviae* (321), have provided the first evidence for sectorial adherence of erythrocytes to mycoplasma colonies, suggesting that hemadsorption correlates with switching of variable surface protein(s). This approach appears to be a valuable tool in identification and cloning of the corresponding cytadherence variable genes of other *Mycoplasma* species.

ANTIGENIC VARIATION

All microorganisms are faced with the perpetual challenge of living in diverse and changing environments. To meet this challenge, microorganism populations as a whole must possess mechanisms and strategies allowing them to sense environmental changes and to rapidly respond and adapt to the new surroundings. In the case of pathogenic bacteria, their adaptive potential is challenged by the host defense mechanisms. Only microorganisms able to exhibit environmentally responsive and adaptive molecular traits, enabling them to enter, adhere to, and replicate within the host will survive. Thus, successful bacterial pathogens are those which have evolved molecular mechanisms to deal with the rigors of the host immune response and the need to be transferred and reestablished in a new host. Such mechanisms include mimicry of host antigens, survival within professional phagocytes, and generation of phenotypic plasticity.

Phenotypic plasticity has been defined as the ability of a single genotype to produce more than one alternative form of morphology, physiological state, and/or behavior in response to environmental conditions. One of the most common ways for phenotypic plasticity is antigenic variation. The term "antigenic variation" or, as it is also named, "phenotypic switching" refers to the ability of a microbial species to alter the antigenic character of its surface components including flagella, pili, outer membrane proteins, and capsules that enhance the colonization of host tissues and evade phagocytosis (109, 350). These surface organelles are the major targets of host antibody response; therefore, the ability of a microorganism to rapidly change the surface antigenic repertoire and consequently to vary the immunogenicity of these structures allows effective avoidance of immune recognition.

Surface antigenic variation is accomplished in two distinct ways. (i) Microbial pathogens may use signal transduction pathways to sense signals in the host environment and respond accordingly by expressing virulence-gene products necessary for survival in the host (381). Alternatively, the microbial population as a whole may spontaneously and randomly generate distinct cell populations with varied antigenic phenotypes, "heterotypes," that will survive the specific host response capable of eliminating the predominant "homotypes." The frequency of occurrence of such genetic variants is strikingly high (10^{-4} to 10^{-2} per cell per generation, compared to 10^{-6} to 10^{-8} for a normally occurring mutation). Thus, the presence of a large repertoire of genetic variants may provide the pathogen with the desired escape variant needed for survival in the event of a sudden environmental change or when confronting the host response. Notably, the molecular switching events leading to the generation of these heterotypes are reversible, and the escape variants produced through random genetic variation must inherit the ability to produce, at high frequency, a wide range of antigenic phenotypes. A considerable evolutionary dividend to the microbial pathogen of such random phenotypic switching can be achieved even before the onset of a specific immune response. For example, by fine-tuning of the specificities of variant receptors or adhesion factors throughout the cell population, there is a better chance that a given variant will be successful in finding the preferred receptors on the mosaic of different tissues displayed by the host. It may also provide the pathogen, during the course of infection, the flexibility within the host to reach and adapt to different niches where distinctive receptors may be required for colonization.

Intrinsic Genomic Limitations and Antigenic Variation

The recently documented complete genomic sequences of *M. genitalium*, *M. pneumoniae* (139, 181), and *Haemophilus influenzae* (130) have provided a unique opportunity for comparative analysis of mycoplasmal antigenic variation systems and pathogenesis. One could expect these wall-less mycoplasmas with minute genomes and a single surface-exposed plasma

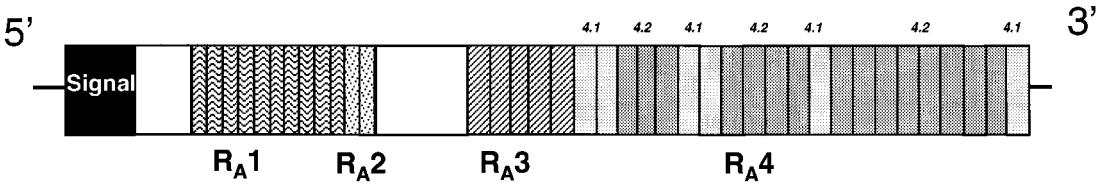

FIG. 5. Schematic representation and structural features of the *M. bovis* VspA protein. The VspA ORF is shown as a rectangle consisting of internal blocks delineating various features of the VspA protein, aligned from the 5′ end to the 3′ end of the *vspA* gene. The solid block, labeled Signal, contains 25 amino acids of a putative lipoprotein signal peptide. Different hatched or shaded blocks designated R_A1, R_A2, R_A3, and R_A4 represent four in-frame repetitive regions encoding distinctive periodic amino acid sequences of 6, 6, 8, and 8 amino acids, respectively. Subrepetitive units within the R_A4 region designated $R_A4.1$ and $R_A4.2$ are also shown. Based on data from Lysnyansky et al. (267).

membrane mediating all physical, physiological, and immunological interactions with the external environment to be severely deficient in adaptive and pathogenic capabilities, in particular when confronting the host immune response. The minimal number of chaperone-encoding genes functioning in protein secretion, the lack of signal peptidase type I (181), and the lack of a periplasmic space could be expected to be associated with a simple and even primitive protein export system and a difficulty in anchoring proteins on the mycoplasma cell surface. In addition, regulatory genes encoding proteins functioning as sensors to environmental stimuli and genes encoding transcriptional activators as well as repressors are scarce in *M. genitalium* and *M. pneumoniae* (139, 181) in comparison to *Haemophilus influenzae* (130) and more so in comparison to *E. coli* (52). The discovery that the minute mycoplasmas possess an impressive capability of maintaining a surface architecture that is antigenically and functionally versatile has placed the mycoplasmas in the "elite" group of bacterial pathogens and parasites distinguished by remarkable antigenic variability (350, 381).

Gene Families as a Common Theme for Surface Diversification

Despite their very limited genetic information, the number of genes in mycoplasmas involved in diversifying the antigenic nature of their cell surface is unexpectedly large. A common theme in pathogenic bacteria and parasites for maintaining surface variability is the utilization of multiple variable genes organized as gene families, allowing the generation of an extensive repertoire of antigenic variants (382, 441). Oscillation of each individual gene between the ON and OFF expression states at high frequency, in conjunction with the ability of each gene to produce distinct size variants of its own product, means that numerous combinatorial antigenic repertoires can be generated on the surface of the organism. In this respect, mycoplasmas are no exception. In the relatively few mollicute species studied, the use of a multiple gene family as a theme for phenotypic switching has been demonstrated.

pMGA family of *M. gallisepticum*. The most remarkable example, in terms of the amount of genetic material used for antigenic variation, was found in the avian pathogen *M. gallisepticum*. This species possesses a large family of related genes, designated the pMGA gene family, encoding variant copies of a major surface lipoprotein (27, 160, 280). The number of pMGA gene copies estimated to be present in the *M. gallisepticum* genome varied from 32 in strain F to 70 in strain R. Assuming that all members of the pMGA family are similar in length to those which have already been characterized, a minimum of 79 kb, or 7.7%, of the genome of strain F and 168 kb, or 16%, of the genome of strain R is dedicated exclusively to the generation of variants of the same surface antigen (27). Moreover, it has been shown that despite the presence of multiple copies of the pMGA gene, only one individual gene is expressed at a time in a given strain (160). These striking findings mean that the pMGA gene family of *M. gallisepticum* is probably the largest known family of translatable mollicute genes.

Vsp family of *M. bovis*. *Mycoplasma bovis*, an important pathogen of cattle, presents another interesting example. A family of proteins undergoing noncoordinate high-frequency phase variation, as well as size variation (designated Vsps), is used to achieve extensive antigenic variation (34, 267, 268, 387). Thirteen Vsp ORFs, not all similarly oriented, were identified within a 16-kb genomic fragment carrying the *vsp* locus (268). These ORFs predicted features typical of the authentic Vsp products characterized so far. Several striking aspects of Vsp structural similarity, sequence divergence, and variability were associated with specific regions of these proteins (Fig. 5). Each *vsp* gene is flanked at its 5′ end by a highly conserved 190-bp noncoding region containing a putative ribosome binding site. The first 29 amino acids also represent a highly homologous N-terminal portion of the Vsp proteins containing a positively charged amino-terminal region and a central hydrophobic region. It ends with a cysteine residue at the predicted acylation site and point of membrane anchorage of a mature processed procaryote lipoprotein (439) (see "Cell membrane" above). A small block of 8 amino acids localized immediately after the cysteine residue is present in all *vsp* gene products. Examination of the deduced Vsp amino acid sequences revealed an unusual structure. About 80% of the Vsp molecules are composed of reiterated sequences of different lengths, extending from the N terminus to the C terminus of the Vsp proteins. Between one and four distinct internal regions of repetitive sequences, usually organized as tandem in-frame blocks, were identified. These regions create a periodic polypeptide structure spanning the entire Vsp molecules (Fig. 5).

Interestingly, however, the potential of *M. bovis* to produce a wide spectrum of Vsp-antigenic phenotypes was found to be more complex than described above. Genetic analysis of the *vsp* gene family in a few *M. bovis* field and clinical isolates revealed that different *M. bovis* strains possess a modified *vsp* gene complex (235). In these strains, major and extensive sequence alterations have occurred throughout the entire *vsp* structural genes. These changes were localized mainly in *vsp* gene regions encoding reiterated sequences of different lengths, extending from the N-terminal to the C-terminal portion of the Vsp proteins. Notably, the 5′ *vsp* promoter region and the N-terminal part encoding the lipoprotein signal peptide remained unaltered and were highly conserved in all *vsp* genes of the particular strain analyzed and essentially in all strains tested. The finding that *M. bovis* isolates possess different versions of the same *vsp* gene family, thereby leading to an

amplified array of phenotypic variants, demonstrates a remarkable and efficient way by which mycoplasmas utilize their limited genetic material to increase their adaptive capability.

Vlp family of *M. hyorhinis*. One of the well-documented examples of a gene family providing an impressive surface variation system is the *vlp* gene family of the swine pathogen *M. hyorhinis*. This system encodes a set of variable lipoproteins (Vlp) that constitute the major coat protein of this mycoplasma (388, 389). By combinatorial expression and high-frequency phase variation and size variation of the Vlps, an extensive array of antigenic variants can be generated. These lipoproteins are products of multiple, related, but divergent *vlp* genes, which occur as single chromosomal gene copies organized in a cluster (485). Structurally, the *vlp* genes are divided into four domains: (i) a highly conserved promoter region; (ii) a highly homologous N-terminal region containing a typical prokaryotic signal peptide sequence, consistent with a prokaryotic lipoprotein signal peptidase recognition site (439); (iii) a region of considerable sequence divergence that contains several short blocks of homologous amino acid sequences recurring at variable locations, within or among different Vlps; and (iv) an external C-terminal domain containing reiterated sequences in the form of tandem, in-frame units encoding 12 to 13 amino acids, undergoing size variation by loss or gain of these repetitive intragenic elements. Interestingly, size variants of a particular Vlp were associated with a characteristic degree of colony opacity, serving as a useful marker in their isolation (388). Each *vlp* gene is subjected to noncoordinate phase variation as well as size variation. The findings that different *M. hyorhinis* strains carry a variable number of *vlp* genes (488) provide additional strong evidence for a vastly expanded potential of structural diversity created by modulation of the *vlp* repertoire.

The ability to modulate the repertoire of the *vsp* and the *vlp* genes provides another important dimension of antigenic variation capability in mycoplasmas. The extensive phenotypic switching observed in mycoplasmas is generated not only by the presence of a cluster of genes undergoing ON/OFF switching but also as a result of an expanded reservoir of genes present in different strains. It is intriguing to speculate that gene clusters, such as the *vlp* or the *vsp* gene families, may be in dynamic flux among propagating individual cells of a certain species, leading to the exchange of genetic material and to the generation of alternative coding sequences.

Vsa family of *M. pulmonis*. Another gene family mediating antigenic variation and displaying features characteristic of the *vlp* and the *vsp* gene systems is that of the murine pathogen *Mycoplasma pulmonis* (45, 421). *M. pulmonis* possesses a genomic region, designated the *vsa* locus, containing multiple *vsa* genes and encoding a variable protein (V-1), which has been implicated as one of the virulence factors in *M. pulmonis*-induced murine respiratory disease. V-1 antigens undergo high-frequency phase switching, and selective patterns of V-1 expression have been correlated with variations in colony opacity. Most of the *vsa* locus from *M. pulmonis* UAB 6510 was cloned, characterized, and shown to possess at least seven distinct *vsa* genes. However, only one *vsa* gene is expressed, while the others are transcriptionally silent (45). Structurally, the expressed *vsa* gene displays features similar to the *vlp* and the *vsp* genes (267, 485). The expressed *vsa* gene is divided into three regions: region I, encoding a putative lipoprotein signal sequence; region II, the region encoding the conserved N terminus of the mature Vsa protein; and region III, a variable region containing the 3′ repetitive elements (45, 421). In contrast, the silent *vsa* genes are truncated and lack regions I and II.

Comparative analysis of the gene structure of the mycoplasmal gene families responsible for antigenic variation demonstrates a remarkable conservation of the 5′-flanking regions and of the N-terminal portion encoding the lipoprotein signal peptide. In contrast, other parts of the structural genes exhibit considerable sequence divergence. Conservation of the 5′ region may offer compelling evidence for the thesis that during evolution, mycoplasmas have recruited their antigenic variation capability through mechanisms of gene duplication while the distinctive host selective pressure could be the driving force for the variable nature of the external repetitive domains. In summary, a common theme in generating surface diversity in mycoplasmas is based on the utilization of a cluster of several related but divergent variable lipoprotein genes. The genes encode a conserved N-terminal region and divergent external domains that consist of reiterated sequences undergoing contraction or expansion, generating size variants (Fig. 5).

Random Genetic Control as a Major Strategy for Phenotypic Switching

The apparent scarcity in mycoplasmas of regulatory genes functioning as sensors to environmental stimuli and of genes encoding transcriptional factors suggests that adaptation of mycoplasmas to the changing environment is not per se a response to signals. In other words, the major survival strategy of the mycoplasmas seems to depend on random and stochastic processes, consisting of various mutational mechanisms which generate high-frequency phenotypic switching (105, 350, 381, 477, 478). Genetic mechanisms of antigenic variation emerging from the mycoplasma studies can be broadly divided into the following three categories (Table 4).

Phase variation via homopolymeric repeats. The essence of this type of variation is the presence of small regions containing reiterated bases (homopolymeric repeats) or oligonucleotide repeats. These "hot spots" provide favorite targets for frequent insertion or deletions of nucleotides and are quite commonly used by several pathogenic bacteria to switch genes ON and OFF (350, 381). Loss or gain of nucleotides is thought to occur due to transient misalignment during DNA replication by a process termed slipped-strand mispairing (252). The location of these homopolymeric repeats within the regulatory region, or within the structural coding region of the corresponding variable genes, determines the level of regulation.

The conserved promoter of the *vlp* genes in *M. hyorhinis* contains a tract of contiguous adenine residues [poly(A) region] immediately upstream of the TATAAT box and downstream of a −35 site or of a directly repeated structure (DR-1) that may accommodate a regulatory DNA binding protein (485). This homopolymeric tract is subjected to frequent mutation, altering its length in exact correspondence to the ON and OFF expression states of individual *vlp* genes (Fig. 6). This mutation, affecting the length of the poly(A) tract from 17 and 18 residues in the ON state to longer stretches of up to 20 residues in the OFF state, is the only sequence change detected during phase transition and correlates well with the expression state of multiple *vlp* genes. These frequent mutations apparently affect the spacing, or secondary structure, between the −10 site and the −35 box or the DR-1 structure, thus influencing the optimal positioning of RNA polymerase as related to the promoter. These findings strongly argue that Vlp phase variation is controlled at least in part at the transcriptional level (82, 485).

In contrast to the products of the *M. hyorhinis vlp* gene family, the variable adherence-associated (Vaa) antigen of *My-*

TABLE 4. Genetic features of surface antigenic variation systems in mollicutes

Organism	System designation	Genes involved	Element involved in variation	Level of regulation	Reference
M. hyorhinis	Vlp	Gene family	Homopolymeric tract, poly(A)	Transcriptional control	485
M. bovis	Vsp	Gene family	DNA transposition	Transcriptional control	267
M. gallisepticum	pMGA	Gene family	Oligonucleotide repeats $(GAA)_n$	Transcriptional control	159
M. pulmonis	Vsa	Gene family	DNA inversion	Transcriptional control	421
M. hominis	Vaa	Single copy	Homopolymeric tract, poly(A)	Translational frameshift	491
M. fermentans	P78	Part of an operon	Homopolymeric tract, poly(A)	Translational frameshift	451

coplasma hominis is controlled at the translational level. The Vaa antigen, which is an abundant surface lipoprotein adhesin that apparently mediates interactions of this mycoplasma with its human host, is subjected to high-frequency phase variation in expression, which correlates precisely with the ability of *M. hominis* to adhere to cultured human cells. It was shown that an oscillating mutation involving a single nucleotide deletion or insertion in a short homopolymeric tract of adenine residues correlates with the Vaa expression state (491). The poly(A) tract was localized near the 5′ end of the sequences encoding mature Vaa sequences, creating a translational frameshift that results in either a complete Vaa ORF or an in-frame UAG stop codon immediately downstream of the poly(A) tract (Fig. 6).

A similar translational control of variable surface lipoproteins was demonstrated for the human pathogen *M. fermentans* (Fig. 6). A putative ABC-type transport operon encoding four gene products was recently identified in this mycoplasma (451). The 3′-distal gene encoding P78, a known surface-exposed antigen undergoing high-frequency phenotypic switching, is the proposed substrate-binding lipoprotein of the ABC transporter. The P78 gene is subjected to localized hypermutation in a short homopolymeric tract of adenine residues located at the N-terminal coding region of the mature product. High-frequency, reversible insertion/deletion frameshift mutations lead to selective phase variation in P78 expression, whereas the P63 protein, encoded by the gene located at the 5′ end of the operon, is continually expressed.

A hot spot for frequent mutation within a structural gene was also found in the major cytadhesin gene (P1) of *M. pneumoniae*. Reversible and spontaneous cytaderence-negative and cytadherence-positive mutants were associated with an insertion or deletion of a single nucleotide in a stretch of seven adenines. This mutation resulted in a frameshift and generation of a termination codon, causing the premature termination of P1 translation (435).

An interesting issue, not yet fully understood, concerns the molecular mechanism mediating high-frequency switching of the major surface lipoprotein pMGA of *M. gallisepticum*. A distinct hot spot region in the form of a trinucleotide GAA repeat motif, $(GAA)_n$, where *n* represents the number of GAA repeats, was identified in the 5′ region of all members of the pMGA gene family, 21 bases upstream from the −35 box of the promoter (Fig. 6). The number of GAA repeats ranges from 10 to 16 (27). Genetic analysis of phenotypically switched clonal isolates, representing the ON or OFF expression state of the pMGA product from a number of *pMGA* genes in *M. gallisepticum* R, revealed that expressible *pMGA* genes always contained a $(GAA)_{12}$ motif. Different numbers of the GAA repeats were correlated with the OFF expression state of the pMGA (159). How does the $(GAA)_{12}$ motif function to initiate pMGA transcription? An intriguing model proposed by Glew (159) suggests that the $(GAA)_{12}$ motif serves as a linker or a spacer for two flanking binding sites necessary for the initiation of pMGA transcription. The first binding site, localized downstream of the $(GAA)_{12}$ motif, is a well-defined promoter site for RNA polymerase. The second site, upstream of the $(GAA)_{12}$ motif, contains a region of sequence homology present in all known pMGA genes. These two binding sites presumably allow the initiation of transcription only when a putative activator protein is in the correct spatial alignment with the RNA polymerase bound to the pMGA promoter region. The correct alignment in this case would correspond to the $(GAA)_{12}$ repeat, acting as a linker between the two binding sites. Interestingly, this model is analogous to activation of transcription by the cyclic AMP receptor protein for class I promoters in *E. coli*.

Phase variation via chromosomal rearrangements. DNA inversions, gene conversions, duplications, or deletions of tandem homologous blocks of DNA, as well as movement of transposable elements, are widely used in bacteria to regulate expression of phase variable surface antigens (see "Chromosomal rearrangements" above). Most of these chromosomal rearrangements are usually considered to be random or spontaneous. Homologous recombination is a major mechanism allowing genetic variation, depending on RecA function, that promotes the annealing of single-stranded DNA to any complementary sequences in double-stranded DNA, with as little 20 to 100 bp of homology (112). Therefore, large gene families, such as those involved in generating surface diversity, containing homologous sequences common to members of a particular gene family, are favorite targets for recombinatorial events. As a result, an extraordinary collection of phenotypic variants can be produced. For example, homologous recombination within the *pil* gene family of *Neisseria gonorrhoeae* results in production of over a million combinations of antigenically variant pili (441). It was therefore reasonable to expect, that mycoplasmal variable-gene families, containing regions of significant sequence similarity, will be subjected to recombinative events regulating the high-frequency ON/OFF switching. Interestingly, however, regulation of variable genes through genomic rearrangements was demonstrated in only two *Mycoplasma* species so far. Studying chromosomal rearrangements associated with phenotypic switching requires the isolation of a lineage of clonal isolates, representing successive generations and exhibiting ON/OFF switching of only the antigen of interest.

Phenotypic switching of the variable surface lipoprotein in *M. bovis* was shown to involve chromosomal rearrangements (267). The nature of these rearrangement events was revealed when a lineage of *M. bovis* PG45 clonal isolates, representing successive generations and exhibiting oscillating phase transitions of only VspA, i.e., ON→OFF→ON, was analyzed. The *vspA* gene, which encodes a representative variable surface lipoprotein of the Vsp family, was found to be subjected to high-frequency reversible DNA rearrangements occurring during oscillating phase transition of VspA (267). Genomic rearrangements during VspA phase variation caused the disappearance of a 1.5-kb *Hin*dIII genomic fragment, carrying the *vspA* ON gene, and the generation of a new restriction frag-

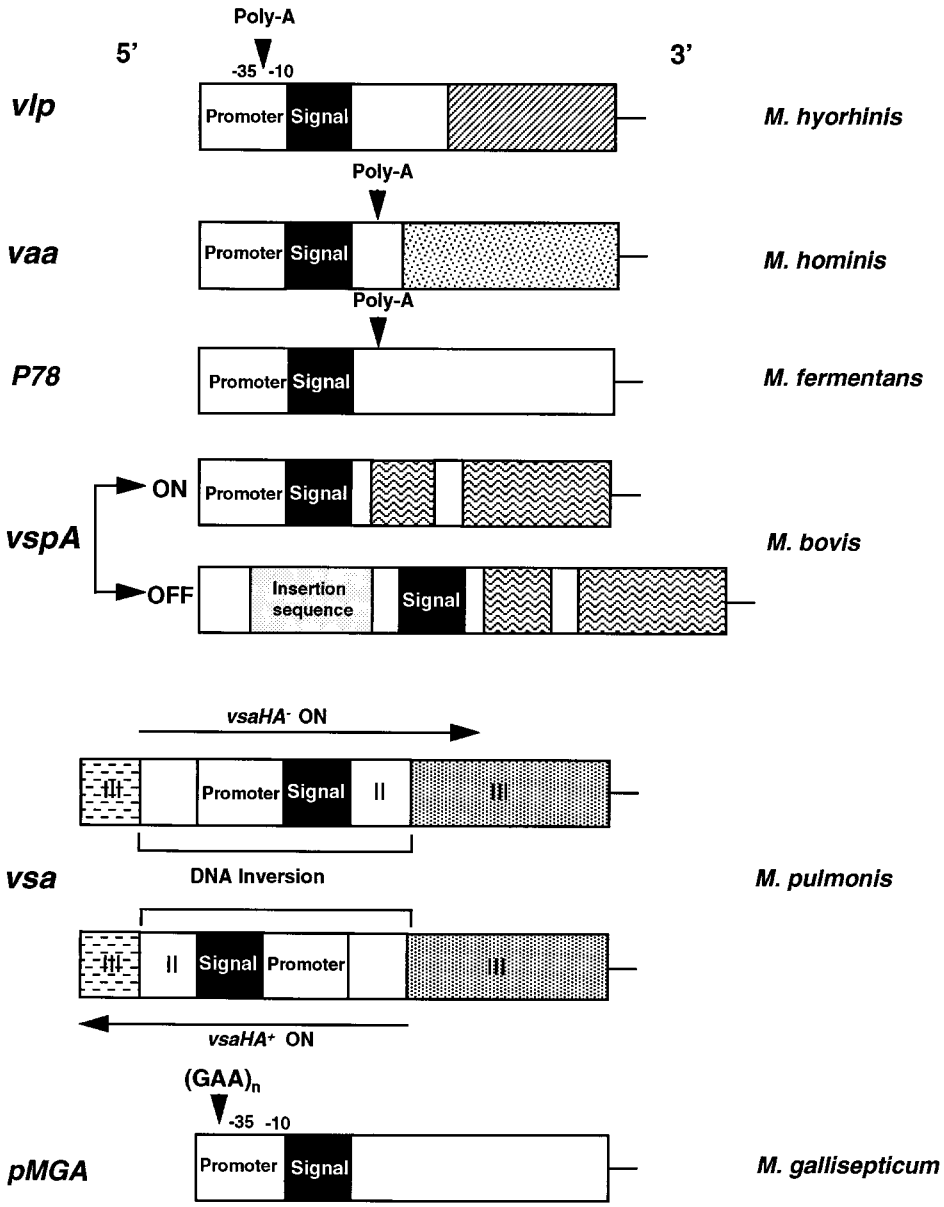

FIG. 6. Schematic representation of regulatory and structural features of antigenic variation systems in mycoplasmas. Representative genes of distinct antigenic variation systems of six mycoplasma species are shown (not to scale) as rectangles consisting of internal blocks aligned from the 5′ end to the 3′ end of each gene. The system designation and the corresponding mycoplasma species are indicated on the left and on the right, respectively. The solid black block, labeled Signal, contains the species-specific amino acid sequence of a putative lipoprotein signal peptide. Different hatched blocks represent system-specific in-frame reiterated sequences. The location of a homopolymeric tract of contiguous adenines (Poly-A) or of oligonucleotide repeats $(GAA)_n$, within the promoter region (*vlp*, *pMGA*) (159, 485) or within the coding-gene region (*vaa*, *P78*) (451, 491) is shown by an arrow. Two *vsp* genes from two *M. bovis* clonal isolates, exhibiting ON or OFF expression states of the variable surface lipoprotein VspA, are shown. A genomic fragment that was inserted within the *vspA* promoter region, leading to its OFF expression state, is shown by a hatched box labelled Insertion sequence. Two *vsa* genes isolated from two *M. pulmonis* variants displaying the $VsaHA^-$ or the $VsaHA^+$ phenotypes are shown (421). A chromosomal fragment that inverted during phase transition ($VsaHA^- \leftrightarrow VsaHA^+$) is indicated by brackets. The direction of expression of the *vsa* gene from each variant is marked by an arrow.

ment of 2.3 kb, present only in the clonal variant in which the *vspA* gene has turned OFF. Sequence analysis of the 2.3-kb *Hin*dIII fragment carrying the *vspA* gene in the OFF expression state revealed that a fragment of about 800 bp was inserted 70 bp upstream of the initiation codon of the *vspA* gene, giving rise to the observed size variation during *vspA* ON/OFF switching (268). Deduced amino acid sequences of this insertion fragment revealed an uninterrupted ORF containing a region of reiterated sequences in the form of 10 tandem, in-frame repeats encoding 11 amino acids each. These repeats were not found in any other member of the *vsp* gene family (Fig. 6).

The precise mechanism of this unique transposition event has not been fully unraveled. Interestingly, however, the finding that this mobile genetic element exists as a single chromosomal copy and undergoes a precise excision and insertion process during ON/OFF switching of the *vspA* gene (268) argues against a replicative mechanism of transposition in which the entire mobile element replicates during transposition, resulting in two copies, and suggests a conservative or cut-and-

paste mechanism. The position of the insertion site within the promoter region of the *vspA* gene also suggests that *vspA* ON/OFF switching is regulated at the transcriptional level (Table 4). An exciting recent finding (268) has shown that adjacent to the *vsp* locus is a gene family displaying significant homology to several bacterial IS elements, including the mobile genetic element IS*30* of *E. coli* (61% amino acid homology), the transposase for insertion sequence element IS*4351* transposon Tn*4551* (58.6%), IS*1161* (57.9%), and IS*1086* (56%) (107). The possibility that transposase enzymes or other regulatory proteins play a role in dictating the genomic rearrangement event responsible for the VspA phase transition, in which environmental signals are involved, is intriguing although not yet experimentally proven.

The second known example in which chromosomal rearrangements regulate the expression of variable surface proteins is that of the V-1 antigens of *M. pulmonis* (43, 45, 421). Identification of *M. pulmonis* isolates exhibiting variation in V-1 expression is rather easy, since expression of the V-1 antigen has been found to correlate with the ability of the mycoplasma colonies to hemadsorb (HA phenotype). Sequence analysis of the V-1 gene from two *M. pulmonis vsa* HA^+ and *vsa* HA^- phenotypes indicated that these two genotypes arose as a result of high-frequency chromosomal inversion (Fig. 6). Silent *vsa* genes lack the 5′-end region containing the promoter and ribosome binding site that is present in the expressed gene. During DNA rearrangements, gene expression is regulated by reassorting of the 5′-end region from an expressed gene with the 3′-end region from a previously silent gene. All *vsa* rearrangements identified so far were shown to be site-specific DNA inversions that occur between copies of a specific 34-bp sequence that is conserved among *vsa* genes (45, 421).

It is worthwhile to examine frequencies of the rearrangement events described in the mycoplasmas in comparison to those described in other microbial genomes. The frequency of the *vspA* transposition mechanism, as deduced from the frequency of colonies oscillating between the ON and OFF expression states of the VspA, VspB, and VspC lipoproteins, was 2×10^{-3} per cell per generation (387). A rate of 10^{-2} to 10^{-3} was observed for the site-specific DNA inversion regulating the switch of the V-1 antigen in *M. pulmonis* (45, 421). Such high frequencies of genomic rearrangements place the mycoplasma chromosome among the most variable genomes known and underscores the efficient way in which mycoplasmas utilize their limited genomic material.

Variation via repetitive domains (size variation). Surface antigens with tandem repetitive domains are being increasingly identified as molecules involved in pathogen-host interactions in a variety of pathogenic microorganisms. The role of repetitive units present within an antigen was shown to be directly associated with ligand binding and to be involved in generating antigenic variation (109, 185, 217). The presence of multiple in-tandem repetitive domains within the antigen-encoding gene generates a highly mutable module subjected to frequent contraction or expansion of these repetitive intragenic coding sequences, resulting in the expression of a distinct size variant of the corresponding protein. Molecular mechanisms capable of precise deletion or insertion of repetitive sequences include homologous recombination and slipped-strand mispairing (252). It was therefore not surprising that the majority of mycoplasmal variable surface antigens identified so far are no exception and contain significant regions of reiterated sequences. These include the Vlps of *M. hyorhinis* (388, 389), the Vsps of *M. bovis* (34, 267, 387), the MB antigen of *Ureaplasma urealyticum* (494), the Vaa adhesin and the Lmp1 antigen of *M. hominis* (490), the P30 and the P1 of *M. pneumoniae* (99), and the V-1 antigen of *M. pulmonis* (421) (Table 4). Notably, in a few phase transitions of distinct Vlps in *M. hyorhinis* or Vsps in *M. bovis*, variation at the DNA level occurred without expression of the corresponding protein (268, 485). This indicates that deletion or addition of repetitive coding sequences in mycoplasmas can be independent of phase variation. It was also established that variation in the number of the reiterated sequences can occur in various locations within the coding region. This was clearly demonstrated in the Vsp system of *M. bovis*, where deletions or additions of repetitive sequences were identified not only within the C-terminal repetitive structures R_A4 (Fig. 5) but also within the N-terminal repetitive domain R_A1 (268, 387).

Perhaps the most intriguing finding associated with antigenic variation in general and size variation in particular, in terms of its functional role in mycoplasmas, was discovered recently in *M. hyorhinis* (80). The Vlp system was shown to play an important role in modulating susceptibility to mycoplasma growth inhibition by host antibodies, thus providing a mutational framework in which a propagating population can escape host antibody inhibitory activity. Variants expressing longer versions of Vlps were completely resistant to host immune serum antibodies, whereas variants expressing shorter allelic versions of each Vlp were susceptible. This study has also shown that the emergence of a prevalent, protective Vlp phenotype in antibody-selected populations is not due to expression of a particular Vlp but, rather, results from optional mutational pathways leading to expression of any long Vlp product. The way the selection of mutational pathways is carried out appears to be determined by the most favorable mutation available to generate a long Vlp in a particular genetic background.

INTERACTIONS WITH THE HOST IMMUNE SYSTEM

The complex network of interactions between mycoplasmas and the host immune system involves mycoplasma-induced specific and nonspecific immune reactions. Specific protective defense mechanisms include the production of systemic as well as local anti-mycoplasmal antibodies of different classes and subclasses, stimulation of cell-mediated immunity, and opsonization and phagocytosis of organisms. The specific reactions elicited by invading mycoplasmas, essential for resistance and protection against mycoplasma infections, have also been shown to play a role in the development of lesions and exacerbation of mycoplasma induced diseases, as described and reviewed previously (46, 72, 93, 189). In addition to eliciting anti-mycoplasmal immune responses, mycoplasmas exert a wide range of nonspecific immunomodulatory effects upon cells making up the immune system. Mycoplasmas affect the immune system by inducing either suppression or polyclonal stimulation of B and T lymphocytes; inducing cytokines; increasing the cytotoxicity of macrophages, natural killer cells, and T cells; enhancing the expression of cell receptors; and activating the complement cascade. The ability of mycoplasmas to immunomodulate host immune responsiveness contributes to their pathogenic properties, enabling them to evade or suppress their host defense mechanisms and establish a chronic, persistent infection.

Mycoplasma-Induced Immune Suppression

Arginine depletion. Perhaps the first to be discovered and the most straightforward mechanism of immunosuppression induced by mycoplasmas has been based on arginine depletion by mycoplasmas possessing the arginine dihydrolase system.

Numerous studies (reviewed in reference 91) have shown that depletion of the essential amino acid L-arginine by these mycoplasmas can induce multiple immunosuppressive effects in immune cell systems in vitro. Reproduction of the immunosuppressive effects by the addition of mycoplasmal arginine deiminase to in vitro systems and reversal of the effects by the addition of L-arginine to the medium (438) has provided direct proof of the possible role of arginine depletion in immunosuppression by mycoplasmas. However, the crucial question is whether infection with arginine-hydrolyzing mycoplasmas is capable of causing significant depletion of the arginine supply in tissues in vivo. While Sugimura et al. (438) proposed that human lymphocytes located in tissues with extremely low concentrations of L-arginine, as a result of infection by arginine-hydrolyzing mycoplasmas, hardly proliferate or even survive, solid experimental evidence to support this proposal is still lacking.

Cytotoxicity toward lymphoid cells. Gabridge et al. (146) showed long ago that injection of freshly isolated *M. fermentans* cells or their membranes into mice caused lethal shock, resembling that induced by endotoxin. Cytotoxic effects could also be demonstrated on mouse thymocytes in vitro. Interest in these early observations was revived following the extensive studies by Lo and coworkers showing that the AIDS-associated *M. fermentans* incognitus and *M. penetrans* induced cytocidal effects on human T lymphocytes in vitro. *M. fermentans* incognitus was also detected in necrotic lesions in lymph nodes and spleens of non-HIV-infected subjects who developed a fulminant fatal respiratory distress syndrome (259).

Some insight into the interactions of *M. fermentans* incognitus with human T lymphocytes was provided by Franzoso et al. (137), who showed that this mycoplasma fused with $CD4^+$ $CD4^-$ T-cell lines and with about 12% of human peripheral blood lymphocytes. The fusion process appeared to leave the lymphocytes intact, and its effects on their functions is still unclear. Fusion was inhibited by pretreatment of *M. fermentans* with proteolytic enzymes, indicating that a surface proteinaceous component was involved in the fusion process. A unique major glyceroglycolipid containing phosphorylcholine, isolated from *M. fermentans* incognitus, was also implicated in the fusogenic activity (see "Cell membrane" above). Work by Rawadi et al. (353) clearly distinguished between the molecular entities of *M. fermentans* that induced proinflammatory cytokines (discussed below) and those that triggered cell death. These investigators showed that a proteinaceous fraction (15 to 30 kDa) of *M. fermentans* incognitus, not associated with lipids, exhibited cytocidal effects on an undifferentiated myelomonocytic cell line but not on differentiated myelomonocytic cells or on freshly separated peripheral blood T and B lymphocytes. The mechanism of cell death resembled apoptosis in the sense that it involved oligonucleosomal DNA fragmentation and chromatinolysis. However, it differed morphologically and functionally from true tumor necrosis factor alpha (TNF-α)-induced apoptosis. Whether these cellular components of *M. fermentans* contribute to the necrosis in lymphoid organs of mice and humans reported by Gabridge et al. (146) and by Lo (259) remains to be clarified.

The second mycoplasma associated with AIDS, *M. penetrans*, was found to adhere to and invade different human and animal cells (259). The organism could penetrate the cytoplasm of $CD4^+$ T lymphocytes and human monocytes and cause variable cytopathic effects, most probably depending on the nature of the lymphoid cell line tested (261). Additional studies confirmed that *M. penetrans* exerted a direct cytopathic effect on human T cells (402) and showed that although the microorganism triggered the expression of Fas (CD95) on a certain population of cultured T cells, the number of apoptotic cells was only moderately increased and could not account for the observed cytopathic effects. Other factors, such as mycoplasma-induced TNF-α, should therefore be considered to play a role in these manifestations (402). Nevertheless, the demonstrated capacity of *M. fermentans* to fuse with T lymphocytes and the ability of *M. penetrans* to invade cells strongly indicate that these two *Mycoplasma* species may affect the host immune system by triggering cell death of infected T cells.

Direct suppression of immune cells. Inhibition of immune responses is not confined to *Mycoplasma* species expressing arginine deiminase or to those exhibiting cytopathic effects on immune cells. Thus, *M. pneumoniae* strains with none of these properties induced transient anergy in most patients during the acute phase of infection by mechanisms yet to be clarified. This anergy was reflected by negative tuberculin skin tests and by reduced in vitro responsiveness of patient lymphocytes (46). Repeated intranasal infection of guinea pigs with *M. pneumoniae* significantly decreased the lymphocyte stimulation by mycoplasmal antigens (204). It has been demonstrated that suppression of lymphocytes from calves infected with *M. bovis* was not caused by arginine depletion or by cytotoxic effect of viable *M. bovis* toward cultured lymphocytes (454). Whereas *M. pneumoniae* and *M. bovis* infections clearly affected host cell-mediated immune responses, infection of rats with *M. pulmonis* caused a reduced humoral antibody response to a T-dependent antigen whereas cell mediated immunity was not affected (1).

M. hyorhinis released into the supernatants of infected cultured cells a protease-sensitive, 200-kDa factor that suppressed the induction of interleukin-2 (IL-2)-dependent cytotoxic T-cell responses to alloantigens. The suppression was not reversed by addition of excess recombinant IL-2 and was more profound when the mycoplasmal suppressive factor was added to cultures during the early stages of the cytotoxic reaction. The suppressive elements from *M. hyorhinis* were also inhibitory for mouse B lymphocytes responding to *E. coli* lipopolysaccharide (LPS) (448).

M. arthritidis, an arginine-utilizing mycoplasma expressing arginine deiminase, is potentially able to induce temporary suppression of lymphocyte responsiveness by arginine depletion. However, studies by Cole et al. (85) clearly demonstrated that arginine deiminase is not the only factor with which *M. arthritidis* modulates its host immune system. Their studies on the in vivo effects of the soluble T-cell mitogen MAM, produced by *M. arthritidis*, showed that high doses of MAM caused a temporary inhibition of cell-mediated immune responses while enhancing humoral immune responses in injected mice. MAM acting as a superantigen (see "The *M. arthritidis* superantigen MAM" below) induced suppression only of lymphocytes from MAM-responsive mouse strains. Suppression was manifested by decreased in vitro response of animal lymphocytes to T-cell mitogens, by partial suppression of contact sensitivity to dinitrofluorobenzene, and by significant prolongation of skin graft survival. The suppressive effects induced by MAM were temporary, and a gradual restoration of T-cell responses was observed.

The suppressor cells stimulated in vivo by MAM belonged to selective MAM-reactive $CD4^+$ $CD8^-$ helper T lymphocytes. These cells were able to act on lymphocytes from normal animals in a non-major histocompatibility complex (MHC)-restricted manner. Spleen cells obtained from mice injected with MAM showed a marked decrease in their ability to produce IL-2 when exposed in vitro to MAM. On the other hand, they secreted increased levels of IL-4 and IL-6. The changes induced by MAM in the cytokine profiles, together with the

observed partial anergy of T cells and enhanced activation of B lymphocytes, suggest that MAM induces an in vivo shift from Th1 to Th2 $CD4^+$ subpopulations of T cells and modifies the animal's T-cell repertoire (85).

Induction of down-regulating cytokines. IL-10 is a down-regulating cytokine, which is produced mainly by Th2 $CD4^+$ cells and macrophages and to a lesser extent by other immune cells. IL-10 inhibits accessory cell functions of antigen-presenting cells (APC), suppresses the production of cytokines such as IL-1β, TNF-α, chemokines, and IL-12 by mononuclear cells, and exhibits inhibitory effects on gamma interferon (IFN-γ) production by Th1 $CD4^+$ cells. Consequently, IL-10 down-regulates T cell activation, mediates a shift from the Th1 phenotype toward the development of a Th2 subset of cells, and stimulates the differentiation of human plasma B cells into immunoglobulin G4-(IgG4)-producing cells.

Pietsch et al. (336) have reported the expression of IL-10 mRNA in the lungs of mice intranasally inoculated with *M. pneumoniae*. They noticed that during a second exposure to the mycoplasma, unlike primary inoculation, a rapid decline in the expression of IL-2 mRNA was accompanied by a parallel rise in the level of IL-10 mRNA. Low but detectable levels of IL-10 were also induced by the *M. arthritidis* superantigen MAM in cultures of human peripheral blood mononuclear cells (PBMC) and mouse splenocytes (379). Additional evidence for induction of this down-regulating cytokine by mycoplasmas was provided by Rawadi et al. (353). Heat-inactivated preparations of *M. fermentans* incognitus triggered the production of IL-10 by freshly isolated human monocytes. It can be speculated that the ability of *M. pneumoniae*, *M. arthritidis*, and *M. fermentans* to stimulate IL-10 production down-regulates and suppresses T-cell proliferation, as well as inhibiting excessive production of proinflammatory cytokines by mononuclear phagocytes. Involvement of IL-10 in the MAM-induced in vivo shift from Th1 to Th2 populations (85) remains to be determined.

Zurita-Salinas et al. (501) demonstrated the expression of mRNA for IL-13 in cultured human fibroblast monolayers contaminated with an unidentified mycoplasma. IL-13 is another cytokine known for its down-regulating activity on macrophages. This cytokine is produced predominantly by $CD4^+$ Th2 cells but also by $CD8^+$ T cells, neutrophils, and nonimmune cells and tissues. IL-13 blocks the production of proinflammatory cytokines by macrophages and inhibits the production of NO, an important factor in macrophage cytotoxic manifestations. On the other hand, IL-13 is stimulatory in B-cell differentiation and triggers mouse plasma cells to produce IgG1 and IgE. Although it is still necessary to identify the *Mycoplasma* species capable of inducing IL-13, there is no doubt that down-regulating cytokines such as IL-10 and IL-13, released in response to interactions of mycoplasmas with host cells, have inhibitory effects on macrophage accessory functions, on the production of proinflammatory cytokines, on T-cell proliferation and on the balance between $CD4^+$ Th1- and Th2-cell phenotypes.

Activation of Immune Cells by Mitogenic Mycoplasmas

Mycoplasma mitogenicity. The discovery by Ginsburg and Nicolet in 1973 (155) that *M. pulmonis* is a potent mitogen for cultured rat lymphocytes was followed by numerous reports on mycoplasma-induced nonspecific activation of immune cells, both in vitro and in vivo. Unlike specific antigenic epitopes that are recognized by the clones of B and T lymphocytes that bear the corresponding antigenic receptors, many mycoplasmas possess the capacity to activate lymphocytes in a nonspecific, polyclonal manner. Mitogenic stimulation of lymphocytes by mycoplasmas is not restricted to lymphocytes of their natural host. Thus, human lymphocytes are stimulated by the human mycoplasmas *M. fermentans*, *M. penetrans*, and *M. pneumoniae* but also by two murine mycoplasmas, *M. arthritidis* and *M. pulmonis*. Similarly, mouse lymphocytes are activated by murine mycoplasmas as well as by many other mycoplasmas which are not commonly isolated from mice.

The nature of interactions between mitogenic mycoplasmas and lymphoid cells was extensively discussed in previous reviews (91, 309, 397, 420). These include the data demonstrating that mitogenic, polyclonal activation of lymphocytes by mycoplasmas, is not simply an anamnestic response of previously sensitized lymphocytes. The observed differences in mitogenic responses of lymphocytes obtained from various animals and the differences between various strains of mice or rats, along with studies documenting MHC gene control of responsiveness to the mitogenic activity of *M. arthritidis* and *M. hyorhinis*, were also discussed in detail in previous reviews (91, 309, 397, 420). Our review will refer only briefly to these summarized data, focusing on more recent observations.

As summarized in Table 5, mycoplasmas are mitogenic toward B lymphocytes, T lymphocytes, or both B and T lymphocytes. Generally, proliferation of B lymphocytes, induced by mitogenic mycoplasmas in vitro, did not require the presence of accessory cells (91, 212, 309). In contrast, accessory cells were essential for *M. arginini*-induced mouse B-cell activation (397) and were crucial for the interactions of *M. arthritidis* with human and mouse T cells (91).

The lymphocyte populations activated by mycoplasmas depend on both the mycoplasmas and the origin of lymphocytes. *M. fermentans* that stimulated human B and T lymphocytes has been shown to bind to 10 to 15% of human blood lymphocytes. The majority of these cells were B lymphocytes, while only a few were T lymphocytes (74). Whereas this mycoplasma induced polyclonal activation of mouse B lymphocytes (reviewed in reference 91), mouse T cells were not activated by membrane lipoproteins of *M. fermentans* incognitus (124). However, concanavalin A-stimulated murine thymocytes exposed to a lipopeptide derived from *M. fermentans* (300) differentiated into cytotoxic T cells (301).

Following the initial phases of mycoplasma-induced, polyclonal activation and proliferation of B lymphocytes, B cells may be triggered to further differentiate into antibody-producing plasma cells. Indeed, secretion of antibodies of different specificities, not related to mycoplasmal antigens, has been found in cultures of spleen cells exposed to various mycoplasmas possessing B-cell mitogens (91, 397), including *M. fermentans* incognitus and *M. penetrans* (124). Polyclonal antibody production induced by mycoplasmas has been observed not only in vitro but also in vivo (91, 309, 397, 420). It is worth noting that there is a clear segregation between mycoplasma-induced DNA synthesis and further differentiation of activated B cells into antibody-producing cells. Thus, stimulation of DNA synthesis was not always a prerequisite for the induction of polyclonal Ig secretion by B lymphocytes exposed to mycoplasmas. On the other hand, stimulation of mitosis did not necessarily trigger subsequent differentiation of the dividing lymphocytes into plasma cells (91, 397).

As mentioned above, accessory cells are probably not essential during the induction of DNA synthesis and proliferation of B lymphocytes. However, the exact roles of macrophages, Th cells, and cytokines produced by these cells in mycoplasma-induced generation of plasma cells and secretion of antibodies of different specificities and various isotypes need further clarification. Polyclonal stimulation of T lymphocytes by mitogenic

TABLE 5. Mycoplasma-induced lymphocyte activation

Organism	Origin of cells	Activated lymphocytes[a]	Nature of mitogenic components	Reference(s)[b]
M. arginini	Mouse (C3H/HeJ)	B (and T?) (macrophages essential)	Heat-stable, proteinase K-sensitive acylated proteins, 88, 84, 74, 70, 61 kDa	134, 397
M. arthritidis	Human	T (Th and CTL) $CD4^+$, $CD8^+$, $CD4^-$ $CD8^-$ (Vβ 3.1, 11.1, 12.1, 13.1, 17.1 = 19.1) (macrophages essential, MHC class II restricted)	MAM superantigen, 26–27 kDa, heat, protease, and acid labile; hydrophobic, soluble, basic protein, 213 amino acids	18, 42, 90, 129, 226, 227, 283
		B and NK	Not defined	97, 108
	Mouse (C3H/HeJ)	T (Th, CTL, and Ts) $CD4^+$, $CD8^+$ (Vβ 5.1, 6, 8.1, 8.2, 8.3) (macrophages essential, MHC class II restricted)	MAM	18, 87, 89, 266, 461
		B	Membranous, heat stable	85, 461
	Rat	T $CD4^+$	MAM	18
M. bovoculi	Cattle	NK	Not defined	322
M. capricolum	Mouse (C3H/HeJ)	B (and T?)	Membranous, heat stable	416
M. fermentans	Human	B and T (macrophages not essential)	Sonicated cells, heat stable	
	Mouse	B (and T?)	LAMPs, heat stable	124, 301
M. gallisepticum	Mouse	B	Membranous, heat stable	416
M. hyorhinis	Mouse	B and T (macrophages not essential) Capping & association to surface alloantigens	Heat-stable, 90-kDa soluble protein and TX-100-extractable proteins, 53, 43, 35 kDa	347
M. neurolyticum	Mouse	B (macrophages not essential)	Partially heat stable, membrane glycoproteins and lipoglycans	309
	Rat	B (macrophages not essential)	Lipoglycans	212, 309
M. orale	Human	NK	Not defined	
	Mouse (C3H/HeJ)	B, (T?), NK	Not defined	295
M. penetrans	Human	T (weak response of both $CD4^+$ and $CD8^+$) CD 69 ↑, HLA-DR ↑, CD 25 ↑, Fas CD 95 ↑	Whole cells	401
	Mouse (C3H/HeJ)	B	LAMPs Glycolipid	6, 124 48
M. pneumoniae	Human	B and (T?)	Crude membranes, heat stable; crude lipids, not stimulatory	46
	Mouse	B (macrophages not essential	Lipid-free polysaccharides and to a lesser extent proteins, heat stable	46
	Guinea pig		Sonicated cells, heat stable	46

Continued on following page

TABLE 5—*Continued*

Organism	Origin of cells	Activated lymphocytes[a]	Nature of mitogenic components	Reference(s)[b]
M. pulmonis	Human	Not defined	Crude membranes	309
	Mouse	B, T, NK	Membranes	243, 420, 442
	Rat	B and T (macrophages partially essential)	Heat-sensitive, external membrane proteins and glycoproteins, 84–113 kDa	91, 247, 248, 309, 420
A. laidlawii	Human	T	Heat stable	228
	Mouse	B	Sonicated cells, heat stable	
Spiroplasma monobiae (MQ-1)	Mouse (C3H/HeJ)	Not defined	Membrane proteins differing from TNF-α inducers	414, 415

[a] CTL, cytotoxic T lymphocyte; Ts, suppressor T cell.
[b] For references published before 1985, see reference 91.

mycoplasmas can theoretically trigger subsequent differentiation and development of cytotoxic T lymphocytes. Nevertheless, clear evidence for the induction of cytotoxic T cells is available only for *M. arthritidis* (91).

Although detailed information on the biochemical nature of mitogens from most species is still very limited, it appears that the wide variety of mycoplasmas displaying mitogenic activity is also reflected in the diversity of their mitogenic elements. It is evident from Table 5 that, except for *M. arthritidis* and *M. pulmonis*, the mitogenic elements of most mycoplasmas resist heating (56°C for 30 or 60 min) or brief ultrasonic treatment. The mitogenicity of mycoplasmas is displayed by live organisms, by nonviable cells, or by crude extracts of lysed cells. The mycoplasmal mitogenic components differ significantly from bacterial LPS in activating lymphocytes from mouse strains that are poor responders to LPS. Taken together, the data summarized in Table 5 not only demonstrate that mycoplasmas differ in the cell constituents which display their mitogenic activity toward murine B cells but also indicate that even a single mycoplasma may carry more than one mitogenic element. Similar observations may also be expected with regard to organisms which activate both T- and B-lymphocyte populations (Table 5). Indeed, studies with *M. pulmonis* showed that surface-exposed membrane proteins were the major cell constituents of this organism inducing proliferation of rat lymphocytes (309). However, more recent studies with monoclonal antibodies that inhibited the mitogenic activity of *M. pulmonis* showed that these monoclonal antibodies recognized several membrane components, including carbohydrate-associated proteins and acylated proteins (247, 248). Unlike *M. pulmonis* mitogenicity (247), the chemoattractive activity of *M. pulmonis* membrane proteins toward rat B cells was not destroyed by solubilization of membranes with detergent (393). The novel mycoplasmal chemotactic proteins were heat stable while being sensitive to trypsin (393). V-1, a major variable membrane antigen of *M. pulmonis* (188), produced a characteristic ladder pattern in SDS-PAGE (188, 471) of repeating subunits that resisted short heat treatment (see "Antigenic variation" above). This antigen appeared to be involved in both adherence of *M. pulmonis* to host tissues (471) and mediation of rat B-lymphocyte chemotaxis (393, 420). Since the mitogenic effects of *M. pulmonis* are carried out by membrane components that differed in heat sensitivity and immunoblotting patterns from those of V-1 (248, 471) and since other mycoplasmas that are polyclonal activators of B cells lack chemoattractants (393), it appears reasonable to assume that mitogenic and chemotactic activities toward B lymphocytes reside in different moieties of the *M. pulmonis* membrane.

The AIDS-associated *M. penetrans* is another species that most probably possesses more than a single mitogenic element acting upon mouse B lymphocytes, as well as stimulating human T cells (Table 5). Whereas antibodies to *M. penetrans* were detected in 40% of HIV-1-positive AIDS patients (466) and in about 20% of HIV-1-positive symptomless persons, only 0.3 to 1.3% of HIV-1-negative individuals had serum antibodies to *M. penetrans* (165, 466, 467). The high prevalence of *M. penetrans* infection in homosexual males and, even more, its probable association with progression of AIDS, has prompted research on the organism's ability to nonspecifically stimulate immune cells. Feng and Lo (124) first demonstrated that a group of lipid-associated membrane proteins (LAMPs), extracted with Triton X-114 from *M. penetrans* or from *M. fermentans* incognitus, stimulated proliferation and polyclonal Ig secretion by murine B lymphocytes. Since spleen cells from athymic nude mice responded to the mycoplasmal mitogenic preparations while splenic-T-cell-enriched populations responded only poorly, it was concluded that both *M. fermentans* incognitus and *M. penetrans* possess T-cell-independent B-cell mitogens. Data obtained by Sasaki et al. (401) showed that viable but not lysed cells of *M. penetrans* activated a certain population of cultured human peripheral blood T lymphocytes to undergo blast transformation and proliferation.

Activated T cells made up about 17% of the mononuclear population in blood samples from healthy donors and a similar portion (15%) of mononuclear cells obtained from HIV-1-positive individuals (401). The lymphocyte population that responded to *M. penetrans* was $CD3^+$ T cells of both $CD4^+$ (helper/inducer) and $CD8^+$ (cytotoxic/suppressor) phenotypes. Exposure of lymphocytes to *M. penetrans* resulted in expression of the early activation molecule CD69 and of the late activation markers HLA-DR and CD25 (the α chain of the receptor for IL-2). Increased levels of Fas (CD95) on the surface of activated human T cells were also noticed (401). Additional attempts by Blanchard (48) to identify the mitogenic elements of *M. penetrans* revealed that this organism is surrounded by a capsule-like material containing a glycolipid. This glycolipid partitioned together with the major surface lipoproteins (and probably free lipids) into the detergent phase

when *M. penetrans* cells were subjected to phase fractionation with Triton X-114. Purified glycolipid stimulated the proliferation of mouse B lymphocytes but not of human B or T lymphocytes (48). The response of murine lymphocytes to purified glycolipid was inhibited by polymyxin B; the results resembled the effects of this cationic polypeptide on proliferation mediated by *E. coli* LPS. However, and unlike LPS, *M. penetrans* glycolipid did not induce detectable levels of IL-1β or IL-6 mRNAs in cultures of murine spleen cells. These data suggest that at least the induction of proinflammatory cytokines (see "Induction of Up-Regulating Cytokines" below) and the apparent activation of human T cells by *M. penetrans* are mediated by cell components that differ from the mycoplasmal glycolipid and that *M. penetrans* exhibits several mitogenic moieties.

In fact, studies by Anaby and Naot (6), in line with the data of Feng and Lo (124), showed that potent mitogenic substances partitioned into the hydrophobic phase following Triton X-114 phase fractionation of *M. penetrans*. Gel electrophoresis and immunoblotting experiments with the mitogenic hydrophobic phase revealed several antigenic bands, including a mitogenic lipoprotein of 35 to 38 kDa. Furthermore, polymyxin B, which inhibited the mitogenic effects of the *M. penetrans* glycolipid (48), had no effect on the mitogenic responses of murine spleen cells to *M. penetrans* lipoprotein (7).

Taken together, the data accumulated so far suggest that *M. penetrans* possesses several mitogenic factors able to stimulate mouse B cells. These elements include a glycolipid as well as a membrane major lipoprotein of 35 to 38 kDa. While the mitogenic lipoprotein is also highly immunogenic in *M. penetrans*-positive AIDS patients, triggering the production of specific antibodies (126, 156, 165, 466), the glycolipid is not recognized by sera obtained from *M. penetrans*-infected persons (48). Although at present the nature of the *M. penetrans* cell components that stimulate human T cells is unknown, additional evidence on the mitogenic potential of mycoplasmal lipoproteins was provided by Brenner et al. (63), who demonstrated that purified spiralin stimulated the in vitro proliferation of human blood mononuclear cells as well as murine B lymphocytes.

Migration, activation, and effector functions of leukocytes depend on adhesion molecules including mucin-like molecules, selectins, integrins, and molecules of the Ig superfamily. These surface adhesion ligands, which bind to their corresponding receptors, mediate interactions between cells of the immune system and between them and the extracellular matrix. The group of costimulatory adhesive β_2-integrins including LFA-1, MAC-1, and CR4, commonly found on lymphocytes, macrophages, neutrophils, and dendritic cells, strongly binds to their intercellular cell adhesion molecule ligands present on activated endothelial cells and on leukocytes, including APC and B cells. Interestingly, Anaby and Naot (6) found that the mitogenic activity of *M. penetrans* hydrophobic components was inhibited, in a dose-dependent manner, by a monoclonal antibody to CD18, the β subunit of β_2-integrins, and that this monoclonal antibody recognized the 35- to 38-kDa mitogenic lipoprotein in immunoblots.

The observation that *M. penetrans* shared an antigenic epitope with CD18 molecules may imply that this antigenic determinant plays a role in stimulation by *M. penetrans* of murine macrophages and/or B lymphocytes. However, additional studies are needed to establish whether an intercellular cell adhesion molecule serves as a receptor for *M. penetrans* mitogenic elements on macrophages or B lymphocytes or both. Giron et al. (156) demonstrated that *M. penetrans* contains a 65-kDa protein that binds to fibronectin, resembling the fibronectin binding activity of the β_1-integrin VLA-5. Together, these observations indicate that some cell constituents of *M. penetrans* may display adhesion-like activities as do those of leukocyte integrins. In this context, it should be mentioned that antigenic epitopes shared by different mycoplasmas and host cells were already demonstrated in the past and proposed as possible candidates involved in either evasion of host defense mechanisms or induction of autoimmune responses observed during the course of infections with different mycoplasmas (46, 93). More recent data reported by Kirchhoff et al. (225) showed the existence of shared epitopes between *M. arthritidis* membrane proteins and chondrocytes obtained from rat and human tissues. It was also reported that homologies exist between certain regions in the adhesin peptides of both *M. genitalium* and *M. pneumoniae* and amino acid sequences contained in the costimulatory CD4 polypeptide of Th cells, as well as with sequences present in class II MHC proteins (384). Elucidation of the roles of these newly identified homologous sequences and cross-reactive epitopes that exist in both mycoplasmas and host cells may be important in future attempts to clarify the mechanisms by which mycoplasmas interact with their host immune cells as well as to elucidate the possible involvement of these cross-reactions in pathogenesis of autoimmune manifestations induced by mycoplasmas.

The *M. arthritidis* superantigen MAM. The most thorough and intriguing investigations on the interactions between mitogenic mycoplasmas and immune system cells were carried out on *M. arthritidis*, an agent of chronic proliferative arthritis of rodents. The results of most of these experiments, extensively reviewed by Cole et al. (83, 84, 86), will be described here only in brief. As summarized in Table 5, viable and sonicated heat-treated cells of *M. arthritidis* are capable of stimulating mouse, human, and rat lymphocytes in a nonspecific mitogenic manner, activating predominantly T cells and to a lesser extent B lymphocytes (91). While mouse B cells were activated by membrane components of *M. arthritidis* that resisted incubation for 2 h at 60°C, T lymphocytes responded to a unique heat-labile soluble exoprotein, found in senescent cell-free culture supernatants of *M. arthritidis* (91). The mitogen, originally termed MAS and later purified and called MAM (90), triggered T-cell proliferation and development of cytotoxic T cells (91). It became evident that responsiveness to the mitogen is controlled at the level of accessory cells and requires presentation of MAM in association with a nonpolymorphic region of the Eα chain of murine H-2E or human HLA-DR MHC class II-encoded molecules (91). Further studies indicated that the *M. arthritidis* mitogen is also able to interact, to some extent, with the Eβ chain of H-2E and HLA-DR (39, 86, 337) and also with MHC class II molecules encoded by H-2A and its human homolog HLA-DQ (92). However, and unlike specific antigens, processing of the *M. arthritidis* mitogen was not required for its presentation by MHC class II-bearing cells (283). MAM could be presented not only by monocytes and macrophages but also by dendritic cells (41), as well as by B lymphocytes (404, 461).

As summarized in Table 5, MAM stimulated T-cell clones displaying variable phenotypes and activities (83, 84, 86, 129, 283). However, regardless of its ability to act upon $CD4^+$ or $CD8^+$ cells, MAM stimulated only selective clones of T lymphocytes (35, 283). These observations led to the postulation that MAM acts as a bivalent molecule, on the one hand depending on expression of MHC class II-encoded molecules, serving as the mitogen binding sites on accessory cells, while on the other hand being controlled by non-MHC genes at the level of the responding T-cell clones (35, 283). Matthes et al. (283) pointed out the similarities between this bivalent activity

of MAM and the activity displayed by staphylococcal enterotoxins, which were shortly thereafter classified as superantigens. The first definite proof showing that MAM is indeed a superantigen and that reactivity of mouse T-cell clones to MAM depends on the variable parts (Vβ) of the αβ T-cell receptor (TCR) was provided by the group of B. C. Cole (89). They showed that T-cell clones expressing the Vβ 8 gene family and Vβ 6 products responded to MAM while T lymphocytes from mice not expressing Vβ 8.1, Vβ 8.2, or Vβ 8.3 alleles (the RIIIS mouse strain) lacked the ability to recognize and respond to the Eα-MAM complex presented to them. Table 5 summarizes the current knowledge on additional Vβ-restricted gene families of human, mouse, and rat T-cell clones stimulated by MAM.

Superantigens are potent activators of T cells and act upon lymphocytes regardless of their antigenic specificities. Unlike classical antigens that are processed by APCs and presented to MHC-restricted T cells bearing the corresponding antigen-specific TCR, superantigens do not require processing and the entire molecule binds to class II MHC molecules. Superantigens presented as a superantigen-MHC II complex to syngeneic as well as to allogeneic lymphocytes do not bind within the conventional peptide binding cleft but interact with the lateral, exposed face of the Vβ domain of TCR. However, the interactions of these bivalent molecules depend upon the Vβ alleles of the β chain of TCR and react with a restricted group of TCR Vβ gene family products. Comparison of MAM with other superantigens showed differences in their relative potentials to stimulate lymphocytes obtained from human versus murine origins. Unlike superantigens derived from the human pathogens *Staphylococcus aureus* and *Streptococcus pyogenes*, which vigorously activate human lymphocytes, MAM acted more effectively upon mouse lymphocytes than upon human lymphocytes (83, 129). This may not be surprising, since *M. arthritidis* is a murine rather than a human pathogen.

Both proliferation of lymphocytes (97) and induction of IFN-γ (60) were more extensive when MAM interacted with murine rather than with human lymphocytes. It should be noted, however, that Brand et al. (60) reported high levels of monocyte-derived proinflammatory cytokines such as IL-1, IL-6, and TNF-α in human peripheral blood mononuclear cell (PBMC) cultures exposed to MAM, in contrast to the low levels of cytokines induced in these cultures by staphylococcal enterotoxin B (SEB). In contrast, Rink et al. (378), who compared the efficiency of MAM toward human and murine cells, reported that although MAM triggered the induction of TNF-α mRNA in human PBMC, only a small portion of the mRNA molecules were translated into protein. On the other hand, high levels of active TNF-α were produced in murine cell cultures stimulated by MAM. Differences in the levels of cytokines produced by human PBMC obtained from genetically different individuals can be expected, and this may explain the discrepancy between the results in the latter reports (60, 378).

Nevertheless, the higher responses of murine lymphocytes to MAM, compared to those of human lymphocytes, were explained by higher affinity of MAM to the murine Eα chain than to the human DRα chain of the class II MHC molecules, leading to more effective presentation of MAM by murine cells (83, 404). Another possible mechanism that can be postulated to explain the differences between murine and human lymphocyte responsiveness to MAM is based on the differential affinities of MAM to murine and human TCRs. This suggestion derives from the observed differences in the affinity of MAM to various Vβ gene family products among human (18, 42) and murine (87, 89) T-cell clones. Moreover, the data suggested that MAM does not react with a single segment of the Vβ domain of TCR, and additional parts of the TCR, including Vα, may be involved in the formation of MAM–T-cell bonding (129). It is therefore possible that the degree of responsiveness to MAM is influenced quantitatively by the affinities of MAM to its receptors—the class II MHC molecules—as well as by the relative affinities of different domains on TCR molecules toward MAM. It has yet to be defined whether other, non-MHC, non-TCR costimulatory adhesion molecules are also involved in the interactions of MAM, APCs, and T cells and whether these molecules influence the extent of these interactions.

In addition to its interactions with T cells, MAM affects B lymphocytes. MAM has been found to enhance the responses of human and mouse B cells (85, 97, 461). Although MAM by itself did not trigger significant stimulation of isolated B lymphocytes, $CD4^+$ T cells bearing a MAM-reactive Vβ allele caused, in the presence of MAM, polyclonal differentiation of B cells into Ig-secreting cells. B lymphocytes expressing class II MHC molecules, bound MAM and presented it to MAM-reactive T-cell clones in a non-MHC restricted manner. These Th–MAM–B-cell interactions resulted in polyclonal activation of both B cells and Th cells. In the presence of a specific antigen, such as sheep erythrocytes (SRBC) these interactions led to enhanced proliferation and differentiation of B cells producing antibodies to SRBC (461). As mentioned above, in vivo administration of MAM to mice caused a temporary anergy of cell-mediated immune responses while enhancing the production of specific antibodies to SRBC and ovalbumin (85). Considering the in vitro and in vivo effects of MAM on B cells, these data suggest that MAM-responsive Th2 cells reacted with MAM bound to class II molecules on B cells. As a result of this cross-linking and formation of Th2-MAM-B complex, IL-4 and IL-6 were secreted by the activated Th cells and Ig production was induced by B lymphocytes linked to MAM.

Early attempts to purify MAM from *M. arthritidis* broth cultures were hampered by its sensitivity to heat (1 h at 56°C) and proteolysis and by its tendency to stick to glass and plastic surfaces and form noncovalent complexes with high-molecular-weight constituents of broth media. MAM was recently purified to homogeneity, and the purified protein (27 kDa) exhibited the biological properties described for the partially purified superantigen. The availability of purified MAM enabled the cloning, sequencing, and expression of the MAM gene, shown to encode 213 amino acids with a calculated molecular mass of 25,193 Da and a deduced pI of 10.1 (90). The MAM gene could be found in 32 different *M. arthritidis* strains but not in human mycoplasmas (90). The MAM sequence showed no homology to other microbial superantigens, indicating that MAM is a unique molecule of *M. arthritidis*. Synthetic oligopeptides corresponding to various regions of MAM, used in inhibition assays, indicated that MAM contains two active domains. One domain shared short amino acid sequences with antigenic determinants of the MHC II binding domain of other superantigens. This domain, included within the region of amino acids 11 to 38 of MAM, is probably active in the interactions of MAM with MHC class II antigens. The second active domain of MAM, containing amino acids 71 to 95, shared the 7-residue motif β, a consensus sequence present in legume lectins, which is important in T-cell activation. Interestingly, high levels of concanavalin A 2–16 (ConA 2–16), containing this motif, inhibited MAM-induced proliferation, while MAM oligopeptide 71–95 partially inhibited proliferation mediated by ConA (90). These cross-reactions between ConA 2–16 and the MAM 71–95 region suggest that an additional secondary pathway for T-cell activation may be exhibited by MAM (90). It should be emphasized that so far, *M. arthri-*

tidis is the only mycoplasma known to produce a superantigen. Searches for putative soluble superantigens in culture supernatants of *M. pneumoniae*, *M. fermentans*, *M. hominis*, *M. arginini* (91), and *M. penetrans* (401) gave no indication for the existence of MAM-like active mitogens acting on human lymphocytes.

Activation of NK cells. The ability of mycoplasmas to interact with immune system cells is not restricted to lymphocytes and is also manifested in other cells comprising the immune system. Augmentation of the lytic activity of mouse and human NK cells was induced in vitro by *M. orale* (47) and in vivo in mice by *M. pulmonis* (243), and in cattle infected with *M. bovoculi* (322). Swing et al. (442), who studied the in vitro NK activity of mouse cells exposed to *M. pulmonis*, found no correlation between in vivo virulence of various *M. pulmonis* strains or resistance of different mouse strains to this mycoplasma and their in vitro stimulation of NK activity. Nevertheless, their study, together with previous data, demonstrated that different mycoplasma species induce enhanced activity of NK cells. These reports did not distinguish between direct activation of NK cells by the mycoplasmas and possible indirect effects induced by NK cell-augmenting cytokines, such as IFN-α (47), IFN-γ (243), TNF-α, IL-1, or IL-2, produced in cultures of unfractionated leukocytes exposed to mycoplasmas. This issue was addressed by D'Orazio et al. (108), who showed that MAM directly affected the activity of isolated NK cells purified from human PBMC. They also showed that IL-2, produced by MAM-activated T cells present in unfractionated cultures of PBMC, contributed to the overall enhancement of NK cell activity mediated by MAM.

Activation of monocytes and polymorphonuclear cells. Similar direct and cytokine-mediated indirect mechanisms may underlie the observed effects of mycoplasmas on expression of class I and class II MHC antigens on the surface of macrophages. Stuart et al. (432) reported the enhanced expression of class II MHC molecules on the surface of a murine myelomonocytic cell line triggered by heat-stable membrane components of *M. arginini*, *M. arthritidis*, *M. hominis*, *M. pneumoniae*, and *M. pulmonis* and the induction of both class I and class II MHC gene expression in mouse bone marrow-derived macrophages exposed to *M. pulmonis* and *M. arginini*. Class II antigens were also induced on mouse and rat B lymphocytes exposed to *M. pulmonis* (392). Further studies showed that TNF-α, but not IFN-γ, IL-4, or granulocyte-monocyte colony-stimulating factor (GM-CSF), was probably involved in the amplified expression of MHC antigens induced by *M. arginini* (431), thus confirming that at least with this mycoplasma, both direct and indirect mechanisms may be responsible for the inducing effects on MHC antigen expression. *M. fermentans* incognitus was found to produce a protein (12 to 15 kDa) that triggered increased transcription and expression of MHC class I and class II antigens in murine cell lines and class II molecules in human monocytic cell lines (434). The proteinaceous factors of *M. fermentans* and *M. arthritidis* that elicited MHC antigens resisted heating (65°C for 1 h) but were sensitive to trypsin (434).

Unlike these stimulatory effects induced by *M. fermentans* protein factor, Frisch et al. (143) reported that the lipopeptide factor (MDHM) isolated from *M. fermentans*, as well as heat-killed organisms, down-regulated the induction of IFN-γ-dependent expression of class II MHC antigens. This effect was mediated by both increased turnover of preexisting molecules and inhibition of newly formed class II antigens on murine macrophages. The investigators claimed that type I IFNs, prostaglandins, nitric oxide, TNF-α, IL-6, IL-10, and transforming growth factor β (TGF-β) were probably not involved in the MDHM-induced suppression. Although they were unable to detect the production of IL-10 in their cultures of MDHM-stimulated murine macrophages, it remains to be clarified whether this cytokine, known to be secreted by human monocytes stimulated by *M. fermentans* (353), is indeed not responsible for the decreased expression of MHC antigens on macrophages. While the conflicting data (143, 434) on the effects of *M. fermentans* on expression of MHC antigens may represent differences in the mycoplasmal strains used (Incognitus versus D15-86), it is also possible that initial interactions of this mycoplasma with monocytes results in augmented expression of MHC antigens, induced by a protein factor, with a subsequent activation of lymphocytes and production of up-regulating cytokines. These stimulatory events are then followed by down-regulation of IFN-γ-induced MHC expression, exerted by the mycoplasmal MDHM.

Modulation of MHC molecules on the surface of macrophages is, in fact, only one of multiple effects that are exerted by mycoplasmas upon monocytes/macrophages. Previous data reviewed in the mid-1980s (91, 93, 189, 263) and numerous studies reported and summarized since then (281) clearly established that mycoplasmas interact both in vivo and in vitro with peritoneal and alveolar macrophages, synovial cells, polymorphonuclear granulocytes, brain astrocytes (64, 65), blood monocytes (31, 349, 413), interdigitating and follicular dendritic cells (41), and different monocytic cell lines. Despite these multiple interactions, the organisms seem to be relatively resistant to phagocytosis in susceptible hosts and to replicate on the surface of phagocytic cells, unless opsonized by specific antibodies (91, 93, 189) or complement components (281). In fact, it has been shown that at early stages of infection, the ability of alveolar macrophages to kill *M. pulmonis* differed among various mouse strains and thereby controlled their susceptibility or resistance to infection (179). Moreover, in vitro studies with *M. pulmonis*, peritoneal macrophages, and lymphocytes obtained from a susceptible mouse strain showed that the cytokines produced during mitogenic stimulation of the lymphocytes did not increase the phagocytic properties of macrophages toward *M. pulmonis* (91). These numerous observations have led to the suggestion that the ability to evade phagocytosis, displayed by certain species and strains of mycoplasmas in susceptible hosts, is at least partially responsible for their capacity to mount a chronic persistent infection (91, 93, 189, 281). Monocytic cells exposed to mycoplasmas are therefore rendered sensitive to suppressive or stimulatory effects induced by the organisms. These effects depend on both the cells and the mycoplasmas involved.

Studies on the interactions between *M. dispar* and bovine alveolar macrophages (4) clearly demonstrated that the capsular polysaccharides surrounding this organism inhibit the activation of macrophages, as attested by decreased glucose consumption and decreased production of TNF-α and IL-1. It should be mentioned in this context that indirect suppression of macrophages and polymorphonuclear phagocytes may also occur due to production of down-regulating cytokines, such as IL-10 (336, 353, 379), IL-13 (501), and possibly also TGF-β, or by the release of prostaglandins that down-regulate host cell functions, as demonstrated for *M. hyopneumoniae* and porcine polymorphonuclear cells (15). These data support the notion that perturbation of phagocyte functions exerted directly and/or indirectly by certain mycoplasmas may contribute to their capacity to evade the host natural defense mechanisms.

In contrast to the down-regulating influences of certain mycoplasmas, an increasing body of evidence shows that interactions of mycoplasmas with monocytic phagocytes and granulocytes often result in activation of these cells. Although murine

macrophages exposed to *M. pulmonis* or *M. arthritidis* are not phagocytizing the organisms efficiently, these macrophages exhibit increased phagocytic capacity toward inert carbon and toward intracellular *Listeria monocytogenes* (91). The activated macrophages secrete hydrolytic enzymes and complement C3a product and display increased levels of acid phosphatase and enhanced uptake of glucosamine (91). Studies with *M. arginini* (480), *M. capricolum* (17), *M. fermentans* (17, 64, 209), *M. mycoides* (209, 386), and *M. bovis* (209) demonstrated the production of nitric oxide by mycoplasma-activated mouse macrophages and by stimulated rat brain astrocytes (64). In addition, formation of oxygen radicals has been noticed following interactions of *M. arthritidis* with rat polymorphonuclear granulocytes (225, 296) and *M. capricolum* with murine macrophages (17). These multiple stimulatory effects, mediated by mycoplasmas, were also reflected in enhanced cytotoxicity of macrophages toward syngeneic, allogeneic, and xenogeneic tumor target cells (230, 263).

Enhancement of tumoricidal activity by mycoplasmas was often carried out in concert with the action of IFN-γ (443, 463, 480), pointing out the synergistic effects of mycoplasmas and cytokines on mononuclear cells. Studies to elucidate the mechanisms by which mycoplasmas trigger macrophage tumoricidal activity have clearly established that the organisms induced the production of TNF-α that subsequently played a major role in toxicity toward tumor cells (12, 149, 413, 414). Mycoplasmas activate macrophages by a mechanism different from that used by LPS inasmuch as they act upon macrophages from LPS-nonresponsive C3H/HeJ mice (263, 299, 413, 414, 443, 463). The effects of mycoplasmas were not inhibited by polymyxin B (463, 480) and synergized with LPS in triggering TNF-α production by macrophages from LPS-responsive mice (413, 414). It appears that while mycoplasmas evade phagocytosis, they interact with mononuclear and polymorphonuclear phagocytes and exert either suppressive or stimulating effects upon them by a combined action of direct and indirect cytokine-mediated effects. Phagocytes activated by mycoplasmas secrete a diverse group of molecules including enzymes, prostaglandins, toxic oxygen radicals and peroxides, nitric oxide, proinflammatory cytokines, and chemokines. All of these mediators have profound local and systemic effects (discussed below). Of interest are the reports (64, 65) on induction of TNF-α, prostaglandin E_2, and nitric oxide in rat glial cells by mycoplasmas, in a manner similar to that observed in several neurological diseases.

Induction of Up-Regulating Cytokines

The ability of mycoplasmas to elicit the production of different types of interferons has been demonstrated long ago (see reference 91 and references therein). However, the dramatic progress in our knowledge of the complex network of cytokines and their multiple regulatory effects on immune and inflammatory host responses and upon nonimmune tissues resulted in extensive accumulation of new data demonstrating the ability of mycoplasmas to induce the production of a wide range of cytokines (147). It became evident that stimulation of host immunocytes by mycoplasmas, as reflected by enhanced expression of surface molecules, proliferation, differentiation, and augmented effector functions, is also manifested by the release of different soluble mediators that play a major role in the outcome of mycoplasma-immune cell interactions. Table 6 summarizes the current information on the cytokines induced by different mollicute species and the nature of mycoplasmal cell components involved in the induction of these cytokines.

Proinflammatory cytokines. A common feature documented for many mycoplasmas (Table 6) is their ability to stimulate macrophages and monocytes to secrete proinflammatory cytokines, which are responsible for local and systemic inflammatory responses, reflecting the host natural defense mechanisms. TNF-α is a principal mediator of inflammation, cachexia, fever, release of acute-phase proteins, septic shock, and hemorrhagic necrosis of tumor cells. This cytokine is produced mainly by mononuclear phagocytes but also by Th cells, NK cells, and other non-immune system cells. TNF-α exerts a broad spectrum of dose-dependent, local and systemic effects upon a wide range of host cells and tissues. Since most of the activities of TNF-α overlap with those exhibited by IL-1, another mediator of inflammation produced by mononuclear phagocytes and other cells, these two cytokines are discussed together. TNF-α and IL-1 are important cofactors in the activation of T and B lymphocytes and promote lymphocyte proliferation and differentiation into effector cells. Both cytokines upregulate the cytocidal activity of macrophages and large granular NK cells and enhance the metabolic activity of polymorphonuclear cells. By inducing increased expression of MHC antigens they potentiate antigen presentation by APCs. Immune system cells costimulated by TNF-α or IL-1 express enhanced levels of receptors for cytokines and produce cytokines, α- and β-chemokines, and prostaglandins. The ability of TNF-α and IL-1 to cause enhanced expression of adhesion molecules on endothelial cells, together with the chemotactic activities of chemokines and TNF-α-mediated expression of adhesion molecules on the surface of neutrophils, triggers the increased recruitment of leukocytes to local sites of inflammation. In addition, TNF-α and IL-1 exert local necrosis and tissue destruction. Although high doses of TNF-α inhibit in vitro replication of bone marrow progenitor cells, both TNF-α and IL-1 stimulate the production of GM-CSFs and enhance in vivo hematopoiesis, thereby increasing the pool of cells to be stimulated and chemoattracted to the site of inflammation. Such effects induced by TNF-α and IL-1, released in response to interactions with mycoplasmas, may provide an explanation for most of the inflammatory and pathologic manifestations observed in mycoplasmal infections.

IL-6 is another pleiotropic, proinflammatory cytokine induced by many mycoplasmas (Table 6). This cytokine is produced by monocytes and macrophages as well as by Th and B lymphocytes and other non-immune system cells. IL-6 exhibits some activities that overlap with those of TNF-α and IL-1. Nevertheless, its major activity is mediated by its ability to serve as a cofactor in B-cell differentiation and maturation into Ig-secreting cells. IL-6 also enhances the expression of IL-2 receptor (IL-2R) on activated cells and induces the production of IL-2 by Th cells. This cytokine promotes the proliferation of T lymphocytes and amplifies hematopoiesis. IL-6, like TNF-α and IL-1, also triggers the production of acute-phase proteins in the liver. It is of interest that except for *M. hyopneumoniae*, *M. mycoides* subsp. *mycoides*, and *M. salivarium*, all the mycoplasmas that induce IL-6 have been identified as mitogens for mouse B lymphocytes, suggesting an active role for IL-6 in the observed mycoplasma-derived B-cell differentiation into plasma cells secreting antibodies of polyclonal specificities. Although many species of mycoplasmas induce TNF-α, IL-1, and IL-6 (Table 6), the nature of mycoplasmal receptors on the surface of mononuclear phagocytes is unknown. It has been established, however, that cross-linking between the *M. arthritidis* superantigen MAM and murine class II Eα chain or human HLA-DR-presenting accessory cells led to the induction of proinflammatory TNF-α, IL-1, and IL-6 in these MAM-stimulated cells (Table 6).

TABLE 6. Mycoplasma-induced cytokine production

Organism	Origin of cells	Cytokine(s) produced	Nature of inducing components	Reference(s)[a]
M. arginini	Human	IL-1β, IL-6, TNF-α	Membrane lipoproteins, heat and proteinase K resistant	178, 231, 352
	Mouse	INF-α, IFN-γ	Heat-stable membrane protein; lipid- or carbohydrate-associated peptide	480
		GM-CSF	Membranous protein, papain sensitive	433
M. arthritidis	Human	IL-1α, IL-1β, IL-6, TNF-α, IL-2, IL-4, IFN-γ, IL-8, IL-10, GM-CSF	MAM *M. arthritidis*-derived superantigen	2, 3, 31, 60, 83, 226, 287, 315, 377–379
	Mouse (C3H/HeJ, SJL and C57BL/6)	IL-6, TNF-α, IL-2, IL-4, INF-α, IFN-γ, IL-10, GM-CSF	MAM Membranes, papain sensitive, heat stable	35, 85, 186, 337, 378, 379, 433
	Rat	IFN-γ	MAM	
	Ovine	IFNα	Not defined	
M. bovis	Bovine	TNF-α	Not defined	209
M. capricolum	Human	TNF-α	Membranous	413
	Mouse (C3H/HeJ)	TNF-α	Membranous, heat stable, alkaline labile, hydrophobic, trypsin resistant	69, 413, 416
	Rat	TNF-α	Not defined	65
M. fermentans	Human	IL-1β, IL-6, TNF-α, IL-10, GM-CSF	MALP-2 (MDHM) 2-kDa lipopeptide; lipoproteins, heat and proteinase K resistant; 48-kDa membrane lipoprotein, proteinase sensitive, partially sensitive to lipoprotein lipase; glycoglycerolipid containing phosphocholine	38, 118, 148, 172, 201, 233, 282, 299, 300, 352, 353, 398
	Mouse (C3H/HeJ)	IL-1β, IL-6, TNF-α, IL-2, IL-4	MALP-2 (MDHM) and heat-stable elements	148, 300–302, 349
	Rat	TNF-α	Not defined	65
	Ovine	INF-α	Not defined	
M. gallisepticum	Human	IL-1β, IL-6, TNF-α, INF-α	Not defined	231
	Mouse (C3H/HeJ)	TNF-α	Membranous, heat, trypsin and acid resistant, alkali labile	416, 443
M. hominis	Human	TNF-α	Not defined	201
M. hyopneumoniae	Porcine	IL-1, IL-6, TNF-α	Not defined	14
M. hyorhinis	Human	IL-1β, IL-6, TNF-α	Water-soluble protein, periodate-sensitive, 69–75-kDa and multiple detergent-soluble proteins	231, 234
	Ovine	IFN-α	Not defined	
M. mycoides subsp. *mycoides*	Mouse (C3H/HeJ)	IL-1α, IL-6, TNF-α	Not defined	386
	Bovine	TNF-α	Not defined	209

Continued on following page

TABLE 6—*Continued*

Organism	Origin of cells	Cytokine(s) produced	Nature of inducing components	Reference(s)[a]
M. neurolyticum	Mouse	GM-CSF	Membranes	154
M. orale	Human	IL-1β, IL-6, TNF-α, IFN-α	Not defined	47, 201, 231
	Mouse	TNF-α	Heat stable	149, 263
	Ovine	IFN-α	Not defined	
M. penetrans	Human	IL-1β, IL-6, TNF-α	Lipoproteins, heat and proteinase K resistant; methanol-soluble, heat-stable, carbohydrate-containing molecule	201, 352, 353, 402
	Mouse (C3H/HeJ)	TNF-α		201
M. pneumoniae	Human	IL-1β, IL-6, TNF-α, IL-2, IFN-α, INF-β, IFN-γ, GM-CSF, sIL-2R	Membrane lipoproteins, heat and proteinase K resistant	13, 70, 200, 231, 253, 270, 307, 437
	Mouse	IL-1β, IL-6, TNF-α, IL-2, IFN-α, IFN-γ, IL-10, sIL-2R	Not defined	12, 336
	Ovine	INF-α	Not defined	
M. pulmonis	Mouse	IL-1α, IL-1β, IL-6, TNF-α, IFN-γ	Not defined	122, 318
	Rat	IL-2	Membranes	154, 309
	Ovine	IFN-α	not defined	
M. salivarium	Human	IL-1β, IL-6, TNF-α, IFN-α, IFN-γ	Not defined	231
M. synoviae	Human	IFN-α	Not defined	
	Ovine	IFN-α	Not defined	
A. laidlawii	Human	IL-1β, IL-6, TNF-α, IFN-α	Not defined	201, 231
	Mouse	TNF-α, IFN-α	Not defined	12
	Ovine	IFN-α	Not defined	
Spiroplasma monobiae (*MQ-1*)	Human	TNF-α	Membranes	414
	Mouse (C3H/HeJ)	TNF-α	Hydrophobic acylated proteins, 68 and 15 kDa, heat and protease sensitive	414, 415

[a] For references published before 1985, see reference 91.

GM-CSF. Immature and committed progenitor bone marrow cells differentiate and proliferate in response to GM-CSFs. These factors were released by human PBMC cultured with *M. pneumoniae* membranes (270) or exposed to partially purified preparations of MAM (310). Human lung fibroblasts infected with *M. fermentans* secreted GM-CSF (118) as well as IL-6. Mouse spleen cells produced GM-CSFs when stimulated by membranes obtained from *M. neurolyticum* (154), *M. arginini*, or *M. arthritidis* (433). Interestingly, these factors, elicited by heat-stable, papain-sensitive components of *M. arginini* and *M. arthritidis*, were also secreted by spleen cells from SJL and C57BL/6 mice (433), which are unresponsive to MAM (91). These data suggest that a protein differing from the highly heat-labile superantigen was responsible for the GM-CSF-

stimulating activity of *M. arthritidis*, at least in cultures of mouse splenocytes. Although a definite identification of cells producing CSFs in unfractionated cell cultures exposed to mycoplasmas is still unavailable, the experiments conducted by Stuart et al. (433) with bone marrow-derived cells strongly indicate that monocytic cells rather than lymphocytic cells are responsible for the production of GM-CSFs. It is worth mentioning that along with a possible direct effect of mycoplasmas upon progenitor cells, TNF-α and IL-1 secreted by mycoplasma-activated monocytes are capable of triggering production of GM-CSFs by monocytes and Th cells. There is no doubt, however, that CSFs enhancing myelopoiesis are produced by cells exposed to certain mycoplasmas, either directly or indirectly via production of other cytokines.

IL-2. Mitogenic interactions of mycoplasmas and T lymphocytes lead to the production of T-cell-derived cytokines, such as IL-2, IFN-γ, or IL-4. These cytokines, exhibiting multiple effects on immune cells, regulate lymphocyte activation and influence the effector phases of immune responses. IL-2 is a major upregulator, stimulating in an autocrine and paracrine manner the proliferation and differentiation of activated T lymphocytes, bearing IL-2 receptors, into effector Th cells or cytotoxic T cells. This cytokine is also effective in stimulating large granular lymphocytes and macrophages and in promoting the activation and growth of B lymphocytes. IL-2 is secreted primarily by naive $CD4^+$ Th cells and by differentiated $CD4^+$ Th1 populations, but it is also secreted by other subpopulations of T lymphocytes and by LGLs. Cells affected by IL-2 and mycoplasmas can theoretically be further stimulated to secrete variable cytokines possessing agonistic or antagonistic properties. The mycoplasmal superantigen MAM elicited the production of IL-2 in cultures of MAM-reactive mouse spleen cells and T-cell hybridomas in the presence of MHC class II-bearing accessory cells (35, 91). Cultured human PBMCs and spleen cells also produced IL-2 in response to MAM. However, injection of MAM into mice caused a transient decrease in the ability of splenocytes to produce IL-2 (85).

IL-2 was produced by rat lymphocytes stimulated by *M. pulmonis* (154) and probably also by concanavalin A-stimulated thymocytes exposed to *M. fermentans* (301). Unlike these T-cell-stimulating mycoplasmas, *M. pneumoniae* is a potent B-cell activator with a questionable mitogenic activity toward human T lymphocytes (91). Conflicting data were reported on the capacity of *M. pneumoniae* to mediate the production of IL-2. Makhool et al. (270) were unable to detect IL-2 in cultures of human PBMC exposed to mitogenic doses of *M. pneumoniae* membranes. On the other hand, Kita et al. (231) reported IL-2 production by *M. pneumoniae*-stimulated human PBMC. These differences may be explained by previous exposure of human blood donors to *M. pneumoniae*, undetected by serologic assays. Alternatively, the conflicting data may be due to the use of different *M. pneumoniae* strains (Eaton FH and MAC, respectively) or may result from differences in the sensitivity of the assays used. More recent observations by Pietsch et al. (336), in agreement with Makhool et al. (270), support the notion that IL-2 is induced by *M. pneumoniae* in previously sensitized lymphocytes only. They reported that genes for IL-2 and IL-2 receptor were expressed only during reinfection of mice with *M. pneumoniae* and not during primary infection. Moreover, they noticed that expression of IL-2 mRNA declined very rapidly, even during a second exposure to *M. pneumoniae*.

IFNs. Another group of potent immunoregulators with multiple effects on immune cells are the IFNs, induced by many species of mycoplasmas (Table 6). IFNs share an ability to interfere with viral replication and inhibit cell growth. They enhance the cytotoxic activity of NK cells and display antiproliferative effects on bone marrow progenitor cells. On the other hand, they promote the differentiation of stimulated immune cells. IFN-α is produced by activated mononuclear phagocytes and leukocytes, including B cells, whereas IFN-β is secreted mainly by virus-infected fibroblasts or by other activated tissues. These two cytokines are discussed together since they exert similar antiviral and other antiproliferative effects on immune and nonimmune tissues. IFN-γ is secreted by activated naive $CD4^+$ T cells, differentiated $CD4^+$ Th1 cells, $CD8^+$ T lymphocytes, and NK cells. IFN-γ is a potent upregulator of immune responses while being less potent than IFN-α and IFN-β in its antiviral activity and its capacity to inhibit tumor growth. IFN-γ and, to a much lesser extent, IFN-α and IFN-β enhance phagocytosis and the release of proinflammatory cytokines and induce the expression of class I MHC antigens, thus amplifying the cytotoxicity of cytotoxic T lymphocytes against virus-infected cells. IFN-γ but not IFN-α or IFN-β also induces class II MHC antigens and promotes the accessory functions of APCs. It should be noted that high levels of IFN-α inhibit class II MHC induction by IFN-γ. Generally, IFN-γ produced by T cells and NK cells promotes the cognitive phase of immune responses and triggers a shift toward the effector stages of immune responses. In this context, IFN-γ also primes macrophages for an additional stimulus that will subsequently trigger the production of mediators, such as TNF-α and nitric oxide, leading to tumor cytotoxicity (see "Activation of monocytes and polymorphonuclear cells" above). IFN-γ promotes the division of Th1 cells while inhibiting the growth of Th2 lymphocytes. It also influences activated B cells and triggers murine plasma cells to switch into plasma cells producing IgG2a and IgG3.

From previously summarized data (91) and from those presented in Table 6, it is evident that a wide variety of mycoplasmas can elicit the production of IFN-α in leukocyte cultures. The most prominent inducer of IFN-γ is MAM, as was revealed in many studies with cultured cells from different origins (91). The data on the in vitro production of IFN-γ by murine splenocytes exposed to *M. arthritidis* are compatible with the demonstrated capacity of this mycoplasma to induce cytotoxic T lymphocytes in vitro (91). IFN-γ production was also observed in cultures of human PBMC exposed to *M. pneumoniae* (13, 231) and *M. salivarium* (231) and probably occurs in cultures of mouse cells exposed to *M. arginini* (91). In vivo studies revealed enhanced expression of IFN-γ mRNA in the lungs and spleens of *M. pneumoniae*-infected mice (336) and in the lungs and bronchoalveolar lavage fluid of mice infected with *M. pulmonis* (122, 318). On the other hand, in mice inoculated with viable and nonviable *M. arthritidis* (91) or with MAM (85), IFN-γ production was not elicited. The lack of in vivo secretion of IFN-γ in MAM-inoculated animals was explained by a possible hyporeactive state induced by high levels of IFN-α (91) or by the in vivo shift from Th1 (IFN-γ-producing) to Th2 (IL-4 producing) populations, induced by MAM. It should be noted that the *M. arthritidis*-derived superantigen mediated differential induction of IFN-γ in human lymphoid cells, depending on the origin of lymphocytes (377). The levels of this cytokine were higher in PBMC than in spleen cells, suggesting that results observed with lymphocytes from one organ do not necessarily reflect the effects exerted upon other lymphoid organs (377).

It is still undetermined whether *M. pulmonis* triggers IFN-γ production by cultured T lymphocytes. Although *M. pulmonis* stimulated IL-2-producing T lymphocytes, cytotoxic T lymphocytes did not develop in response to mitogenic stimulation with *M. pulmonis* in cultures of rat lymph node cells (309). On the

other hand the ability of this *Mycoplasma* species to augment the activity of NK cells, known producers of IFN-γ (243), may explain the observed in vivo production of IFN-γ mRNA (122, 318) in cells obtained from lung tissues. As mentioned above, it is doubtful whether T lymphocytes are mitogenically stimulated by *M. pneumoniae*, and it is still not clear whether *M. arginini* (397) stimulates T cells. Therefore, the observed capacity of these mycoplasmas to trigger IFN-γ points to the possibility that mycoplasma-stimulated NK cells are responsible for IFN-γ production.

IL-4. IL-4 is another T-cell-derived cytokine, secreted by Th cells (especially the Th2 population) and by NK cells. This cytokine is a costimulator of B lymphocytes and mediates B-cell proliferation and differentiation into plasma cells secreting IgE. It also activates class II MHC molecules upon B cells and monocytes and enhances Th2 proliferation. IL-4 is a growth factor for mast cells and basophils and triggers chemoattraction of monocytes and eosinophils. IFN-γ and IL-4 have some opposing activities. The production of IFN-γ by Th1 cells is inhibited by IL-4, while IFN-γ interferes with IL-4 induction of class II antigens on B lymphocytes. In contrast to IFN-γ, IL-4 inhibits the production of proinflammatory IL-1 and TNF-α by monocytes, thereby exerting its anti-inflammatory effects on monocytes. At present, only *M. arthritidis* and possibly *M. fermentans* are known to mediate the production of IL-4. Rink et al. (377) showed that human PBMC and spleen cells secreted IL-4 when responding to MAM. However, in contrast to IFN-γ, IL-4 levels were higher in spleen cultures than in PBMC cultures. In agreement with these data are the observations of Cole et al. (85), who showed that spleen cells from MAM-inoculated animals produced increased levels of IL-4, a cytokine characteristic of Th2 subsets. Further experiments reported by the group of Mehindate (287) clearly established the anti-inflammatory nature of IL-4. This cytokine, when added exogenously, down-regulated MAM-induced expression of IL-1β and TNF-α genes and induced strong expression of IL-1R antagonist (IL-1Ra) mRNA in MAM-stimulated monocyte cell lines. It should be mentioned, however, that both Al-Daccak et al. (3) and Rink et al. (379) reported that human and mouse leukocytes exposed to MAM (without external addition of IL-4) produced high levels of proinflammatory cytokines rather than the antagonist IL-1Ra. IL-4 is probably also produced in cultures of murine ConA-stimulated thymocytes, exposed to sonicated *M. fermentans* cells (301). This conclusion is based on the observation that monoclonal antibodies to IL-4 abrogated the generation of cytotoxic T lymphocytes in these cultures. Unlike *M. arthritidis* and *M. fermentans*, which trigger the production of IL-4 under certain conditions, other mycoplasmas (*M. arginini*, *M. gallisepticum*, *M. hyorhinis*, *M. orale*, *M. pneumoniae*, *M. salivarium*, and *A. laidlawii*) tested by Kita et al. (231) failed to induce IL-4 in human PBMC.

In vivo cytokine effects. The documented differences in cytokine levels induced in cells obtained from different lymphoid organs, combined with the multifunctional properties described for individual cytokines, as well as the complex network of synergistic or antagonistic influences among cytokines, hinder our ability to extrapolate in vitro data obtained with a single mediator to the in vivo situation where a combined panel of cytokines may be produced in parallel and act simultaneously. Obviously, the overall effects of a set of cytokines depends on the time course by which each cytokine is produced and consequently on their relative concentrations at each time point. In addition, it should be noted that mycoplasma-mediated release of soluble cytokine receptors, such as soluble IL-2R (sIL-2R) observed in patients infected with *M. pneumoniae* (200, 437), or production of cytokine receptor antagonists like IL-1Ra (3, 287, 379) can affect the overall effects of cytokines. These molecules bind to their corresponding cytokine or to the cytokine surface receptors, respectively, and thereby block the action of cytokines upon target cells. Nevertheless, despite these limitations, certain conclusions can be drawn about the major significance of cytokines during interactions of mycoplasmas with host immune cells. As mentioned above, at least part of the tumoricidal activity of macrophages acquired in response to stimulation by mycoplasmas can be attributed to the release of TNF, toxic oxygen species, and nitric oxide by activated macrophages (147, 149).

In vivo elicitation of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 occurred following infection of pigs with *M. hyopneumoniae* (14) and inoculation of mice with either *M. pneumoniae* (336) or with *M. pulmonis* (122, 318). In addition, mRNAs encoding GM-CSF (336) and the up-regulating cytokine IFN-γ were detected in lung tissues and in cells present in bronchoalveolar lavage fluid from animals exposed to *M. pneumoniae* or *M. pulmonis* (122, 307, 318, 336). Levels of IL-1β in serum (253) and of IFN-α in both nasopharyngeal secretions and sera (307) were elevated in most patients infected with *M. pneumoniae*. Lymphocytes isolated from *M. pneumoniae* patients produced IFN-γ in response to *M. pneumoniae* antigens, especially if they were obtained during convalescence (307). It should be mentioned, however, that in human subjects, *M. pneumoniae* caused a transient anergy of cell-mediated immune responses during the acute phase of disease. Patient PBMC produced decreased levels of IFN-γ in response to the specific recall antigen purified protein derivative (46).

Faulkner et al. (122) demonstrated that TNF-α was the first cytokine produced in mice exposed to *M. pulmonis*. IL-1 and IL-6, were detected later, followed by IFN-γ. Moreover, studies by Pietsch et al. (336) revealed that the levels of TNF-α and IL-6 secreted following a second exposure of mice to *M. pneumoniae* were 10-fold higher than those secreted after primary inoculation and that the expression of IFN-γ mRNA was 50-fold higher during the anamnestic response. Unlike the enhanced expression of these cytokines, expression of IL-2 and IL-2R genes in lung tissue occurred only during reinfection of mice with *M. pneumoniae* and for a very limited period. Parallel to the decline of IL-2, a rapid increase in expression of the gene for the down-regulating cytokine IL-10 was observed (336). Interestingly, when mice depleted of $CD4^+$ T cells were reinfected with *M. pneumoniae*, reduced lung infiltration was accompanied by decreased expression of TNF-α, IL-1β, and IL-6 and by complete abrogation of IL-2 and IL-2R mRNA expression (327). Elevated levels of sIL-2R were detected in patients infected with *M. pneumoniae* (437). Ito et al. (200) suggested that high levels of sIL-2R in serum correlate with disease severity. These observations strongly suggest an active role for lung-infiltrating $CD4^+$ T cells, monocytes, and neutrophils, secreting toxic molecules, as well as TNF-α, IL-1β, IL-6, GM-CSFs, IL-2, IL-2R, and IFN-γ, in the local inflammatory lesions and in the pathogenesis of mycoplasma-induced respiratory diseases.

From the data reported so far, a sequence of events can be proposed. Following respiratory tract infection, mycoplasmas interact with mononuclear phagocytes and induce production of proinflammatory cytokines as well as α and β chemokines, such as IL-8 and MCP-1 (419). These potent mediators of inflammation enhance the expression of adhesion molecules on the surface of endothelial cells and neutrophils. Leukocytes are then attracted by mycoplasmal cell components (393) and by chemokines and accumulate at the site of inflammation. Lymphocytes, including B and T lymphocytes, are activated in

a polyclonal manner by the combined effects of mycoplasmal mitogens (91, 309, 420) and cytokines. The activated lymphocytes proliferate, differentiate, and secrete Igs of multiple specificities. An additional panel of up-regulating cytokines, such as IFN-γ, T-cell-derived-chemokines, MIP-1α, and MIP-1β, secreted by activated cells, amplifies the inflammatory responses (419). It is still undetermined whether IL-2 is produced in vivo at late stages of primary infection or only after reinfection with respiratory tract pathogens. Down-regulation of acute respiratory tract inflammatory responses may occur when cytokines such as IL-10 (336) or IFN-α (307), together with soluble cytokine receptors (200, 437) or possibly with other down-regulating, anti-inflammatory cytokines such as IL-4, TGF-β, and IL-13 or cytokine antagonists, accumulate and exert their anti-inflammatory effects. Differential killing of invading organisms by alveolar macrophages from various hosts (179, 281), differences in the capacity of various *Mycoplasma* strains to induce the production of cytokines by monocytes (209) and to polyclonally stimulate lymphocytes (309, 420), and variation in the potentials of different host strains and species to mount an inflammatory response (122) may have a major impact on the resulting virulence of mycoplasmas infecting the respiratory organs and on disease manifestation in different hosts.

At present, there is only limited information about in vivo secretion of cytokines and chemokines during mycoplasma-induced joint diseases, genitourinary tract inflammatory diseases, and autoimmune manifestations and following infections with AIDS-associated mycoplasmas. As described above (see "The *M. arthritidis* superantigen MAM"), only part of the panel of cytokines induced by MAM in vitro have also been examined in mice injected with MAM. Synergistic effects between *M. orale* cell components and TNF-α have been shown to cause hemorragic necrosis and lethal shock in mice (394). These data and the demonstration that *M. fermentans* (including incognitus) induced TNF-α, IL-1β, and IL-6 in vitro (Table 6) provide a possible explanation for the early observations of Gabridge et al. (146), who demonstrated toxic shock in mice inoculated with *M. fermentans* K10. Furthermore, the multifunctional proinflammatory cytokines induced by *M. fermentans* and *M. penetrans* in cell cultures most probably play a major role in the cytopathogenic effects of these AIDS-associated mycoplasmas. There is little doubt that future studies on the time course and relative levels of in vivo-produced cytokines and chemokines will clarify the importance of these mediators in different mycoplasma-induced manifestations.

As summarized in Table 6, cytokines are induced by viable and nonviable mycoplasma cells and by mycoplasmal membrane fractions. Hydrophobic *M. gallisepticum* and *M. capricolum* membrane components, alkali labile, but heat and trypsin resistant (69, 443), were found to be responsible for augmentation of murine macrophage tumoricidal activity and release of TNF-α. Isolated cell components of *M. arginini* were shown to synergize with IFN-γ in enhancing macrophage tumoricidal activity. Peptides (2.5 to 4 kDa), probably linked to either lipid or carbohydrate and resistant to heat and protease treatment, were apparently responsible for the effect (480). In agreement with these data, membrane lipoproteins of *M. arginini*, resistant to heat and proteinase K, elicited proinflammatory cytokines in human monocytes (178, 352). Heat-stable components from *M. arginini* also induced enhanced expression of MHC molecules on murine bone marrow cells (433). However, the membranous elements of *M. arginini* that induced GM-CSFs were sensitive to digestion with papain, although they were thermostable (433). It thus appears that MHC antigens and proinflammatory cytokines are elicited by membrane entities of *M. arginini* that differ from those inducing GM-CSFs.

Interestingly, partially purified MAM induced the production of GM-CSFs in cultures of human PBMC (310) whereas a heat-stable protein was responsible for GM-CSF production in murine bone marrow cells (433). These results indicate that MAM-induced TNF-α and IL-1 trigger the subsequent production of GM-CSFs by MAM-activated cells, present in cultures of human blood mononuclear cells. An additional protein in *M. arthritidis* membranes is probably responsible for the direct stimulation and cell division of bone marrow cells that secrete GM-CSFs (433). Differentiation between membrane entities of *Spiroplasma monobiae* (MQ-1) possessing mitogenic potentials and those eliciting TNF-α production by murine cells was reported by Sher et al. (414, 415). Evidence for the presence of multiple TNF-α-inducing molecules in *M. hyorhinis* was provided by Koystal et al. (234), who showed that following Triton X-114 extraction of mycoplasmal cells, proteinase-K labile TNF-α-inducing proteins were recovered in both the aqueous and detergent phases (Table 6). It should be noted that the molecules identified by Koystal et al. (234) as TNF-α inducers in human monocytes are not necessarily identical to the factors of *M. hyorhinis* that stimulated the proliferation of mouse B lymphocytes and were reported to have different molecular weights from these factors (347). Taken together, these data suggest the existence of several active cytokine inducers in a single *Mycoplasma* species.

Investigations performed in several laboratories indicate that *M. fermentans* is yet another species possessing more than a single molecule interacting with immune cells. Extensive studies by Muhlradt et al. (300) revealed a nonmitogenic membrane-associated hydrophobic lipopeptide, originally designated MDHM by them and later renamed macrophage-activating lipopeptide-2 (MALP-2). The 2-kDa molecule consists of a 14-amino-acid chain starting with *S*-(2,3-bisacyloxypropyl)cysteine and two fatty acid residues in ester linkage. Whereas MDHM could be isolated from delipidated cells of *M. fermentans* D15-86, a unique major immunogenic phospholipid was isolated by Salman et al. (398) from *M. fermentans* incognitus. This peculiar choline-containing phosphoglycolipid was an effective immunogen (38, 282) and displayed a capacity to trigger TNF-α production by human PBMC (398). Koystal et al. (233) found an additional TNF-α inducer in *M. fermentans* incognitus identified as a 48-kDa lipoprotein, highly sensitive to proteinase K and only partially sensitive to digestion by lipoprotein lipase. In accordance with these data, an *M. fermentans* gene encoding a 48-kDa monocyte differentiation/activation factor was later cloned and sequenced by Hall et al. (172). *M. fermentans* incognitus also contains membrane lipoproteins which, unlike the 48-kDa lipoprotein (233), resisted heat and proteinase K treatments (352, 353). These latter lipoproteins also differed from MDHM in their action on the human myelomonocytic cell line THP-1 and were postulated by Rawadi and Roman-Roman (352) to represent yet another panel of active cytokine inducers contained in *M. fermentans*. The cytokine-inducing molecules differ from the mycoplasmal proteins of 15 to 30 kDa that exhibited cytocidal activity and were not associated with lipid.

Similarly, *M. penetrans* contains heat- and proteinase K-resistant lipoproteins that mediated the production of proinflammatory cytokines in human monocytes (352). In addition, extraction of *M. penetrans* membranes with chloroform-methanol yielded, in the methanol layer, a carbohydrate-containing molecule capable of triggering TNF-α in murine cell lines (201). The activity of this molecule was inhibited by ConA-sepharose but not by the LPS inhibitor polymyxin B. Appar-

ently, *M. penetrans* lipoproteins (352) and the carbohydrate-containing factor (201) that induced cytokine differ from the mitogenic capsular glycolipid of *M. penetrans* (48), which was unable to elicit IL-1β or TNF-α in murine splenocytes and was inhibited by polymyxin B.

Evidently, further studies are required to identify and isolate the cytokine-inducing molecules of most *Mycoplasma* species. The data reported so far indicate that membrane lipoproteins play a major but not necessarily an exclusive role in the induction of proinflammatory cytokines by mycoplasmas. The active domain of membrane lipoproteins may vary among different mycoplasmas in its sensitivity to proteinase K or lipoprotein lipase. Generally, the activity of the mycoplasmal cytokine-inducing elements differs from that of LPS. Mycoplasmal products induce cytokines in cells from C3H/HeJ mice that respond poorly to LPS. Furthermore, cytokine induction is not inhibited by the LPS inhibitor polymyxin B (178, 201, 352, 353). The results also show that the same *Mycoplasma* species may carry several cytokine-inducing molecules that are not necessarily identical to the mycoplasmal mitogenic elements.

Autoimmune Manifestations

Several mechanisms were postulated to operate in perturbation of tolerance to self antigens and development of autoimmune disorders. These mechanisms include the sharing of antigenic determinants by the infectious agent and host tissues and/or modified self antigens that stimulate autoimmune responses. Activation of autoreactive T cells as a result of a nonspecific stimulus or due to a breakdown in down-regulating mechanisms, as well as stimulation of B lymphocytes by polyclonal B-cell activators, can also lead to development of autoimmune manifestations. The ability of many mycoplasmas to modulate immune responses by inducing nonspecific suppressive or stimulatory effects upon host macrophages and lymphocytes, and the ability of mycoplasmas to elicit up- and down-regulating cytokines may lead to perturbation of host immune homeostasis. A previous review described in detail the variety of cross-reactive antigens and autoantibodies to host tissues, commonly produced during *M. pneumoniae* infections, and discussed the possible mechanisms that underly these systemic autoimmune reactions (46). More recent studies (29, 123, 319) provide some new information on the nature of the autoantibodies and the possible mechanisms for their induction in *M. pneumoniae* infections. However, there are still more questions than answers concerning the pathophysiology of the postinfection sequelae, mostly neurological disorders affecting some of the patients.

The possible involvement of mycoplasmas in the pathogenesis of organ-specific, autoimmune rheumatic diseases was reviewed by Cole et al. (93) and later by Barile et al. (23). Although extensive efforts to isolate viable mycoplasmas, ureaplasmas, spiroplasmas, or chlamydiae from synovial fluids of rheumatoid arthritis (RA) patients have generally failed (23), infection of different hosts with mycoplasmas can cause acute and chronic joint diseases that in many aspects resemble human inflammatory arthropathies, including RA (23, 93). The introduction of PCR into mycoplasma diagnostics (363) has provided a very sensitive tool to test for the presence of mycoplasmal genomic DNA in synovial fluids of RA patients. *M. fermentans* was detected by PCR in the joints of 8 of 38 RA patients, leading to the conclusion that the presence of *M. fermentans* in the joints is associated with inflammatory rheumatic diseases, including RA (405). However, in more recent PCR studies of synovial fluids of a larger group of RA patients (406) and in studies with *Mycoplasma* genus-specific primers (183), the results were much less conclusive. Whether *M. fermentans* or any other mycoplasma triggers or perpetuates an existing disease or behaves as a passenger remains conjectural.

Following the identification of microbial superantigens, these potent T-cell activators, acting upon selective Vβ TCR-bearing clones, were proposed as possible agents involved in the induction of autoimmune responses. The potential of *M. arthritidis* to cause acute and chronic inflammatory arthritis in rodents, together with the discovery that this mycoplasma produces the potent superantigen MAM (83), prompted Cole and associates to investigate the effects of MAM in vivo. Although MAM by itself failed to induce chronic arthritis in mice (85), the investigators found that intra-articular administration of MAM to DA rats produced local joint inflammation and swelling that resolved a week later (67). Systemic injection of MAM into mice caused a temporary anergy of cell-mediated immune responses with a simultaneous enhancement of B-cell activation and differentiation (85). The exposure to MAM caused increased secretion of IL-4 and IL-6 cytokines, known to augment B-cell growth and antibody production (85). In vitro experiments demonstrated the formation of B-cell–MAM–Th-cell bridges (142, 461) that resulted in activation of both MAM-reactive Th-cell clones and MAM-linked B lymphocytes that differentiated into plasma cells producing polyclonal IgM and IgG. In the presence of a specific antigen, such cognate interactions between antigen-specific B-lymphocyte MAM and MAM-reactive Th cells may trigger an enhanced production of antigen-specific antibodies by activated B cells that received the required help from Th cells stimulated by MAM (142, 461). These data, combined with the observed cross-reactivity between *M. arthritidis* antigens and rat chondrocytes (225) and the numerous reports on the ability of MAM to induce inflammatory cytokines (Table 6), strongly support the postulated role of MAM in the development of autoimmune inflammations (142, 343, 461). In fact, Cole and Griffiths (88) were able to show that MAM influenced the course of murine arthritis produced by immunization of mice with porcine type II collagen. When MAM, SEB, or staphylococcal enterotoxin A (SEA) were administered to mice previously immunized with suboptimal doses of porcine collagen that did not by themselves produce arthritis, the superantigens MAM and SEB but not SEA triggered the onset of disease in these animals. In addition, MAM exacerbated the disease in mice convalescing from collagen-induced arthritis (88). In view of these data and the common Vβ TCR used by MAM, SEB, and the T-cell clones activated in murine collagen-induced arthritis, as well as the similar human T-cell clones reactive to MAM and those found in synovial tissues of RA patients, Cole and Griffiths (88) concluded that superantigens similar to MAM may play a role in human RA. Whether this notion can be substantiated will depend on the results of future studies.

Molecular Approaches to Vaccine Development

The frequent failure of antibiotics and other therapeutic approaches to eradicate mycoplasmas and abort the infectious disease process has led to the conclusion that development of effective vaccines is the most promising approach to control mycoplasma infections in humans and animals. The many efforts to develop effective and safe vaccines to mycoplasmas have been only partially successful. The overall success of formalin-inactivated *M. pneumoniae* vaccines in humans has been extremely disappointing, although successful inactivated vaccines and live attenuated vaccines have had some success in controlling some mycoplasma diseases in animals. No accept-

able live vaccine has been developed for human use (reviewed in reference 117).

The molecular characterization of P1, the major adhesin and a dominant antigen of *M. pneumoniae*, prompted trials to use purified P1 as a molecularly defined vaccinogen, assuming that antibodies to this protein will block attachment of the parasite and in this way prevent colonization and infection. The results of immunization of guinea pigs with P1 by Jacobs et al. were disappointing (368). Although the number of mycoplasmas in bronchial washings from the immunized animals challenged with virulent *M. pneumoniae* was much smaller than for unvaccinated animals, the lung tissues of the immunized guinea pigs showed no reduction of lymphohistiocyte infiltration and in some cases the immunized animals revealed an exacerbation of the cell-mediated response, making vaccination with purified P1 protein potentially harmful.

Genetic immunization (DNA vaccines) is a novel promising approach to vaccine production that has many of the advantages of live attenuated vaccines but with no risk of infection. There is no need for confounding factors, such as adjuvant, hapten carriers, or denaturing agents. It involves introducing DNA encoding a pathogen protein into host cells by using an expression library of the pathogen. The DNA vaccine effectively induces antibodies as well as cytotoxic T lymphocytes. Thus, both humoral and cell-mediated responses are induced—a distinct advantage (462). Barry et al. (26) prepared an *M. pulmonis* library by fusing digested mycoplasmal DNA onto the last exon of the gene encoding human growth hormone (hGH), using cytomegalovirus as a vector, so that hGH-*M. pulmonis* antigens might be secreted. Theoretically, a library expressing the entire genome of a pathogen could be used as a vaccine. Barry et al. (26) found that about 1 ng of DNA was required to produce an immune response by genetic immunization into the skin. Mice immunized with *M. pulmonis* libraries and challenged had no culturable mycoplasmas and no lung lesions.

Lai et al. (244) constructed an *M. pulmonis* genomic library cloned in λgt11 transfected into *E. coli*. Clones of *E. coli* expressing a fusion protein reactive with anti-*M. pulmonis* antibodies were transferred orally or intravenously into mice. Expression of fusion proteins in the mice was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to the drinking water, resulting in local and systemic antibody production and effective protection of the animals against large numbers of virulent *M. pulmonis*.

It is possible to select the epitope important as an immunogen by isolating the dominant antigen, prepare a monoclonal antibody to it, and use this antibody to select the *E. coli* clone producing the specific fusion protein carrying this epitope. Thus, the DNA vaccine is controllable. Such a specific DNA vaccine was developed for *M. hyopneumoniae*. Following screening of the mycoplasmal clone bank with hyperimmune pig serum, Fagan et al. (119) identified a clone with a sequence homology to the R2 subunit of ribonucleotide reductase. This clone was expressed in *E. coli* as an 11-kDa protein fused to β-galactosidase. Assessment of the vaccine potential of the fusion protein in pig trials showed the vaccinated pigs to be significantly protected against challenge with a virulent *M. hyopneumoniae* strain. Use of an attenuated *Salmonella typhimurium aroA* strain as a vector for delivery of the above immunogenic *M. hyopneumoniae* fusion protein protected mice against experimental infection with *M. hyopneumoniae* and thus has the potential of providing a cheap and easily adminstrable oral vaccine for swine (120).

There can be little doubt about the great potential of DNA vaccines, so that one may expect the rapid development of this new generation of vaccines. Whether the high hopes will be substantiated will have to await the results of large-scale field trials.

CONCLUSIONS

The recent mycoplasmal genomic projects have brought us much closer to achieving the goal of completely deciphering, in molecular terms, the machinery of a self-replicating cell. These projects have contributed most conspicuously to our understanding of the molecular biology of mycoplasmas and have opened new ways of looking at the evolutionary history of these organisms. There is now solid genetic support for the thesis that mollicutes evolved as a branch of gram-positive bacteria by a process of reductive or degenerative evolution. During this process, the mycoplasmas lost considerable portions of their ancestors' chromosomes but retained the genes essential for life. Thus, the mycoplasmal genomes carry a high percentage of conserved genes, a fact that considerably facilitates gene identification.

The significant genome compaction that occurred in mollicutes was made possible by adoption of a parasitic mode of life. The supply of many nutrients from their hosts resulted in the loss, during mycoplasma evolution, of the genes for many assimilative processess. The marked dependency on the exogenous supply of nutrients has hampered the in vitro cultivation of mollicutes, as reflected by the total inability to grow many mycoplasmas in axenic cultures. To keep to the parasitic mode of life, mycoplasmas have developed rather sophisticated mechanisms to colonize their hosts and resist the host immune system. A genomic price had to be paid for these functions, as reflected by the significant number of genes devoted to adhesion and to the generation of antigenic variation systems in mycoplasmas.

There can be little doubt that mycoplasmas are successful evolutionary creations; their wide distribution in nature points to this conclusion. Mycoplasmas are commensals or benign pathogens, causing mostly mild and chronic infections but rarely killing their host. It is possible that mycoplasma evolution is advancing toward symbiosis. Thus, spiroplasmas and phytoplasmas have little or no pathogenic effects on their insect vectors, although when transmitted to plants, they usually cause disease.

Do mycoplasmas represent minimal cells? It appears that *M. genitalium* is, thus far, the organism closest to the theoretical minimal cell capable of self-replication. It would be of interest to define the minimal set of essential genes in *M. genitalium* by selective inactivation or deletion of genes, testing the effects of each of these manipulations on survival and replication under defined conditions. It has been suggested that in the case of DNA replication, transcription, and translation, the minimal set of genes has already been established in *M. genitalium* and *M. pneumoniae*. Clearly, definition of the minimal set of essential genes depends to some extent on growth conditions, e.g., whether the minimal cell is growing in vitro or in the host, where adhesion and antigenic variation genes are expected to play an essential role. When discussing mycoplasmas as minimal cells, it should be emphasized that many mollicutes (acholeplasmas and spiroplasmas) have genomes two to three times the size of the *M. genitalium* and *M. pneumoniae* genomes. Acholeplasmas and spiroplasmas thus carry a definitely more complex genetic complement, equivalent in gene numbers to genomes of many wall-covered bacteria, such as *H. influenzae*.

Although the mycoplasmal cytoskeletal elements, associated with cell shape, cell division, motility, and adhesion to host cells, are expected to be relatively simple, our knowledge of

these elements is still very fragmentary. The mycoplasmal genome projects did not significantly advance our knowledge in these areas, apparently because most of the genes involved are unique and could not be annotated by comparison to known gene sequences available in data banks. It should be emphasized, in this context, that even with the small sequenced genomes of *M. genitalium* and *M. pneumoniae*, there is still a great deal to do in order to identify the unclassified ORFs that have no database match, prove the DNA-based predictions experimentally, and assign functions to proposed ORFs with hitherto unknown functions. Cell protein analysis, applying the proteome approach, and extensive tests of metabolic activities, are obviously some of the directions to take.

The relative structural simplicity and lack of a cell wall have been major factors favoring the use of mycoplasmas as models in studies on membrane structure and function. The easy manipulation of the composition of mycoplasmal membrane lipids has been exploited most effectively to elucidate the mode of organization and physical state of membrane lipids. The recent initiation of studies directed to the molecular definition of enzymes involved in glycolipid and phosphpholipid synthesis is expected to facilitate our understanding of the regulatory controls of membrane lipid composition in mollicutes under different growth conditions.

During their evolution and adaptation to a parasitic mode of life, the mycoplasmas have developed various genetic systems providing a highly plastic set of variable surface proteins. The uniqueness of the mycoplasmal systems is manifested by the presence of highly mutable modules combined with an ability to expand the antigenic repertoire by generating structural alternatives, all compressed into limited genomic sequences. In the absence of a cell wall and a periplasmic space, the majority of surface proteins involved in generating antigenic variation in mycoplasmas are lipoproteins. These surface components, anchored to the cell membrane via acyl chains, are the most dominant antigens, and their abundance in the mycoplasma membrane is remarkable. The generation of a versatile surface coat through high-frequency phase and size variation provides the organism with a useful tool for immune system avoidance, allowing the mycoplasmas to escape antibody attack.

Apart from providing a specific anti-mycoplasmal defense, the host immune system is also involved in the development of pathogenic lesions and exacerbation of mycoplasma-induced diseases. Mycoplasmas are able to stimulate as well as suppress lymphocytes in a nonspecific, polyclonal manner, both in vitro and in vivo. In addition to affecting various subsets of lymphocytes, mycoplasmas and mycoplasma-derived cell components modulate the activities of monocytes/macrophages and NK cells and trigger the production of a wide variety of up-regulating and down-regulating cytokines and chemokines. Mycoplasma-mediated secretion of proinflammatory cytokines, such as TNF-α, IL-1, and IL-6, by macrophages and of up-regulating cytokines by mitogenically stimulated lymphocytes plays a major role in mycoplasma-induced immune system modulation and inflammatory responses. On the other hand, the ability of some mycoplasmas to induce down-regulating cytokines, such as IL-10, IL-13, and possibly TGF-β, may contribute to the ability to evade host immune mechanisms by perturbation of macrophage accessory and phagocytic functions, thus decreasing the production of proinflammatory cytokines and T-cell proliferation and affecting the balance between different Th-cell subsets. The potential to either stimulate or suppress the immune system would impart a distinct advantage to any pathogen attempting to survive in the hostile and changing environment of the infected host.

It appears that certain mycoplasmas possess more than a single cell component displaying mitogenic activity and that mycoplasmal mitogens are not necessarily identical to mycoplasmal cytokine-inducing elements. However, except for the chemically characterized *M. arthritidis* superantigen MAM and the macrophage-stimulating lipopeptide from *M. fermentans*, there is an urgent need to identify and isolate the cell components that exhibit mitogenic and/or cytokine-inducing activities from the other mycoplasmas. At present, we are still unable to discern between the direct and indirect cytokine-mediated modulatory effects induced by mycoplasmas. This issue may be resolved by future studies with anti-cytokine specific antibodies and defined mycoplasmal cell components eliciting the various cytokines and those directly affecting immune cells.

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