The variable internal structure of the *Mycoplasma penetrans* attachment organelle revealed by biochemical and microscopic analyses: implications for attachment organelle mechanism and evolution

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Abstract

Although mycoplasmas have small genomes, many of them, including the HIV-associated opportunist *Mycoplasma penetrans*, construct a polar attachment organelle (AO) used for both adherence to host cells and gliding motility. However, the irregular phylogenetic distribution of similar structures within the mycoplasmas as well as compositional and ultrastructural differences among these AOs suggest that AOs have arisen several times by convergent evolution. We investigated the ultrastructure and protein composition of the cytoskeleton-like material of the *M. penetrans* AO by several forms of microscopy and biochemical analysis to discern whether the *M. penetrans* AO was constructed at the molecular level on principles similar to those of other mycoplasmas like *Mycoplasma pneumoniae* and *Mycoplasma mobile*. We found that the *M. penetrans* AO interior was generally dissimilar from other mycoplasmas in that it exhibited considerable heterogeneity in size and shape, suggestive of a gel-like nature. In contrast, several of the twelve potential protein components identified by mass spectrometry of *M. penetrans* detergent-insoluble proteins shared certain distinctive biochemical characteristics with *M. pneumoniae* AO proteins, though not with *M. mobile* ones. We conclude that convergence between *M. penetrans* and *M. pneumoniae* AOs extends to the molecular level, leading to the possibility that the less organized material in both *M. pneumoniae* and *M. penetrans* is the substance principally responsible for organization and function of the AO.
**Importance**

*Mycoplasma penetrans* is a bacterium that infects HIV-positive patients and may contribute to the progression of AIDS. It attaches to host cells through a structure called an attachment organelle (AO), but it is not clear how it builds this structure. Our research is significant not only because it identifies the novel protein components that make up the material within the AO that give it its structure, but also because we find that the *M. penetrans* AO is organized unlike AOs from other mycoplasmas, suggesting that similar structures have evolved multiple times. From this work we derive some basic principles by which mycoplasmas, and in fact, potentially all organisms, build structures at the subcellular level.

**Introduction**

Mycoplasmas, which are genomically reduced, cell wall-lacking bacteria that live parasitically or commensally in nature but can be cultured axenically, occupy a unique space within the field of bacterial cell polarization that makes them potentially informative concerning mechanisms by which polarization is achieved as well as the evolutionary origins of these mechanisms. Some mycoplasmas have a differentiated, prosthecal tip structure known as an attachment organelle (AO) at one pole (reviewed in 1). The AO houses adhesin proteins for interaction with host cells and surfaces, as well as machinery that carries out unidirectional gliding motility, and in some cases appears to be physically associated with the
chromosome (reviewed in 2). The interior of the AO contains a complex of structural proteins that serves as a cytoskeletal scaffold for AO assembly (reviewed in 2). In the sense that the AO is constructed at a cell pole and involved in functions associated with polarity, this structure parallels other polar structures of bacteria like the *Caulobacter crescentus* stalk (2).

The majority of information regarding the components, assembly, and substructures of mycoplasma AOs comes from studies of two different species, *Mycoplasma pneumoniae* and *Mycoplasma mobile*. These two species are members of two distantly related phylogenetic clusters within the genus (3). Characterization of the structures and proteins that make up the interior of the AO of each of these species has shown them to be very different both ultrastructurally and compositionally, with no AO protein homologs shared between these organisms 4; reviewed in 1), suggesting that like the *C. crescentus* stalk, these structures have evolved independently and recently as compared with other members of their respective phyla.

*Mycoplasma penetrans* is another species that has a polar AO, representing a third mycoplasma phylogenetic cluster (5-7). *M. penetrans* is best known for its association with AIDS patients, in whom it is commonly found in the urogenital tract, but it has also been isolated from an HIV-negative patient with antiphospholipid syndrome (5, 6, 8). The major focus of studies of *M. penetrans* has been its putative role as an AIDS cofactor (9-12). We previously showed that the *M. penetrans* AO contains material distinct in structure to that of *M. pneumoniae* and *M. mobile* (7). Furthermore, *M. penetrans* lacks any homologs of AO structural proteins.
of *M. pneumoniae* or *M. mobile* (4, 13, 14). Special difficulty in working with this organism stems from its genetic intractability, leaving biochemical and cell biological routes as the best options for its study.

The ability to establish cellular polarization and to localize specific biomolecules to non-uniformly distributed subcellular positions is critical for many bacteria. Many fundamental processes such as DNA segregation, cell division, and placement of specialized structures rely on morphological polarity as a cue for appropriate spatial organization. The cytoskeleton, composed of a network of polymeric proteins, plays an integral role in maintaining the morphological and functional integrity of bacterial cells by establishing polarity (reviewed in 15). In addition to the widely phylogenetically distributed MreB and FtsZ, which form dynamic filaments that contribute to the nearly universal bacterial processes of peptidoglycan synthesis and cell division (reviewed in 16), there is increasing evidence of a diverse array of other cytoskeletal elements with narrow phylogenetic distribution that facilitate more recently evolved functions (reviewed in 17). In contrast to the highly dynamic MreB and FtsZ, many of these proteins form static structures. The alpha-helical coiled coil-rich, intermediate filament-like protein crescentin from *C. crescentus* is associated with the establishment of cell shape (18, 19). Many bacterial scaffold proteins contain an abundance of coiled coil domains, important for oligomerization and close protein-protein interactions, that are referred to as coiled coil-rich proteins (CCRPs) (reviewed in 20). CCRPs serve in an array of functions such as maintaining cell morphology and enabling cell motility, often by participating in polar localization of proteins (reviewed in 19). The widely
distributed bactofilins are characterized by the conserved DUF583 domain, and form stable nucleotide-independent filaments that serve as spatial landmarks (21, 22). Other bacterial cytoskeletal proteins include DivIVA and PopZ, which assemble into 2- or 3-dimensional arrays at extant or nascent cell poles and interact with proteins involved in pole-associated processes (reviewed in 19). It is essential to understand processes that rely on bacterial cell polarization, and this understanding can be achieved by establishing an inventory of cytoskeletal proteins across bacterial phylogeny and determining their characteristics and how these properties enable them to carry out their functions.

We sought to identify the proteins of the interior of the *M. penetrans* AO and examine their properties in relation to other proteins of mycoplasma AOs. Because mycoplasma AOs seem to have evolved in a convergent manner we hypothesized that the proteins involved in structure and assembly of the *M. penetrans* AO would be distinct from those of other mycoplasmas, and we predicted that their composition would reveal important information about the properties of the AO. To test these ideas we investigated the protein content of the detergent-insoluble material of *M. penetrans*, which comprises the AO interior, and characterized the structure of this material. We found that although many of the proteins share some properties resembling corresponding proteins of *M. pneumoniae*, the *M. penetrans* AO is quite distinct from its counterparts in other mycoplasmas, consistent with convergent evolution of AOs and of mechanisms of polarization of bacterial cells. We also examined changes in gene expression of *M. penetrans* upon incubation with human cervical epithelioid HeLa cells to test whether AO and other genes were...
subject to transcriptional regulation when encountering the host, and found that they were not.

Materials and Methods

Bacterial culture

Hyperadherent *Mycoplasma penetrans* strain HP88 cells (23) were grown to mid-log phase at 37°C in plastic tissue culture flasks (Thermo Scientific BioLite) containing SP-4 broth (24).

Detergent extraction and protein analysis

Cultures of *M. penetrans* were decanted and washed twice with warm potassium-free phosphate-buffered saline (PBS; 145 mM NaCl, 2.83 mM NaH$_2$PO$_4$·H$_2$O, 7.20 mM NaHPO$_4$·7H$_2$O, pH 7.2). For detergent extraction, Triton X-100 or Tween-20 in 20 mM Tris-HCl –150 mM NaCl (pH 7.2) was added to the cultures to a final concentration of 1% in 25 ml PBS. Detergent extracts were then incubated for 30 minutes at 37°C, scraped, and centrifuged for 20 minutes at 17,050 x g. Whole cell lysates were treated the same without the addition of detergent. Following centrifugation the pellets were washed three times and resuspended in PBS. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce). Equal amounts of protein of each fraction were subjected to 9% SDS-PAGE (25) and stained with Coomassie Brilliant Blue or Sypro Ruby (Molecular Probes). Detergent-extracted proteins were separated by SDS-PAGE and select bands, chosen on the
basis of being abundant or enriched compared to total cell lysate protein, were excised, reduced and alkylated, digested with trypsin, and analyzed by ABSciex 4800 matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)/TOF at the University of Cincinnati Proteomics Laboratory as previously described (26). MultiCoil (27) was used to test predicted amino acid sequences for the presence of coiled coil domains; the presence of regions with >50% probability of forming alpha-helical coiled coils was recorded as positive.

Scanning electron microscopy (SEM)

Cells were prepared for scanning electron microscopy as previously described (28). Briefly, cells were grown on glass coverslips for 6 to 24 h in SP-4 media supplemented with 3% gelatin at 37°C. For examination of Triton X-100-insoluble (TXI) structures or Tween-20-insoluble (TWI) structures, detergent in 20 mM Tris-HCl, pH 7.5/150 mM NaCl was added to cells at a final concentration of 1% and incubated at 37°C for 30 min. The samples were fixed for 30 minutes at room temperature in 1.5% glutaraldehyde/1% paraformaldehyde/0.1 M sodium cacodylate, pH 7.2. Following fixation the coverslips were washed four times in 0.1 M sodium cacodylate, pH 7.2 and dehydrated through a series of ethanol washes from 25% to 100%. The coverslips were then critical point-dried, gold sputter-coated, and viewed on a Zeiss Supra 35 FEG-VP scanning electron microscope at the Miami University Center for Advanced Microscopy and Imaging.
**Electron cryotomography (ECT)**

*M. penetrans* was grown on Quantifoil Au-finder grids (Quantifoil Micro Tools GmbH) in SP-4 broth at 37°C until mid-log growth, indicated by a color change in the media from red to orange. The EM grids with attached *M. penetrans* cells were removed from the media, loaded onto tweezers, and washed with fresh media. Colloidal gold (10 nM) was added to the grids and they were blotted (Whatman, grade 40) prior to being plunge-frozen in liquid ethane on a gravity plunger freezer. Grids were loaded into a 300 kV FEI Polara G2 electro cryo-transmission electron microscope equipped with a field emission gun, a lens-coupled 4k x 4k Gatan UltraCam, and a Gatan energy filter (GIF). Samples were maintained in liquid nitrogen temperature as tilt series were captured of whole cells. The tilt-series was recorded from -60° to +60° with 1° increments at a 10-µm defocus using Leginon (29). All tilt series were collected through GIF around zero-loss energy with a slit-width of 20 keV for a cumulative dose of 180 e/Å² used for each tilt series.

**Measurements and analysis**

To measure the lengths of nucleoid-free zones, *M. penetrans* cells were grown overnight on cover slips, fixed as previously described (7), and mounted on slides with Vectashield containing DAPI (Vector Laboratories). Fields were imaged using a 100X objective as previously described (7). Phase-contrast and DAPI fluorescence images were overlaid and the lengths of 84 polar nucleoid-free zones were measured using SPOT software. To measure the lengths of TXI and TWI objects, SEM images of the objects from multiple fields, captured as described above, were
measured along their long axes. Objects that appeared to be clusters of individual objects were excluded. For TXI objects, 70 were measured, and for TWI objects, 79 were measured. Statistical comparison of lengths was done by one-way ANOVA after using a Shannon-Wilk test to establish that the distributions of lengths were not normal. Statistical comparison of the lengths of the cell and the nucleoid-free zones was performed using least squares regression analysis.

RNA extraction

RNA was harvested as previously described (30). *M. penetrans* cells were harvested by centrifugation at 17,400 \( x \) \( g \) for 20 min, followed by three washes in PBS. The cell pellet was resuspended in 1 mL of TRI reagent solution (Ambion). Total RNA was then purified using the Direct-zol RNA MiniPrep kit (Zymo Research) according to the manufacturer’s instructions. Remaining DNA was removed from the sample by treating with DNase I twice using the DNA-free kit (Applied Biosystems) according to the manufacturer’s instructions. Quantitative PCR was performed to ensure the RNA was free of DNA. The quality of the RNA was then examined using the Agilent 2100 Bioanalyzer (Agilent Technologies).

RNA sequencing (RNA-Seq)

*M. penetrans* cells were grown for 6 h in SP-4 broth at 37°C either alone or together with HeLa cells grown to 70-80% confluency at an MOI of 100. Three biological replicates were used for each of these conditions. For each biological replicate, RNA was extracted as described above and divided into three technical
replicates. Purified bacterial RNA was enriched by performing poly(A) depletion using the NEBNext Poly(A) mRNA magnetic isolation module (New England Biolabs) on all samples as previously described (30). The RNA was then depleted of both prokaryotic and eukaryotic ribosomal RNA using the Ribo-Zero Gold rRNA removal kit (Illumina) as directed by the manufacturer’s instructions and then cleaned and concentrated using the Zymo RNA Clean and Concentrator-25 kit (Zymo Research) as described previously (30). The RNA was eluted in 25 μl RNase-free water, examined for quality using an Agilent 2100 Bioanalyzer (Agilent Technologies), and used for cDNA synthesis.

cDNA libraries were created using the Illumina TruSeq stranded mRNA library preparation kit (Illumina) following the manufacturer’s protocol, as previously described (30). The cDNA libraries were examined for quality using the Agilent 2100 Bioanalyzer and then quantified using a library quantification kit (Kapa Biosystems). Libraries were then normalized to 10 nM, pooled, diluted, denatured, and loaded on to the MiSeq reagent kit v3 cartridge as directed by the manufacturer (Illumina). The samples were sequenced on a MiSeq system using a 75-bp paired-end approach.

Data from the RNA-Seq experiment were analyzed as described previously for *Mycoplasma gallisepticum* data (30). Briefly, the fastq files were assembled, mapped, and analyzed for differential gene expression using Rockhopper ([http://cs.wellesley.edu/~btjaden/Rockhopper/](http://cs.wellesley.edu/~btjaden/Rockhopper/)) with the *M. penetrans* HF-2 genome as the reference genome. Parameters were as described previously (30). For comparison of gene expression values the data were first normalized by
determining the ratio of reads per kilobase per million (RPKM) and then the fold change was calculated by taking the log₂ transformation of the RPKM data between the two biological samples.

Reverse transcriptase (RT)-coupled polymerase chain reaction (PCR) The RT reaction was carried out as previously described for *Mycoplasma iowae* (32). Briefly, cDNA synthesis was performed with 100 ng of total RNA from *M. penetrans* and random hexamers using the Verso cDNA synthesis kit (Thermo Scientific) according to the manufacturer’s instructions. One microliter of cDNA was then used as template in a 50-μl PCR with Taq polymerase (New England Biolabs) using EasyStart PCR tubes (Molecular Bioproducts) according to the manufacturer’s instructions. The primers used in each reaction were designed to span the adjacent gene junctions (Table 1). Reactions were amplified by incubating at 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 53°C for 1 minute, and 68°C for 1 minute, with a final extension of 5 minutes at 68°C. The PCR products were then subject to gel electrophoresis. Reactions with genomic DNA as well as without addition of RT or template were performed as positive and negative controls, respectively.
Results

Internal structure of the *M. penetrans* AO

As previously described, *M. penetrans* cells are elongated (Fig. 1B), with a polar tip structure that functions as an AO. The AO confers attachment and gliding motility, which contributes to cell division; dividing cells often take the form of two cell bodies linked by a membranous connecting filament (Fig. 1A; 7). *M. penetrans* cells attach to host cells by means of a pole, whose interior is distinctly differentiated from the cytoplasm of the rest of the cell despite being adjacent to it, as previously established by transmission electron microscopy (TEM) (5, 6).

Treatment of *M. penetrans* cells attached to plastic with the nonionic detergent Triton X-100 results in solubilization of a large amount of cellular material, leaving behind discrete detergent-insoluble objects with a range of dimensions as visualized by SEM (Fig. 1C; 7). Unlike the AO structures obtained similarly from species of the *M. pneumoniae* cluster (33), treatment with DNase had no effect on the appearance of these objects (not shown), indicating that they did not contain significant amounts of DNA.

Close examination of these TXI structures revealed that despite considerable heterogeneity in size and shape, they generally consisted of a wider, irregular, ball-like object, from which emanated one or more rod-like filaments which were often periodically punctuated with other material along the filaments. The structures had an average length of 320 ± 100 nm (Fig. 2A; n=70). This range of lengths was significantly ~15% smaller than that of the polar nucleoid-free zones of *M.*
penetrans cells (Fig. 2B; n=84; p<0.001), as revealed by overlaying phase-contrast images and images of the DNA stained by DAPI (Fig. 2G; 7). The mean length of the nucleoid-free zones was 380 ± 140 nm. In cells with single nucleoid-free zones, there was a positive correlation between the lengths of the cell and the nucleoid-free zone (Fig. 2D; n=52; p<0.0001), suggesting that growth of this structure is coupled to cell growth. In cells with two such zones at each pole, the length of the smaller one was correlated with cell length (Fig. 2E; n=16; p=0.04), whereas that of the larger one was not (Fig. 2F; n=16; p=0.52), consistent with the larger one being a pre-existing structure whose net growth has slowed or stopped.

To determine whether another nonionic detergent might yield similar structures, we used SEM to examine the structures that remained following extraction of cells grown under identical conditions with the chemically distinct Tween-20 (Fig. 1D). These objects were generally similar to those obtained using Triton X-100, although they were larger and had a generally smoother appearance. Indeed, their mean length of 380 ± 130 nm was identical to that of the nucleoid-free zones (Fig. 2C; n=79; p>0.8). The variability in sizes and shapes of the detergent-insoluble objects was recapitulated by ECT images, which showed ribosome-containing zones in the central area of the cell that were distinct from ribosome-free zones at both cell poles. These ribosome-free zones had irregular shapes and boundaries (Fig. 3A, B), often extending into the filament connecting cells undergoing division (Fig. 3C; 7). Given the similarities in size distribution, previous TEM images of similar material at M. penetrans poles (5, 6, 34), and the limited amount of space in an M. penetrans cell for large objects, we conclude that the polar
nucleoid-free zones visualized by DAPI staining, the polar ribosome-free zones visualized by ECT, and the DNA-lacking, detergent-insoluble objects visualized by SEM are the same objects, with Triton X-100 solubilizing some component that Tween-20 does not. Interestingly, whereas the poles of whole *M. penetrans* cells as visualized by SEM (Fig. 1B) or by light microscopy (Fig. 2G) were distinctly narrower than the cell bodies, in cells imaged by ECT there was no abrupt narrowing at the poles, with cells exhibiting a more ovoid shape (Fig. 3).

**Identification and characterization of detergent-insoluble proteins**

*M. penetrans* lacks homologs of the AO cytoskeletal proteins of other mycoplasmas (14). To determine what proteins make up the TXI and TWI material, we examined the protein profiles of whole-cell lysates, TXI proteins, and TWI proteins using SDS-PAGE. The detergent-insoluble material was generated by extracting attached *M. penetrans* cells in flasks and scraping the material that remained attached to the flasks, ensuring that it was identical to the material that was visualized by SEM, which consisted almost entirely of structures similar in shape but heterogeneous in size, as described above (Fig. 1C, 1D). Several proteins were prominent in the TXI and TWI fractions, and some were enriched compared to the whole-cell lysate (Fig. 4). Consistent with the similar appearances of the structures, the profile of TWI proteins was similar to that of the TXI ones, with the principal exception of one prominent TWI band migrating at ~40 kDa that was absent in the TXI fraction (Fig. 4). In general, the profiles featured mostly the same proteins but often in different relative amounts.
We selected eight bands from SDS-polyacrylamide gels that appeared enriched in both detergent-insoluble fractions compared to whole cell lysate, as well as the one band that was present in the TWI fraction but not the TXI fraction, for identification using MALDI-TOF (Fig. 4). The twelve proteins that were identified were associated with a variety of functional characteristics, including four with well-established biochemical roles, three putative lipoproteins, and five of unknown function (Table 2). The four with characterized biochemical roles were lactate dehydrogenase, ribosomal protein S3, pyruvate dehydrogenase subunit E2, and OppF, an oligopeptide ABC transporter ATP-binding protein. Two out of the three putative lipoproteins were identified as P35 and P42, both members of the P35 lipoprotein family; P42 was the protein that was present only in the TWI fraction. The third lipoprotein is a homolog of *Mycoplasma genitalium* G37 MG309. Of the five identified proteins that were uncharacterized, four are encoded by genes found very close to one another in the genome, MYPE1530, 1550, 1560, 1570. Together with MYPE4000, they share no specific sequence homology with other proteins except in the close relative of *M. penetrans*, *M. iowae* (35), but the first four were labeled as predicted cytoskeletal proteins in the original annotation of the *M. penetrans* genome, based on predicted extensive alpha-helical coiled-coil structure, reminiscent of *Mycoplasma pneumoniae* AO protein HMW2 (14), which is predicted to constitute a major structural element within the *M. pneumoniae* AO cytoskeleton (36-38).

The absence of sequence homology of these five proteins to the proteins of the AO cytoskeleton of *M. pneumoniae* (38) or the analogous structure in *M. mobile*
(39), together with the disparity in the detergent-insoluble structures between \textit{M. penetrans} and mycoplasmas from other phylogenetic clusters, is consistent with a model in which attachment organelles arose independently during the course of mycoplasma evolution multiple times (7, 40). We examined whether there were nonetheless general features shared in common among the cytoskeletal proteins of \textit{M. penetrans}, \textit{M. pneumoniae}, and \textit{M. mobile}. Interestingly, the five putative \textit{M. penetrans} cytoskeletal proteins have molecular weights $\geq$93 kDa, isoelectric points of $\sim$4.5, and predicted alpha-helical coiled-coil regions, all but MYPE4000 with extensive ones. Many of the proteins of the \textit{M. pneumoniae} attachment organelle, but not of the tip structure of \textit{M. mobile}, share these features (Table 3).

\section*{Cotranscription of putative cytoskeletal genes}

Examination of the genomic organization of the genes encoding proteins MYPE1530, 1550, 1560, and 1570, as well as predicted structural and compositional similarities among their predicted protein products, suggested that along with the genes encoding the structurally and compositionally similar proteins MYPE1520 and 1540 they might form a transcriptional unit. We performed RT-PCR to test whether these genes, each separated by no more than 30 bp, are cotranscribed. We used a primer complementary to a region near the 3' end of MYPE1570, which is positioned downstream of the other genes in question, to create cDNA. From this cDNA we amplified regions spanning the junctions between each of the six candidate genes (MYPE1520-1570) as well as MYPE1510, which is $\sim$180 bp away from MYPE1520 and on the opposite strand. We also made cDNA using a primer
complementary to MYPE1580, which is also ~180 bp away and attempted to
amplify cDNA linking MYPE1570 and MYPE1580. There were RT-PCR products for
all of the junctions between MYPE1520-1570, but no products for the MYPE1510-
1520 junction (Fig. 5A). Amplification of the junction between MYPE1570 and
MYPE1580 sometimes yielded a faint, poorly reproducible product, suggestive of
partial read-through (not shown). These results suggest that MYPE1520-1570
constitute an operon (Fig. 5B).

Transcriptomics of *M. penetrans* grown with HeLa cells

Because the major function of the AO is adherence to host cells, which is
expected to occur at the initial stages of infection, we hypothesized that the genes
involved in AO formation and function, including those encoding the proteins
identified in the TXI fraction, would be up-regulated during early stages of infection.
The interactions between *M. penetrans* and HeLa cells, including the events that
occur during invasion, have been documented (41-44). To identify genes that
undergo differential expression upon interaction with host cells, we performed
RNA-Seq on *M. penetrans* cells when grown alone and when grown in the presence
of HeLa cells 6 h after infection, representing a time point at which cytadherence
and invasion have occurred (43), and compared global gene expression profiles
between the two conditions. Surprisingly, there was almost no statistically
significant difference in gene expression between the two conditions, including the
AO genes, with the exception of three genes that were up-regulated and one gene
that was down-regulated by two-fold (Suppl. Table 1). The three up-regulated genes
were MYPE1035, 3985, and 8260, all of which encode small hypothetical proteins of less than 70 amino acids. The single down-regulated gene was MYPE20130, which codes for a tRNA for arginine whose corresponding codon is predicted to be commonly used; its expression was three-fold lower in mycoplasmas incubated with HeLa cells.

Discussion

Convergent evolution of attachment organelles

The overall organization of the AO-associated structures of *M. penetrans* was quite distinct from those of *M. pneumoniae* (45, 46), *Mycoplasma mobile* (39), and *Mycoplasma insons* (40), which represent three other phylogenetic lineages or clusters within the genus. Despite superficial similarities in morphology and function of the AOs of *M. pneumoniae* and *M. mobile*, dissimilarities in both organization and composition of their internal structures are well-documented (47). In contrast, the structure and composition of AO cytoskeletal structures are shared among the close relatives of *M. pneumoniae* in the *M. pneumoniae* cluster (33). These differences have been taken to suggest that the AOs of *M. pneumoniae* and *M. mobile* arose through convergent evolution (2), which is not altogether surprising considering that the two species are separated by one of the deepest phylogenetic divisions within the genus, with *M. pneumoniae* in the pneumoniae group and *M. mobile* in the hominis group (3). However, the subsequent finding that *Mycoplasma*
insons, a species in the pneumoniae group that is not a close relative of M. pneumoniae, is unidirectionally motile but lacks a distinct tip structure and instead contains cytoskeletal elements throughout its entire rod-shaped body (40) raised the possibility that even within the pneumoniae group there has been independent evolution of polar attachment and motility. If so, then these structures have an even more recent evolutionary origin. Alternatively, the M. insons organization could represent a variation of the M. pneumoniae organization whose homology is not obvious, pending further studies of this organism. M. penetrans is also in the pneumoniae group, but within a different cluster than either M. pneumoniae or M. insons (48). The AO of this species, whose motility is driven by a fundamentally different mechanism than that of M. mobile (23), provided an opportunity to test whether there were detectable, conserved elements of AO organization within the pneumoniae group.

The absence of homology between M. penetrans and M. pneumoniae AO proteins strongly suggests that these parallel systems evolved independently. On the other hand, M. mobile, with no apparent equivalent of a ribosome-free space (39), and with its cytoskeletal components lacking the hallmark characteristics of the M. pneumoniae and M. penetrans AO proteins identified in the present work, appears to have evolved a different approach to generating a protrusive and therefore superficially similar structure. M. insons achieves polarization of adherence and motility without a protrusive structure (40), representing a completely different model for polarization.
Properties of the *M. penetrans* AO interior

As previously described (7), there can be little doubt that the detergent-insoluble, DNA-free objects observed by SEM, whose abundance on cover slips is routinely parallel to that of unextracted *M. penetrans* cells grown from the same inoculum (not shown), are what occupy the space in the nucleoid-free zones of *M. penetrans* cells and are the material observed in thin sections at cell poles, also observed here by ECT. Indeed, the material underlying the cell poles in ECT images, which closely resemble the material at the cell poles observed in TEM images of thin sections (5, 6, 34), are strikingly reminiscent of ribosome-free PopZ-containing structures at the poles of *C. crescentus* cells when PopZ is overproduced (49). Our deeper study of these objects reveals that the organization of the TXI and TWI structures of the *M. penetrans* AO is completely unlike that of *M. pneumoniae*. Whereas the dimensions of the *M. pneumoniae* structure appear to be tightly constrained (33), in *M. penetrans*, as visualized in a number of ways, the dimensions are highly variable. Nonetheless, SEM images of these variable objects revealed some commonalities, including a wider and a narrower portion, the latter often containing one or more flexible rod-like elements. This heterogeneity, which is distinct from the highly regular *M. pneumoniae* AO structures, suggests the possibility of growth by accretion of material onto, or absorption of material into, a pre-existing structure. ECT images suggest that both poles contain the same differentiated material, and this material even extends into the filaments connecting dividing *M. penetrans* cells. These facts are consistent with a model in which nucleation and biogenesis of the AO underlying structure are linked to the cell cycle,
perhaps with new material being deposited into spaces in the cytoplasm vacated during chromosome condensation and segregation. The broader size distribution of the nucleoid-free zones and the detergent-insoluble structures suggests that growth of the *M. penetrans* AO, though capped, is not capped at a uniform size.

In addition to the heterogeneity in size, the pleomorphy of the detergent-insoluble objects is striking. We noted that whereas our SEM images of whole *M. penetrans* cells and previously described TEM images of thin sections (5-7, 34) show that the region of the cell at the pole is constricted along the long axis, this was not the case in ECT images. One possible interpretation of this discrepancy is that the material within the *M. penetrans* AO is highly hydrated and therefore subject to shrinkage during the dehydration steps associated with standard SEM and TEM processing techniques, whereas ECT lacks a dehydration step. Conceivably this hydration results from attraction of water molecules to the highly negatively charged proteins present in the structure. If the structural elements are principally organized parallel to the long axis, dehydration would result predominantly in a decrease in width rather than length. Another possibility is that because the cell bodies visualized by ECT are not attached firmly to surfaces, but instead are present in the holes of the carbon-coated grids, they are in a relaxed conformation. In either case, we predict that the material within the AO is flexible like a gel.

*M. penetrans* AO composition and gene expression

Of the proteins we identified in the TXI and similar TWI fractions of *M. penetrans* by MALDI-TOF, four have characterized biochemical roles. Whether any
or all of them are part of the internal structure of the AO is unclear. Pyruvate dehydrogenase subunit E2 normally functions as part of a large structure that could be insoluble in nonionic detergents; on the other hand, none of the other subunits of pyruvate dehydrogenase were identified, suggesting a possible alternative or additional role for this protein, which also moonlights as a surface adhesin in other mycoplasmas (50). OppF is normally a peripheral membrane component of the oligopeptide ABC transporter (51), including in mycoplasmas (52). The substrate-binding protein component of this transporter, OppA, doubles as an adhesin in *Mycoplasma hominis* (53, 54), raising the possibility that *M. penetrans* OppF anchors the material underlying the AO to OppA acting as an adhesin. However, none of the proteins of this complex other than OppF were identified in the *M. penetrans* detergent-insoluble fractions. Surprisingly, there were three lipoproteins identified in the detergent-insoluble fraction. Two of these lipoproteins are members of the P35 lipoprotein family, which comprises 44 total *M. penetrans* genes (14). The P35 lipoprotein and its paralogs, which are distributed across the surface of *M. penetrans* cells, are immunodominant (55-58). Expression of genes encoding these proteins undergoes extensive antigenic variation, which is believed to be a major factor for immune evasion (59). The presence of two of these P35 homologs in the detergent-insoluble fractions may suggest that they indirectly interact with the AO interior, although it is also possible given their extensive distribution across the cell surface that they aggregate or form multiple interactions with each other that prevents their solubilization in nonionic detergents. Indeed, the difference in appearance between
the TWI and TXI fractions might be entirely attributable to the presence of P42 in
the former. The third lipoprotein identified, MYPE6960, has no known function and
its distribution and presence at the cell surface of *M. penetrans* cells is unknown.
However, this protein is a homolog of MG309, a protein in *M. genitalium* that binds
and activates Toll-like receptors (60), making MYPE6960 a particularly interesting
candidate for further study with respect to pathogenesis. Conceivably, this
lipoprotein plays a role in adherence of *M. penetrans* to host cells and is associated
with AO proteins.

The remaining five proteins that were identified in this study had no
predicted function or conserved domains, making them ideal candidates for
principal structural elements of the AO. The genes encoding four of these proteins
are located very close together in the genome of *M. penetrans* and were found to be
transcribed as a polycistronic message with two other genes. Three of these,
MYPE1550, 1560, and 1570, are among the most abundant proteins in the
detergent-insoluble fractions. All six of these proteins, as well as MYPE4000, share
an unusual set of characteristics: predicted alpha-helical coiled-coil regions,
molecular weights greater than 90 kDa, and pIs of approximately 4.5 (Table 3).
Interestingly, these characteristics are shared with many of the *M. pneumoniae* AO
cytoskeletal proteins (Table 3) despite lacking sequence homology. It is highly
unlikely, given that the *M. penetrans* proteins lack characteristics of *M. pneumoniae*
AO proteins like proline-rich domains and sequence elements like the EAGR box,
that the *M. pneumoniae* and *M. penetrans* AO proteins share common ancestry.
However, the shared properties are intriguing when considering the functional
analogy of the respective structures that they build. These shared properties suggest that one solution to forming a polar cytoskeletal structure, arrived at independently in *M. pneumoniae* and *M. penetrans*, involves assembly of large, highly charged proteins that interact with each other over an extensive surface area via alpha-helical coiled coils.

Although another group reported changes in expression of a specific set of phase-variable genes in response to infection of a host (30), this study is the first to our knowledge to report on expression of an entire mycoplasma transcriptome upon exposure to host cells. Particularly striking was the absence of major differences in the transcript profile of *M. penetrans* cells grown independently and grown for 6 h in the presence of HeLa cells, which is enough time for interactions to occur between the two, including invasion (41, 43). We anticipate that, whether or not all *M. penetrans* cells have adhered and invaded HeLa cells, after 6 h in an environment filled with signals from host cells, *M. penetrans* cells will have had ample opportunity to adjust their gene expression profile to prepare for interactions. Therefore, even if, as a consequence of using HeLa cells at 70-80% confluency, only ~75% of *M. penetrans* cells interacted with host cells, we would expect to see a significant change in the overall gene expression pattern if a change were occurring. The absence of transcriptional regulation is a formal but unlikely possibility, as mycoplasmas, despite having limited machinery for transcriptional regulation, nonetheless exhibit it (61-63). A more likely explanation is that *M. penetrans*, which naturally occurs as an obligate commensal or parasite like all mycoplasmas, has no evolutionary adaptation to living in the absence of host cells,
and therefore is always in a mode in which it is deployed for interaction with the host. Relatedly, it is possible that the fetal bovine serum endows SP-4 broth with all the same relevant factors provided by HeLa cells.

Implications for mechanisms of AO function

Unlike the *M. penetrans* AO, which consists entirely of a poorly organized matrix, the corresponding *M. pneumoniae* feature is visually dominated by a dense, highly organized structure flanked by a ribosome-excluding zone in contact with the cell membrane (45). The structure, which has been called the electron-dense core because of its prominent staining with metal-based dyes (64), has been the focus of considerable study (reviewed in Balish, 2). Because the ribosome-excluding zone is directly adjacent to the core, even in cells in which the core is detached from the membrane (45, 65, 66), it is likely formed by some fine substance linked to the core, probably regions of the same proteins that make up the core itself. Indeed, the failure to identify any non-core-associated proteins in the *M. pneumoniae* AO (66) supports this model. In contrast to *M. pneumoniae*, the *M. penetrans* AO consists solely of material whose lack of obvious organization is more reminiscent of the lucent area of *M. pneumoniae* than to the core. Because both AOs, one with a core and one without, function in adherence and motility, it is possible that in *M. pneumoniae*, the core itself is dispensable for these functions, as previously suggested (33, 67), although this assertion is in opposition to some models (45, 66). We therefore propose that the less organized material, constituting the ribosome-excluding zone in *M. pneumoniae* and perhaps the entire AO interior in *M. penetrans,
is the principal organizer of the AO, driving concentration and organization of adhesins at the AO in both species, leaving motility to be carried out directly by adhesins with motor properties, as described for the more distantly related \( \text{M.} \) mobile (47).

Regarding \( \text{M. pneumoniae} \), if it is the lucent material that organizes the AO, then the core may be dispensable for adherence and motility. Superficially this statement appears to be counter to observations (reviewed in 68). However, if the lucent and core regions of the \( \text{M. pneumoniae} \) AO are actually composed of different regions of the same proteins, then mutants in which core proteins are lost would also be defective in the functions associated with the lucent region. Indeed, if this model is correct, then \( \text{M. pneumoniae} \) mutants affected only in the lucent area and not in the core have never been studied. The physical association of DNA with the core in \( \text{M. pneumoniae} \) relatives (33) raises the possibility that the \( \text{M. pneumoniae} \) core itself is primarily involved in chromosome segregation. In \( \text{M. pneumoniae} \) a newly formed AO is displaced from the pre-existing pole to the pole created during binary fission during cell division (69), possibly carrying the newly replicated chromosome with it to the daughter cell (2). Because \( \text{M. penetrans} \) cells appear to acquire a mass of AO material opposite the pole of the functioning AO, and not adjacent to it, during the process of division, and because DNA is not associated with the \( \text{M. penetrans} \) detergent-insoluble material, it is reasonable to propose that the \( \text{M. penetrans} \) AO is not directly involved in chromosome segregation.
Acknowledgments

This work was supported by the National Institutes of Health (Public Health Service grant R15 AI073994) to MFB. We thank A. Kiss (Miami University Center for Bioinformatics and Functional Genomics) and K. Pflaum (University of Connecticut) for help with RNA-Seq. We thank R.J. Hickey (Miami University), W. Ambrosius (Wake Forest University), and members of the Balish laboratory for insightful discussions. This work was done in partial fulfillment of SLD’s doctoral dissertation requirements.
Table 1. Primers used for RT-PCR

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Note: RT = Reverse Transcriptase
Table 2. TXI and TWI proteins identified by MALDI-TOF

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Table 3. Comparison of AO protein features

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* M. penetrans

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Figure 1. SEM images of *M. penetrans* whole cells and detergent-insoluble structures. A) Field of *M. penetrans* cells; black arrows, connecting filaments; scale bar, 1 μm. B) Individual *M. penetrans* cell; white arrow, AO; scale bar, 200 nm. C) and D), detergent-insoluble structures from *M. penetrans* cells extracted with Triton X-100 (C) or Tween-20 (D). *, wide end of structure; scale bar, 200 nm.

Figure 2. AO-associated objects and their lengths. A) Distribution of length of TXI objects observed by SEM; B) distribution of lengths of nucleoid-free zones observed by light and fluorescence microscopy; C) distribution of lengths of TWI objects observed by SEM; D) correspondence of lengths of nucleoid-free zones to fraction of the cell length occupied by the nucleoid-free zones in 52 cells with a single nucleoid-free zone; E) and F) correspondence of lengths of nucleoid-free zones to fraction of the cell length occupied by the nucleoid-free zones in 16 cells with two nucleoid-free zones – the shorter one (E) and the longer one (F). G) Example of nucleoid free zones. Black, cell outline viewed by phase-contrast microscopy; white, nucleoid stained by DAPI. Arrows, nucleoid-free zones, measured in (B) along the long axis of the cell; scale bar, 500 nm.

Figure 3. Internal organization of *M. penetrans* observed by ECT. A-C, sections of different whole cells; D- F, corresponding schematics. A-C, ~20-nm granules are ribosomes (reviewed in 70). The insertion site of a connecting filament was
observed at one cell pole (C, F). Black lines, cell membranes and boundaries of ribosome-free zones; circles, ribosomes (positions are schematic and do not represent actual ribosomes). Scale bar, 100 nm.

Figure 4. SDS-PAGE of *M. penetrans* whole-cell lysate, TWI, and TXI proteins. A total of 22 μg each of protein from whole cell lysate (lane 2), TWI (lane 3), and TXI (lane 4) were separated by SDS-PAGE for comparison and for excision of candidate bands chosen for identification. Lane 1 contains protein standards, with sizes in kDa indicated to the left. A 66-kDa band in lane 4 is only present in some preparations. Symbols indicate specific bands chosen for MALDI-TOF. Numbers in gel correspond to protein identifications in Table 2.

Figure 5. RT-PCR analysis of putative cytoskeletal operon of *M. penetrans*. A) Agarose gel containing PCR products from RT-generated transcripts that span across the gene junctions numbered in (B). Top, amplified products from reverse transcribed RNA using a primer complementary to a region at the 3’ end of *mype1570*; bottom, products amplified from genomic DNA used as a positive control. B) Schematic of genes encoding MYPE1520-1570 showing genomic orientation and relative size, including the 5’ and 3’ flanking genes. Numbers 1-7 refer to position of primer pairs used to span gene junctions for RT-PCR.
References


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