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Identification and Biochemical Investigations of Rhamnose and Glycoprotein in Mycoplasmas

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IDENTIFICATION AND BIOCHEMICAL INVESTIGATIONS OF RHAMNOSE AND GLYCOPROTEIN IN MYCOPLASMAS

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MICROBIOLOGY

ABSTRACT

Mycoplasmas are host-specific human and animal pathogens within the class of wall-deficient bacteria named *Mollicutes* and have smaller sizes and genomes than the walled bacteria. Mycoplasma genome sequences reveal few if any genes for synthesis of a glycocalyx. Nevertheless, we find that mycoplasmas produce glycolipids, glycoprotein, and polysaccharides. Using gas chromatography and mass spectrometry, glucose, mannose, rhamnose, and galactose were detected in the glycolytic species *Mycoplasma pneumoniae* and *Mycoplasma pulmonis*. All of these sugars except galactose was found in *Mycoplasma arthritidis*. Rhamnose was in the rare D configuration in the glycolytic species and in both D and L forms in *M. arthritidis*. Since there is no known source of rhamnose in the culture media or animal hosts, we speculated that mycoplasmas synthesize rhamnose and pursued this idea further by tracing rhamnose labeling with ^{13}C isotopes. Surprisingly, ^{13}C-glucose did not label glycoconjugates in any species. However, ^{13}C-labeled starch, a glucose polymer, labeled rhamnose, glucose, *N*-acetylglucosamine, and galactose. Mycoplasmas indeed synthesize rhamnose. Supporting this claim are observations that supplementing growth medium with starch increased the amount of rhamnose produced by the mycoplasmas over 10-fold and resulted in a higher L-rhamnose to D-rhamnose ratio. Methanol/chloroform extraction showed that the majority of rhamnose and glucose partitioned in the aqueous fraction or the protein-concentrated

interphase, suggesting the presence of glycoprotein. All species tested reacted lightly with a glycoprotein stain, and heavily stained bands were observed in concentrated lipoprotein extracts from *M. arthritidis*. Using high resolution mass spectrometry, threonine and serine residues that were *O*-glycosylated with a hexose were identified. Using ^{13}C-labeled starch, we confirmed that glucose was attached to protein. This is the first definitive characterization of any glycan or glycosylation site in any mollicutes. These studies show that although mycoplasmas do not have common glycoconjugate-producing machinery, they do produce glycoconjugates. Identifying and characterizing this novel machinery would reveal protein functions that could extend well beyond this group of bacteria.

Keywords: FT-ICR, methanolysis, butanolysis, serum-free medium, trimethylsilylation

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DEDICATION

I dedicate this work those who left the world during the course of this work: my grandmothers, Vivian Jordan and Wilma Hall, and my great-grandmother, Amy Hall. I also dedicate this to my daughter, Amellya Jordan, who entered the world during the course of this work.

ACKNOWLEDGMENTS

This work would not have been possible without the mentorship of Dr. Kevin Dybvig and the guidance and technical expertise of Dr. James Daubenspeck.

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INTRODUCTION

Sporadic reports of the presence of glycoconjugates in mycoplasmas have been mostly ignored due to the complex media required to cultivate this fastidious genus of bacteria. Two mycoplasma growth media components, animal serum and yeast extract, contain a rich abundance of diverse glycomoieties in the forms of glycoproteins, glycosaminoglycans, polysaccharides and cell wall fragments. These molecules have made extensive glycome studies of mycoplasmas nearly impossible because they adhere to cells and interfere with subsequent analysis.

As genome data became available for mycoplasmas, the existence of an intricate mycoplasma glycome appeared improbable. For example, most species lack recognizable nucleotidyltransferase genes. These gene products convert phosphate sugars into nucleotide sugars that can then be used by glycosyltransferases to build glycomoieties. The few nucleotidyltransferases that are found in the entire mollicute class are associated with glycolipid synthesis (Klement, Ojemyr, Tagscherer, Widmalm, & Wieslander, 2007; Smith, 1969, 1971). There is also a paucity of recognizable glycosyltransferase genes within mycoplasmas. For example, both *Mycoplasma arthritidis* and *Mycoplasma pulmonis* have only one gene annotated as a glycosyltransferase, suggesting that these species have a limited glycome. *M. arthritidis* is known to make glucolipids and must have a glycosyltransferase for glycolipid synthesis (Li, Matsuda, Takagi, & Yamamoto, 1997). Perhaps the *M. pulmonis* glycosyltransferase is used for glycolipid production as well.

Despite the lack of traditional machinery, mycoplasmas produce a diversity of glycomoieties. The first published report of mycoplasma polysaccharide described a ga-

lactan produced by *Mycoplasma mycoides* (Buttery & Plackett, 1960; Plackett & Buttery, 1958). Only recently it was shown that *M. pulmonis* produces a capsular polysaccharide comprised of equimolar amounts of glucose and galactose (Daubenspeck, Bolland, Luo, Simmons, & Dybvig, 2009). Strains of *Mycoplasma pneumoniae* produce distinct biofilms and adhesive polysaccharide that is thought to contain galactose- and *N*-acetylglucosamine (Simmons et al., 2013). *M. pneumoniae* polysaccharide is cross-reactive to antisera produced from some serotypes of *Streptococcus pneumoniae* (Allen & Prescott, 1978). *Mycoplasma gallisepticum* has two proteins that react with a carbohydrate-specific stain (Demina et al., 2009), but the glycans and glycosylation sites have not been identified. Hence, scant studies on the glycobiology of mycoplasmas have been reported, but are only now receiving attention.

A recent formulation of serum-free medium for *M. pneumoniae* provided the opportunity to make advances in our understanding of mycoplasma glycobiology. By eliminating all of the glycomoieties, save that from bovine serum albumin and peptone, we discovered that several if not all mycoplasmas synthesize D-rhamnose and some even synthesize both D- and L-rhamnose. Also, *M. arthritidis* produced glycoproteins that contained either mannose or glucose. Three distinct glycosylation sites have been identified thus far. The work presented here is significant due to the lack of recognizable rhamnose synthesis machinery encoded by mycoplasma genomes. This work is also the first to definitively describe any glycosylation site and the carbohydrate composition of glycoproteins in any mollicutes.

Mycoplasmas, the Nonconformists of the Kingdom

Mycoplasmas belong to a class of bacteria known as *Mollicutes* and are vastly different than their bacterial counterparts. This is largely due to the fact that the Gram-positive ancestors of mycoplasmas ejected much of their genomic content as they lost their cell wall and evolved to become more dependent on specific hosts.

Due to their small genomes the mycoplasmas were among the first bacteria to be fully sequenced (Bork et al., 1995; Chambaud et al., 2001; Dybvig et al., 2008; Himmelreich et al., 1996; Muto, Yamao, & Osawa, 1987; Papazisi et al., 2003; Peterson, Hu, Bott, & Hutchinson, 1993; Vasconcelos et al., 2005; Westberg et al., 2004). Interestingly, the mycoplasmas have had a large impact on the entire field of synthetic biology, which is focused on designing the minimal set of genes for life and designing synthetic genomes to allow optimal synthesis and purification of cloned gene products (Glass et al., 2006). Synthetic biologists have fully synthesized a bacterial genome and transplanted it into a mycoplasma cell, creating the first synthetic self-replicating cell (Gibson et al., 2010).

The streamlined nature of most mycoplasmas results in these species lacking the genetic machinery for numerous biosynthetic pathways responsible for forming molecules and organelles common in other bacteria. The best known feature and phylogenic identity of mycoplasmas is their lack of a cell wall. This lack of a cell wall is often hypothesized to be responsible for *Mycoplasma* species' requirement for cholesterol in growth media, as cholesterol is thought to be essential for plasma membrane integrity (D. G. Edward, 1953; D. G. A. Edward, 1971; Grimellec, Cardinal, Giocondi, & Carriere, 1981; Rottem, 1981; Rottem, Cirillo, deKruyff, Shinitzky, & Razin, 1973).

Another striking feature of mycoplasmas involves their methods of making adenosine triphosphate (ATP), the molecule that supplies the energy for biochemical reactions in most, if not all, organisms. Some mycoplasmas synthesize ATP from urea, some from arginine while others use the more common pathway of glycolysis (Cunin, Glansdorff, Pierard, & Stalon, 1986; Romano, LaLicata, & Alesi, 1986; Tully & Razin, 1977). One thing in common among the different ATP-synthesis strategies is that all use substrate-level phosphorylation. Mycoplasmas have also lost genes encoding the proteins necessary for oxidative phosphorylation, cytochromes, and enzymes needed for the tricarboxylic acid (TCA) cycle (Pollack, 1992).

Another factor differentiating the mycoplasmas from most other bacteria is that they cannot maintain known plasmids within their populations. There also is little horizontal gene transfer, no natural transformation and no known conjugation from mycoplasma donors. Mycoplasma can receive genomic material from other bacteria, as demonstrated by the conjugational transfer of transposon Tn*916* from *Enterococcus faecalis* to *Mycoplasma hominis* and *M. pulmonis* (Dybvig, 1992; Roberts & Kenny, 1987). The mycoplasmas have altered codon usage to translate messenger RNA to protein. In other bacteria the UGA codon signals an end to the peptide sequence, but in mycoplasmas this codon is a signal to add a tryptophan to the growing peptide chain. Also, the genomes of mycoplasmas have relatively low G + C content when compared to other bacteria (Muto, Andachi, Yamao, Tanaka, & Osawa, 1992).

As mycoplasmas are vastly different from other bacteria, it would not be surprising to find previously unknown biochemical pathways in mycoplasmas as genes are removed and new pathways arise from side reactions of the gene products that remain.

These potentially novel pathways may be limited to mycoplasmas or could simply have been overlooked in other bacteria. Studying mycoplasmas could potentially lead to discovery of alternative protein functions in numerous bacterial species.

The Known Pathways of Rhamnose Synthesis

The biosynthetic pathways for production of D- and L-rhamnose are similar but not identical. Bacteria that produce L-rhamnose are thought to do so by the Rml pathway. This pathway employs nucleotide sugars in a series of four reactions that converts dTDP-D-glucose to dTDP-L-rhamnose. RmlA converts glucose-1-phosphate to dTDP-glucose in a nucleotidyltransferase reaction, RmlB dehydrates dTDP-glucose to dTDP-4-keto-6-deoxyglucose, RmlC epimerizes this molecule to dTDP-L-lyxo-6-deoxy-4-hexulose and RmlD reduces this finally to dTDP-L-rhamnose (Giraud & Naismith, 2000). *Pseudomonas aeruginosa* can synthesize D-rhamnose by converting fructose-6-phosphate to mannose-6-phosphate, which is isomerized to mannose-1-phosphate and then converted to GDP-D-mannose. This molecule is dehydrated to GDP-4-keto-6-deoxymannose from which it is then reduced to GDP-D-rhamnose (Rocchetta, Pacan, & Lam, 1998). Both configurations of rhamnose are finally transferred to a saccharide chain using the energy stored within the phosphate bonds binding the sugar to the nucleotide. Mycoplasmas have no homologues of any of these enzymes and the enzymes involved in rhamnose synthesis are currently unknown.

Glycoproteins

Glycoproteins contain covalently-linked glycomoieties and are found throughout many forms of life. These sugar-decorated proteins are highly abundant in eukaryotic cells, estimated to comprise two-thirds of all eukaryotic proteins (Apweiler, Hermjakob, & Sharon, 1999; Brooks, 2009). The consistent and abundant nature of eukaryotic glycoprotein structures and direct health relevance in humans has led to an intense scrutiny and elucidation of these glycoconjugates. Perhaps the failure to find eukaryotic-type glycoproteins in bacteria led to the initial assumption that prokaryotic glycosylation was a rare event. In recent years, however, technology and research focus has revealed that bacterial glycoproteins are prevalent and function in essential roles of protein stability and are important for host colonization.

Assembly

Glycomoieties are linked to proteins generally through an asparagine in *N*-linkages and threonine or serine in *O*-linkages. Similar to eukaryotes, bacterial *O*-linked and *N*-linked glycoproteins have been found. The two mechanisms of glycan assembly are oligosaccharyltransferase-mediated assembly, which involves prebuilding the glycan before transferring to protein and stepwise assembly, in which sugar residues are added to the protein one at a time. Both mechanisms have also been found within the bacterial realm. Oligosaccharyltransferase-mediated assembly is the mechanism used with *N*-linkages in most organisms and also in the *O*-linkages found within some Gram-negative bacteria. All *O*-linkages are assembled in a stepwise manner in eukaryotes and in the Gram-positive bacterial glycoproteins characterized so far (Dell, Galadari, Sastre, &

Hitchen, 2010). Glycans of bacterial glycoproteins are generally smaller than eukaryotes and often incorporate rare or exotic sugar residues such as anthrose in *Bacillus anthracis* (Daubenspeck et al., 2004), 2,4-diacetamido-2,4,6-trideoxyhexose in *Neisseria* species (Hegge et al., 2004) and derivatives of the nine-carbon pseudaminic acid in *Campylobacter* and *Helicobacter* species (Logan, 2006; Schirm et al., 2003; Thibault et al., 2001).

Functions

Studies of the function of protein glycosylation must identify a distinct difference between the function of the protein itself, regardless of glycosylation, and functions that can only be observed in the fully glycosylated form. Recent studies indicate that glycosylation of proteins can play intimate roles in protein stability or efficient colonization of the host. For example, the serine-rich fimbria-associated adhesion glycoprotein, Fap1, of the tooth colonizer *Streptococcus parasanguinis* can still adhere to saliva-coated hydroxylapatite beads even with altered glycosylation. Altering the glycosylation, however, can result in the production of a less stable Fap1 precursor. All the downstream functions that would be observed in mature Fap1 are then affected, including less adherence, loss of long fimbriae assembly, and decreased biofilm mass (Peng et al., 2008; Wu, Zeng, & Fives-Taylor, 2007).

Glycosylation of flagellin produced by the human gastric pathogen *Helicobacter pylori* has also been shown to stabilize the protein. Insertional mutations in glycosylation genes of flagellin result in the bacteria's inability to produce intact flagella even though flagellin is still produced at normal levels (Schirm et al., 2003). This lack of flagella

causes a defect in motility. Gene disruption of a deglycosylation enzyme in *H. pylori* result in heavier glycosylation of flagellin, causing hypermotility (Asakura et al., 2010).

Another function of bacterial protein glycosylation is efficient colonization of the host. *Campylobacter jejuni*, a chicken colonizer and human gastric pathogen, makes a glycosylated flagellin protein. Disrupting glycosylation genes do not affect motility as is the case for *H. pylori*. Autoagglutination and biofilm production, however, are affected in *C. jejuni* and are thought to be responsible for the reduction in colonization of the chicken gastrointestinal tract (Howard et al., 2009).

The abundant colonizer of the human intestines, *Bacteriodes fragilis*, has a general glycosylation system that produces numerous glycoproteins. Deleting this glycosylation system dramatically reduces its ability to competitively colonize a murine host, perhaps due to documented defects in bacterial growth. (Fletcher, Coyne, Villa, Chatzidaki-Livanis, & Comstock, 2009).

Phylogenic Relationship of *M. arthritidis*, *M. pulmonis* and *M. pneumoniae*

The studies presented here focus predominantly on *M. arthritidis*, *M. pulmonis*, and *M. pneumoniae*, and it is imperative to examine their phylogenic positions to understand the magnitude of their common features. As of 2007, the International Committee on Systematics of Prokaryotes (ICSP) further codified the minimal requirements for distinguishing species within the *Mollicutes* class. The requirements are differences in, with this order of priority: (1) 16S rRNA gene sequences, (2) serological reactions, (3) phenotype and (4) ecological niches. Only then are species defined by previously established taxonomic rules. For many mycoplasmas, the 16S rRNA gene sequences are enough for

identification purposes and the other rules are necessary for species where 16S rRNA has a "gene sequence matrix similarity" score greater than 0.94 (Brown, Whitcomb, & Bradbury, 2007). Obviously, total genome sequence comparisons are ultimately the final step in species comparisons and these data are available for *M. arthritidis*, *M. pulmonis* and *M. pneumoniae* as well as many other mycoplasmas. It is expected that as genome sequencing becomes more prevalent these rules will be unnecessary, however they are useful for establishing evolutionary relationships among all the mycoplasmas identified thus far, sequenced or not.

The curators of the website www.molligen.org have assembled a phylogeny tree showing the evolutionary relationship of 51 mycoplasma species based on 16S rRNA sequences. One of the first major branches from the common Gram-positive bacterial ancestor contains the phytoplasmas and acholeplasmas. This clade has members who can maintain plasmids and some who are dependent on plant hosts. The alternate branch is further divided into two clades. One clade contains further branching, eventually delineating *M. pneumoniae* and several mycoplasma species that infect humans and animals, many of them responsible for respiratory and urinary tract infections. The other clade is further split separating the corkscrew-shaped spiroplasmas from the remaining mycoplasmas that include *M. pulmonis* and *M. arthritidis*. This latter clade includes species that are pathogenic in humans and animals, infecting various tissues such as the lungs, urinary tract and synovia. We can conclude from this that *M. pneumoniae* is more evolutionarily distant than are *M. pulmonis* and *M. arthritidis* from each other. Any common features shared by all three are more likely to be shared by most members of one of the first clades, more evolutionarily ancient and perhaps even essential.

Technical Significance

This work makes use of two recent developments in the microbiology field. Within mycoplasma research specifically, the formulation of a serum-free medium has greatly enhanced the ability to study the glycocalyx. Animal serum is an abundant source of glycoproteins, glycosaminoglycans and other glycomoieties, which has long interfered with mycoplasma glycobiology assays. The application of the serum-free medium, initially designed for *M. pneumoniae* (Yus et al., 2009), to several other species is significantly impacting our understanding of mycoplasmas as described in later chapters.

The other development within the microbiology field is the use of high resolution mass spectrometry to identify *O*-linked glycosylation sites in bacteria (Hitchen & Dell, 2006). This technology is frequently used to study post-translational modifications of purified proteins and we have used it to study mycoplasma glycoproteins that have been separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Summary

This dissertation presents evidence that rhamnose synthesis is a genus-wide trait of mycoplasmas. This synthesis can be traced with the ^{13}C-labeled glucose polysaccharide starch, but not from monosaccharide glucose. *M. arthritidis* has almost equal amounts of D- and L-rhamnose when grown in serum-free medium, but this ratio can be modulated by supplementing the medium with polysaccharide.

Also described is the first evidence of *O*-linked protein glycosylation of any mycoplasma. Three distinct glycosylation sites are identified. This, too, is probably a genus-wide feature as glycoprotein staining is shown for three species.

RHAMNOSE BIOSYNTHESIS IN MYCOPLASMAS REQUIRES PRECURSOR GLYCANS LARGER THAN MONOSACCHARIDE

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Rhamnose Biosynthesis in Mycoplasmas Requires Precursor Glycans Larger than Monosaccharide

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Summary

Despite the apparent absence of genes coding for the known pathways for biosynthesis, the monosaccharide rhamnose was detected in the D configuration in *Mycoplasma pneumoniae* and *Mycoplasma pulmonis*, and in both the D and L configurations in *Mycoplasma arthritidis*. Surprisingly, the monosaccharide glucose was not a precursor for rhamnose biosynthesis and was not incorporated at detectable levels in glucose-containing polysaccharides or glycoconjugates. In contrast, carbon atoms from starch, a polymer of glucose, were incorporated into rhamnose in each of the three species examined. When grown in a serum-free medium supplemented with starch, *M. arthritidis* synthesized higher levels of rhamnose, with a shift in the relative amounts of the D and L configurations. Our findings suggest the presence of a novel pathway for rhamnose synthesis that is widespread in the genus *Mycoplasma*.

Introduction

Mycoplasmas (Class *Mollicutes*) are significant pathogens of humans and animals, causing chronic diseases of the respiratory system, genital tract, and joints. Factors that contribute to disease chronicity include polysaccharides that protect the mycoplasma from host defenses and have roles in biofilm formation (Bolland *et al.*, 2012, Shaw *et al.*, 2013, Shaw *et al.*, 2012, Daubenspeck *et al.*, 2009). The mycoplasmas lack a cell wall but have descended from A+T-rich *Firmicutes* through a process that resulted in a streamlined genome containing about 600-800 protein-coding regions. Extensive bioinformatic analysis of each gene in *Mycoplasma arthritidis* and *Mycoplasma pulmonis* suggests that these species have limited machinery for synthesis of monosaccharides and glycoconjugates (Dybvig *et al.*, 2008, French *et al.*, 2008, Daubenspeck *et al.*, 2014). These murine pathogens have only a single annotated gene (Marth_orf849 and MYPU_7700) coding for a glycosyltransferase and no strong candidate for a nucleotidyltransferase gene. Nevertheless, the mycoplasmas produce glycolipids, polysaccharides, and glycoproteins (Klement *et al.*, 2007, Razin *et al.*, 1970, Chandler *et al.*, 1989, Buttery and Plackett, 1960, Plackett and Buttery, 1958, Daubenspeck *et al.*, 2009, Demina *et al.*, 2009). *M. pulmonis* and probably *Mycoplasma pneumoniae* produce a polysaccharide that contains glucose (Daubenspeck *et al.*, 2009, Simmons *et al.*, 2013).

The pathways for synthesis of D- and L-rhamnose have been extensively studied in bacteria and generally involve nucleotide sugar intermediates (Giraud and Naismith, 2000). Of the twenty or so mycoplasma genomes that have been sequenced, no rhamnose synthesis genes have been identified. Nevertheless, we find rhamnose in several species

of mycoplasma including the human pathogen *M. pneumoniae* and the murine pathogens *M. arthritidis* and *M. pulmonis*. The presence of rhamnose was unexpected. These mycoplasmas are unlikely to acquire rhamnose from the environment because their mammalian hosts neither synthesize rhamnose nor incorporate it into glycoconjugates. Rhamnose was in the rare D configuration in all three species, and *M. arthritidis* also contained L-rhamnose. When grown in the presence of [U-^{13}C]starch, the carbon atoms of rhamnose were labeled with ^{13}C, demonstrating rhamnose synthesis. Surprisingly, when the medium was supplemented with D-[U-^{13}C]glucose, no labeling of rhamnose was detected. Hence, rhamnose is not synthesized through a traditional pathway beginning with the conversion of glucose to glucose-6-phosphate (G6P), G6P to glucose-1-phosphate (G1P), and then G1P to UDP-glucose as generally found for L-rhamnose. Similarly, D-rhamnose is not synthesized by the usual pathway in which glucose is converted to G6P and eventually to GDP-mannose via mannose-6-phosphate and mannose-1-phosphate (M1P) (Giraud and Naismith, 2000). Because starch, a polymer of glucose, supports rhamnose synthesis but glucose monosaccharide does not, we propose that the energy from the glycosidic bonds between the glucose residues of starch is a requirement for synthesis.

Results

Mycoplasmas contain D-rhamnose

The monosaccharide composition of the glycomoieties (glycolipids, glycoproteins, and polysaccharides) of mycoplasmas was examined. In pilot experiments, lysates of 11 species of *Mollicutes* were dialyzed extensively to remove monosaccharides, and the

remaining glycomoieties were analyzed by GC/MS. The dialyzed lysates of each species (*Acholeplasma laidlawii, Mycoplasma arginini, M. arthritidis, Mycoplasma capricolum, Mycoplasma fermentans, Mycoplasma genitalium, Mycoplasma gallisepticum, Mycoplasma hyorhinis, Mycoplasma mycoides* subsp. *capri*, *M. pneumoniae*, and *M. pulmonis*) contained the 6-deoxyhexose rhamnose. *M. arthritidis* was chosen for further study because initial experiments indicated that this species had a relatively high level of rhamnose. In addition to rhamnose, this species also had significant levels of glucose and mannose (Fig. 1). Standard curves of known amounts of these sugars were used to quantitate the amount of each sugar in the lysates (Fig. S1). Most of the rhamnose in nature is in the L configuration, but D-rhamnose has been reported in a few organisms

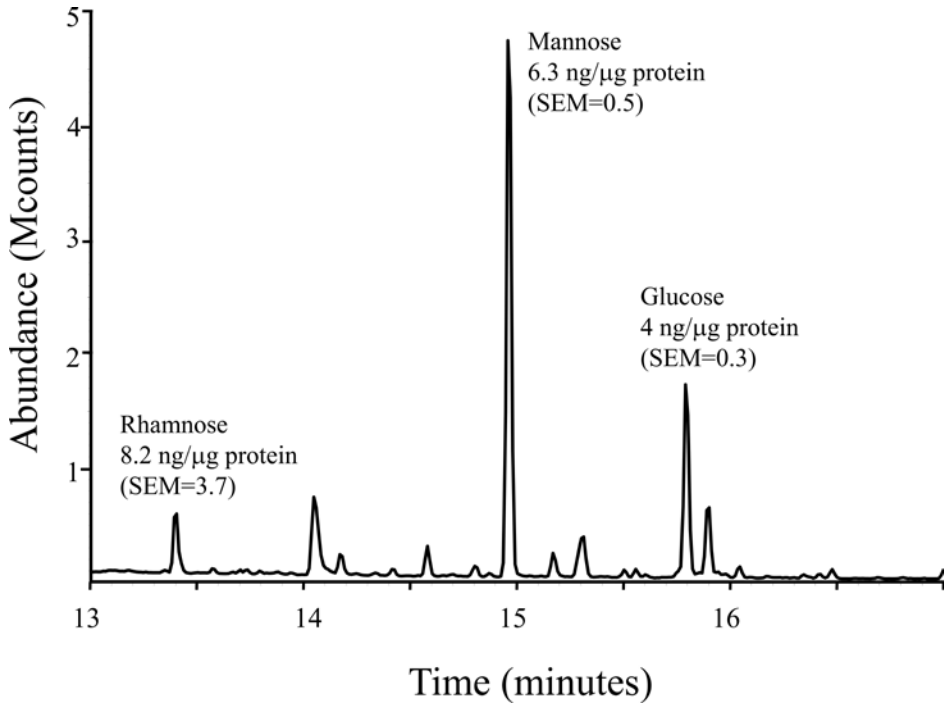

Fig. 1. Representative chromatogram of dialyzed lysates of *M. arthritidis* indicating the amount of each sugar per μg protein with standard error of the mean (SEM). Lysates (n =3) contained 20-μg protein.

such as *Pseudomonas* (Rocchetta *et al.*, 1998). The trimethylsilyl (TMS) derivatives of the methyl glycosides of D- and L-rhamnose cannot be resolved by GC/MS, but the TMS derivatives of the butyl glycosides can be. Hence, lysates were subjected to butanolysis and compared to the butyl glycosides generated from standards of D- and L-rhamnose. Both D- and L-rhamnose were detected in *M. arthritidis* (Fig. 2A). Because the finding of both enantiomers of rhamnose was surprising, the TMS derivatives of the butyl glycosides of *M. pneumoniae* and *M. pulmonis* were also analyzed. The rare D configuration predominated in these two species (Fig. 2B and C).

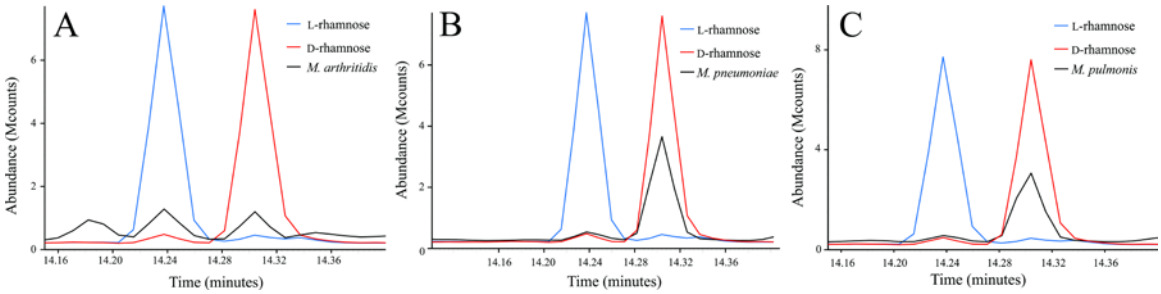

Fig. 2. Gas chromatograms of dialyzed lysates of *M. arthritidis* (A), *M. pneumoniae* (B), and *M. pulmonis* (C), subjected to butanolysis to resolve and D- and L-rhamnose. The enantiomers of rhamnose were identified by comparison to standards of each configuration.

^{13}C labeling

To confirm rhamnose biosynthesis, mycoplasmas were grown in medium supplemented with carbohydrates containing ^{13}C isotopes (universally labeled). When the TMS derivatives of the methyl glycosides of ^{12}C glucose, mannose and rhamnose are fragmented by electron ionization, ions at *m/z* 133 containing one carbon atom (C_1), 204 containing two carbon atoms (C_2—C_3 or C_3—C_4) and 217 containing three carbon atoms (C_2—C_3—C_4) are detected by MS (DeJongh *et al.*, 1969). The increased mass of ^{13}C

isotopes shifts the more abundant ions to *m/z* 134, 206 and 220. Also abundant are ions at *m/z* 147, a known adduct of the TMS reagent used for methanolysis (DeJongh *et al.*, 1969). The ^{13}C labeling of mycoplasmal glycomoieties was examined by GC/MS analysis of dialyzed lysates. The results are summarized in Table 1. When grown in medium supplemented with D-[U-^{13}C]glucose monosaccharide, the abundant ions from glucose, mannose and rhamnose are found at *m/z* 133, 204 and 217, not *m/z* 134, 206 and 220 for the three species *M. arthritidis*, *M. pneumoniae* and *M. pulmonis* (Figs. 3, S2 and S3). Thus, the glucose, mannose and rhamnose residues of the glycomoieties were not labeled with ^{13}C. The mass spectra of TMS derivatives of the methyl glycosides of ribose standards were analyzed and had ions at *m/z* 133, 204 and 217, as expected for fragmentation of pentose sugars (Kochetkov and Chizhov, 1965, Lawson *et al.*, 1971). Ribose was detected in the mycoplasmal samples grown in D-[U-^{13}C]glucose, suggesting that some nucleic acids were not removed by nuclease digestion and dialysis. The ions from the TMS derivatives of the methyl glycosides of ribose were found at m/z 133, 204 and 217 in the case of *M. arthritidis* but at 134, 206 and 220 for *M. pneumoniae* and *M. pulmonis*. Thus, ribose was labeled with ^{13}C in the glycolytic species of mycoplasma only, indicating that glucose was imported and metabolized in these species but not in the non-glycolytic *M. arthritidis*.

Table 1. ^{13}C labeling (ratio of ^{13}C to ^{12}C ions) of the indicated sugars in *M. arthritidis*, *M. pulmonis*, and *M. pneumoniae* when medium is supplemented with ^{13}C-glucose or ^{13}C-starch.

		M. arthritidis			*M. pulmonis*			*M. pneumoniae*		
	Ion ratios	134/133	206/204	220/217	134/133	206/204	220/217	134/133	206/204	220/217
^{13}C-glucose supplement	Rhamnose	1[a]	1	1	2	1	3[b]	2	1	7[b]
	Glucose	1	1	2	1	1	1	1	1	1
	Mannose	1	1	1	1	1	1	1	1	1
	Ribose	1	1	1	4	6	17	17	6	17
	Galactose	NA[c]	NA	NA	1	1	1	1	1	1
^{13}C-starch supplement	Rhamnose	9	290	600	7	25	467	7	21	43
	Glucose	27	2,300	16,000	26	820	6,000	26	1,100	8,300
	Mannose	2	3	7	1	1	1	1	1	2
	Ribose[d]	1	2	1	2	2	3	2	5	2
	Galactose	NA	NA	NA	5	5	13	5	27	7

[a]Each ion ratio for the indicated sugar was divided by the ion ratio of the ^{12}C standard to normalize. For both ^{12}C glucose and ^{12}C starch, the ratio of 134 to 133 ions, the ratio of 206 to 204 ions, and the ratio of 220 to 217 ions was 0.1, 0.1, and 0.03, respectively. For comparison, the ratio of 134 to 133 ions, 206 to 204 ions, and 220 to 217 ions was 2.7, 220, and 530, respectively, for ^{13}C glucose. The ratio of 134 to 133 ions, 206 to 204 ions, and 220 to 217 ions was 2.9, 160, and 300, respectively, for ^{13}C starch.

[b]Apparent increase in ion ratio attributed to labeled ribose (major peak) overlapping the major rhamnose peak in the chromatogram.

[c]NA, Not applicable, galactose not detected in this species.

[d]There is no issue of labeled rhamnose overlapping the ribose peak because the ribose minor peak was used for this calculation.

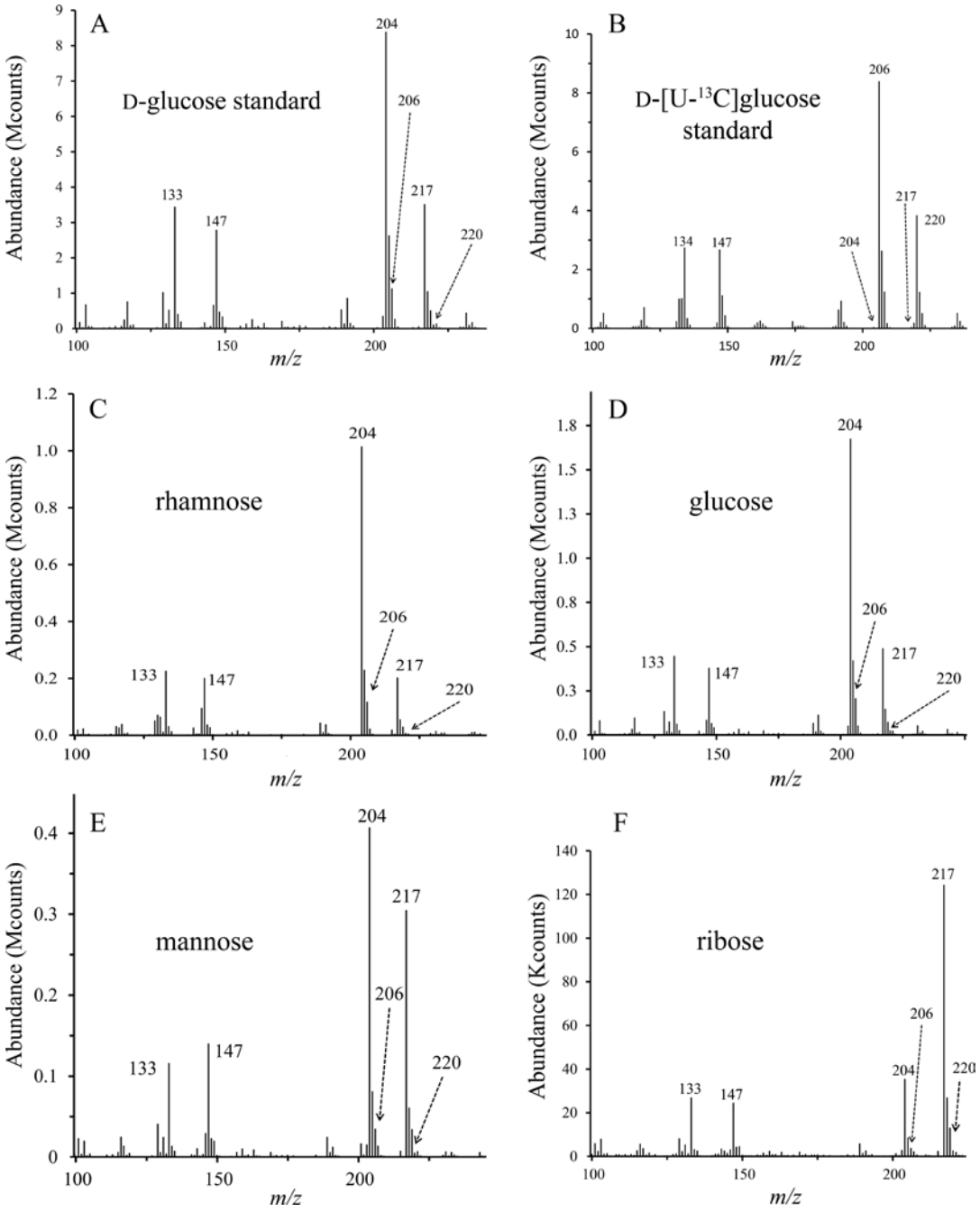

Fig. 3. Mass spectra of TMS derivatives of the methyl glycosides from *M. arthritidis* cells grown in medium supplemented with D-[U-^{13}C]glucose. (A) ^{12}C glucose standard, (B) D-[U-^{13}C]glucose standard, (C) rhamnose, (D) glucose, (E) mannose, (F) ribose.

The failure of even the glucose residues of the glycomoieties of these species to become labeled when the medium was supplemented with D-[U-^{13}C]glucose demonstrated that monosaccharides were not utilized for synthesis of these macromolecules. The commercial availability of [U-^{13}C]starch provided a resource to determine whether glucose polymers would support the synthesis of rhamnose. In contrast to the results obtained with D-[U-^{13}C]glucose monosaccharide, cells grown in the presence of [U-^{13}C]starch yielded ^{13}C-labeled rhamnose as evidenced by the abundant ions at *m/z* 134, 206 and 220 (Fig. 4C, and panel C of Figs. S4 and S5). We believe that [U-^{13}C]starch also labeled glucose (Fig. 4D, and panel D of Figs. S4 and S5) but no firm conclusion could be reached because some of the starch molecules did not remain in solution during the course of the experiment and were harvested along with the mycoplasma cells. [U-^{13}C]starch did not label mannose in *M. pneumoniae* and *M. pulmonis* but perhaps slightly did so in *M. arthritidis* (Fig. 4E, and panel E of Figs. S4 and S5). In contrast to D-[U-^{13}C]glucose, ribose was not labeled appreciably by [U-^{13}C]starch (Fig. 4F, and panel F of Figs. S4 and S5), suggesting that monosaccharides and polysaccharides are metabolized distinctly. Galactose was absent in lysates of *M. arthritidis* but found in *M. pneumoniae* and *M. pulmonis*. Galactose in both *M. pneumoniae* and *M. pulmonis* was labeled in cultures supplemented with [U-^{13}C]starch but not D-[U-^{13}C]glucose (panel G of Figs. S2-S5).

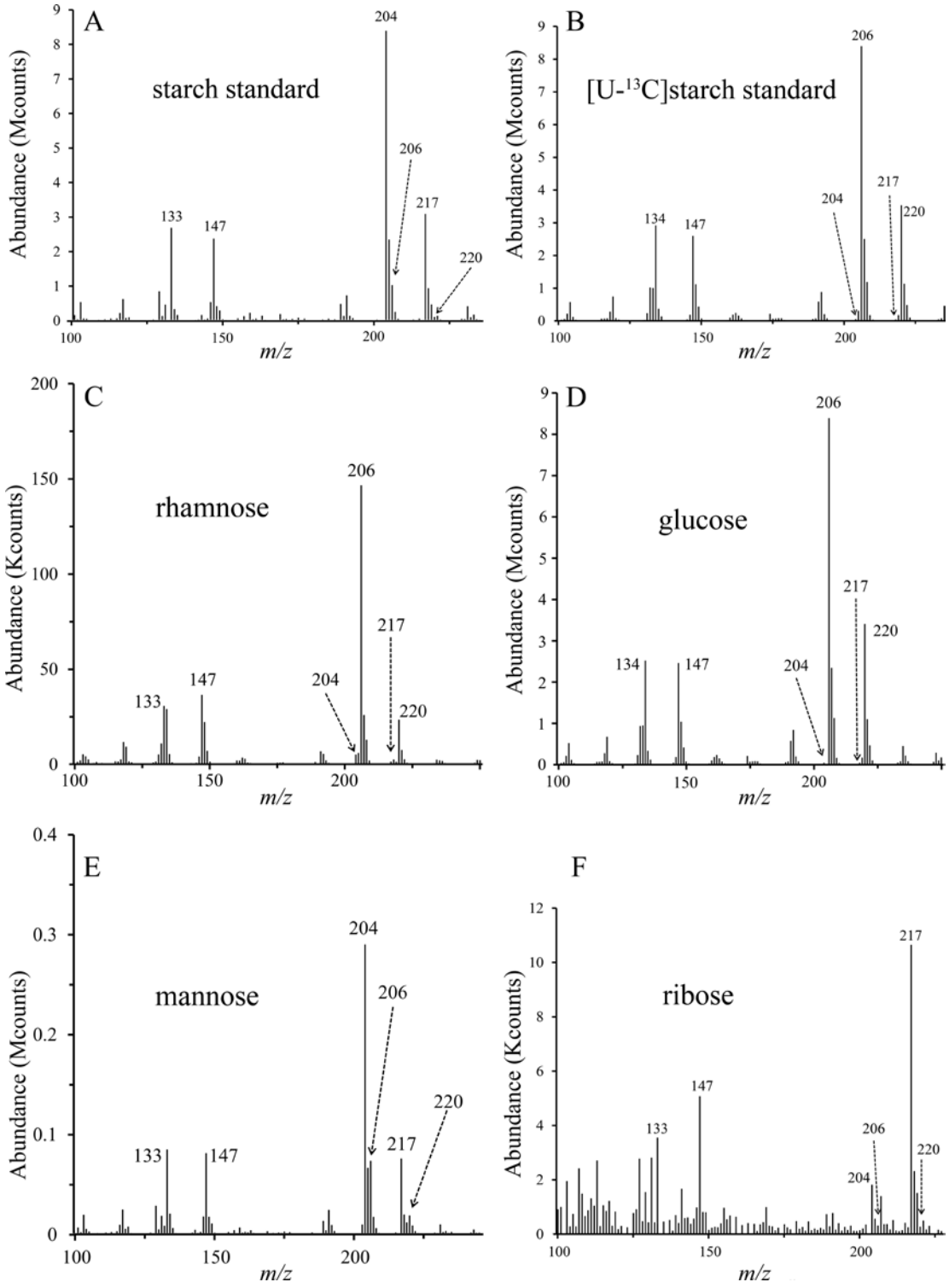

Fig. 4. Mass spectra of TMS derivatives of the methyl glycosides from *M. arthritidis* cells grown in medium supplemented with [U-^{13}C]starch. (A) ^{12}C starch standard, (B) [U-^{13}C]starch standard, (C) rhamnose, (D) glucose, (E) mannose, (F) ribose.

Relative amounts of D- and L-rhamnose are dependent on medium composition

The amount of rhamnose in *M. arthritidis* increased by about 10-fold when serum-free medium (SFM) was supplemented with starch (Fig. 5), with an increase in the relative abundance of L-rhamnose (Fig. 6). These results are in contrast to the finding of nearly equal amounts of D- and L-rhamnose in *M. arthritidis* cells grown in medium without starch (Figs. 2A and 6A). A medium supplement commonly used for growth of mycoplasmas is yeast extract. When yeast extract was added to SFM, there was a similar increase in the relative amount of L-rhamnose (Fig. 6C).

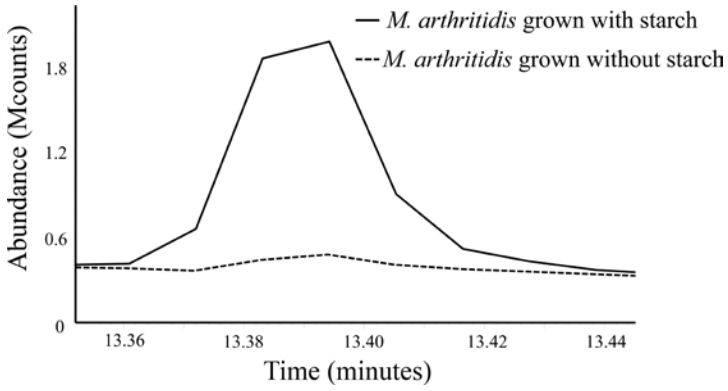

Fig. 5. Representative chromatogram showing the abundance of rhamnose from cells grown in medium with and without starch. Dialyzed lysates containing 20-μg protein were analyzed by GC/MS.

Medium glycomoieties

Rhamnose was detected in all species of mycoplasma examined, even when starch was absent from the growth medium. Because the mycoplasmas did not convert glucose monosaccharide into rhamnose, the growth medium must supply oligosaccharides or glycoconjugates that would support rhamnose biosynthesis. Some of the media formulations used in this study contained serum and yeast extract, which is a component

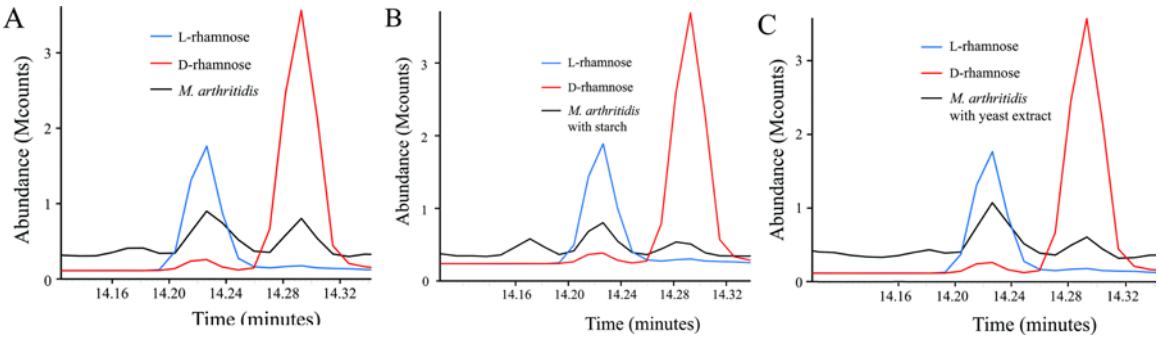

Fig. 6. Gas chromatograms of dialyzed lysates of *M. arthritidis* grown in SFM (A), SFM supplemented with starch (B), or SFM supplemented with yeast extract (C) and subjected to butanolysis to resolve D- and L-rhamnose. The enantiomers of rhamnose were identified by comparison to standards of each configuration.

of mycoplasma broth base. Serum and yeast extract have an abundance of glycoproteins that could serve as starting material for rhamnose synthesis. SFM would lack these glycoproteins. Peptone and bovine serum albumin (BSA) were major ingredients of the SFM. All SFM components were dialyzed to remove monosaccharides and analyzed by GC/MS to examine the composition of any glycoconjugates that might be present. Dialyzed peptone and BSA were the only reagents that contained significant levels of glucose and mannose, and the peptone also contained an abundant level of galactose (Fig. 7A and B). Hence, the SFM did contain glycoconjugates of mammalian origin that might support a minimal level of rhamnose synthesis in the absence of a glucose polymer (starch). Also analyzed by GC/MS was the yeast extract that had been used in some experiments to supplement SFM. Yeast extract contained substantial quantities of mannose and glucose (Fig. 7C). We note that no rhamnose whatsoever was detected in BSA, peptone, yeast extract, or any of the other reagents used in the media formulations for study.

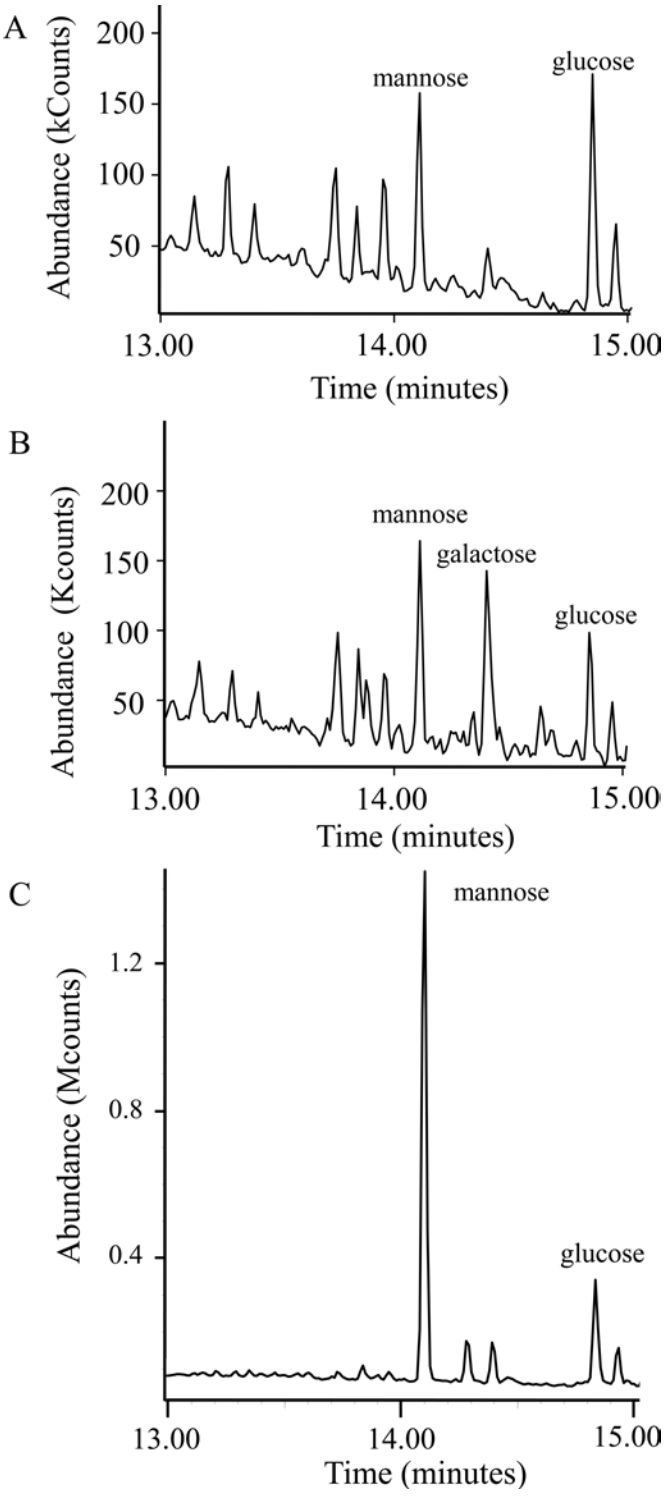

Fig. 7. Gas chromatograms of dialyzed (A) BSA (1 mg), (B) peptone (2.5 mg), and (C) yeast extract (3 mg). Due to a column replacement, the retention times of the TMS derivatives of the glycosides in this figure vary from other figures.

Rhamnose partitioning in chloroform-methanol

Dialyzed lysates of *M. arthritidis* were extracted with chloroform-methanol. GC analysis was performed on the material that partitioned into the aqueous and organic phases as well as the insoluble material at the interphase. With the extraction protocol, glycolipids partition into the organic phase and polysaccharide into the aqueous phase. Some protein should be found in the aqueous phase but most of it is in the interphase (Wessel and Flugge, 1984). No rhamnose was detected in the organic phase, indicating that rhamnose is not a component of glycolipid (Fig. 8). Glucose was found in the organic phase, consistent with a previous report of glucolipid in this species (Li *et al.*, 1997). Glucose, mannose and rhamnose were found in both the aqueous phase and the interphase.

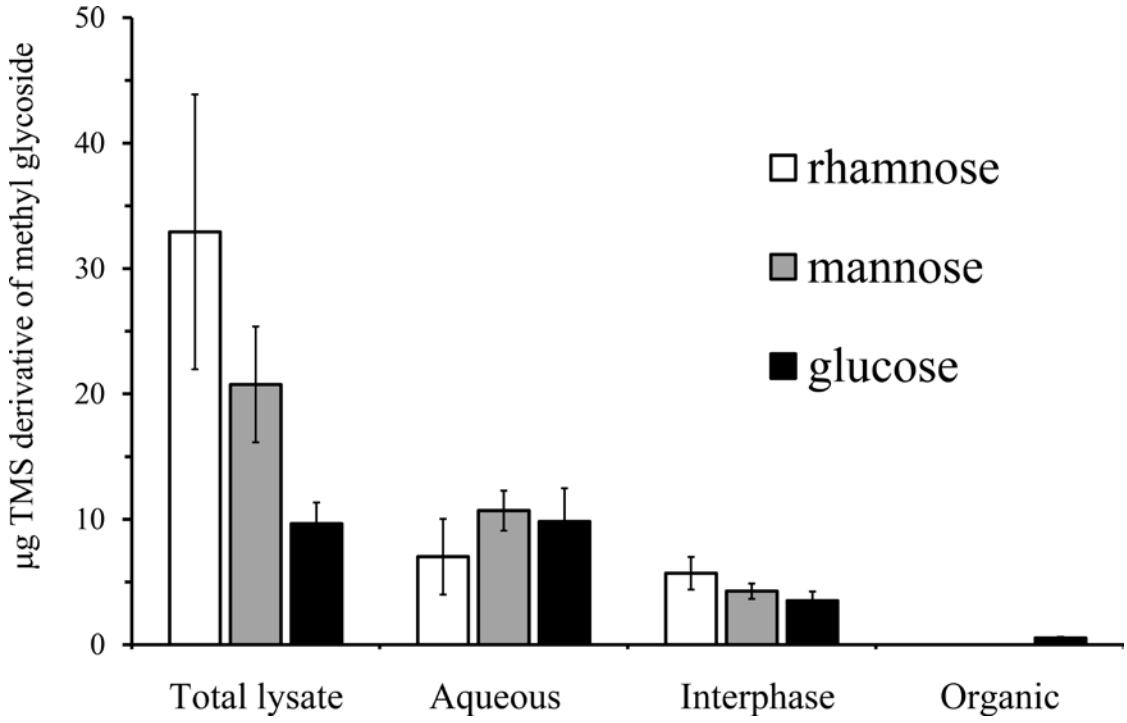

Fig. 8. Phase partitioning of *M. arthritidis* lysate in chloroform-methanol (n = 4). Error bars are standard error of the mean.

Discussion

Over 20 species of mycoplasma are listed in the NCBI genome projects database (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html). There are few genes annotated as coding for nucleotidyltransferases, glycosyltransferases, and other enzymes associated with sugar synthesis (Daubenspeck *et al.*, 2014). However, it is clear that mycoplasmas synthesize polysaccharides and glycolipids. *M. mycoides* synthesizes a galactan (Plackett and Buttery, 1958), *M. pulmonis* synthesizes a capsule that is antiphagocytic and protects from complement (Daubenspeck *et al.*, 2009, Bolland *et al.*, 2012, Shaw *et al.*, 2013), and *M. pneumoniae* synthesizes an adhesive polysaccharide necessary for robust biofilm formation (Simmons *et al.*, 2013). Possibly all species of mycoplasma produce glycolipids. For *M. pneumoniae*, the glycolipids contain galactose or glucose (Klement *et al.*, 2007). These glycolipids are thought to be important for pathogenesis through their involvement in molecular mimicry (Ang *et al.*, 2002, Yuki, 2007, Kitazawa *et al.*, 1998, Kusunoki *et al.*, 2001).

Rhamnose biosynthesis is well studied in bacteria (Giraud and Naismith, 2000). This 6-deoxy monosaccharide is common in bacteria and also found in some plants and eukaryotic microbes but not in mammals. The stereoisomers D- and L-rhamnose are mirror images, with the L-form being significantly more common. Generally, these enantiomers are synthesized by two distinct pathways. G6P is converted to G1P, which is used for production of dTDP-glucose to initiate the pathway for synthesis of L-rhamnose. Synthesis of D-rhamnose involves the production of GDP-mannose from M1P. A hydrolase converts the nucleotide sugar to a 4-keto-6-deoxyhexose. For L-rhamnose synthesis, the RmlC enzyme catalyzes an isomerase reaction that generates dTDP-L-lyxo-

6-deoxy-4-hexulose. The final step in synthesis is shared between the two pathways and is a NADPH-dependent reaction that reduces the ketone at the C_4 position, giving rise to dTDP-L-rhamnose or GDP-D-rhamnose. None of the species of mycoplasma for which the genome sequence is available contain any homologs of known rhamnose synthesis machinery.

Although rhamnose was previously reported to be present in *M. pneumoniae* (Allen and Prescott, 1978), it was unexpected to find rhamnose throughout the *Mycoplasma* genus because of the lack of recognizable rhamnose synthesis genes in any species, including *M. pneumoniae*. A bigger surprise was that the mycoplasmas produce D-rhamnose, an exceedingly rare carbohydrate. The glycolytic species *M. pneumoniae* and *M. pulmonis* had the D-form only while the non-glycolytic *M. arthritidis* had both D- and L-rhamnose. The failure of carbon isotopes from D-[U-^{13}C]glucose monosaccharide to be incorporated into rhamnose suggested a novel pathway for synthesis that did not involve the phosphorylation of glucose to G6P followed by its conversion to G1P or M1P. The glycolytic species do import glucose monosaccharide, converting it to G6P (Pollack *et al.*, 1983). In these species, ribose was labeled by D-[U-^{13}C]glucose monosaccharide, demonstrating that hexoses can be converted to pentoses as suggested by bioinformatic analysis. In contrast, ribose was not labeled with D-[U-^{13}C]glucose monosaccharide in *M. arthritidis*, and this species might not import monosaccharides.

[U-^{13}C]starch labeled rhamnose and galactose but not mannose and ribose in *M. pneumoniae* and *M. pulmonis*. Bioinformatic analysis indicates that these species are missing phosphoglucomutase and glucose phosphatases. Hence, the mycoplasmas should not be able to interconvert G6P and G1P or generate glucose from either hexose

phosphate. The mycoplasmas presumably hydrolyze starch to oligosaccharides that are imported. Perhaps G1P is produced in mycoplasmas only by transfer of phosphate to the hexose as the glycosidic bond between the sugar residues of an imported oligosaccharide is broken, similar to the proposed production of galactose-1-P in bifidobacteria (Yamamoto, 2012). The resulting G1P is then used to support synthesis of rhamnose and glycoconjugates. Because ribose was not labeled with [U-^{13}C]starch, glycolysis and synthesis of glycoconjugates may occur by separate pathways with no interconversion of G6P and G1P as predicted from bioinformatics. It is also possible that the mycoplasmas transfer the energy from the glycosidic bond of oligosaccharides to something other than G1P, such as a lipid-linked sugar (Lairson *et al.*, 2008). The failure of mannose to be labeled by D-[U-^{13}C]glucose or [U-^{13}C]starch suggests that there is no interconversion of glucose and mannose, the two primary sugars found in the glycomoieties of SFM. The requirement for oligosaccharides as substrate for glycosyltransferase reactions instead of nucleotide sugars would explain the failure of homology searches to identify the synthesis machinery (Henrissat *et al.*, 2008).

BSA and peptone are the only possible sources of mannose and glucose glycoconjugates in the SFM and must contain the appropriate precursors to synthesize both L- and D-rhamnose in *M. arthritidis*. The ability of medium supplements such as yeast extract and starch to vary the relative amounts of D- and L-rhamnose in *M. arthritidis* suggests that some glycomoieties in the medium support synthesis of L-rhamnose and others support D-rhamnose. Perhaps oligosaccharides, such as those derived from α-linked polymers of starch, primarily support the synthesis of L-rhamnose via a pathway that does not involve nucleotide sugars but nevertheless includes

dehydratase, epimerase and reductase reactions similar to the conversion of UDP-glucose to dTDP-L-rhamnose in other organisms. D-rhamnose in mycoplasmas may be synthesized from a different glycan, such as a mannose glycoconjugate, similar to the conversion of GDP-mannose to GDP-D-rhamnose in other organisms. In this scenario, one might have predicted that yeast extract, which contains high levels of mannose, would primarily support the synthesis of D-rhamnose, which was not the case. The mannose-containing oligosaccharides in yeast extract might have linkages that are not used or used only inefficiently by the mycoplasma.

Although bioinformatic analysis suggests *M. arthritidis* and *M. pulmonis* cannot convert G1P to UDP-glucose, *M. pneumoniae* has the required nucleotidyltransferase for this reaction. *M. pneumoniae*, but not the other two species, also has the GalE enzyme that can interconvert UDP-glucose and UDP-galactose. *M. pneumoniae* has three annotated glycosyltransferases while the other species have only one. One of the glycosyltransferases of *M. pneumoniae* is known to catalyze synthesis of glycolipids and can use UDP-glucose and UDP-galactose as a substrate (Klement *et al.*, 2007). The pathway for glycolipid synthesis in species of mycoplasma that lack recognizable nucleotidyltransferases has not been investigated.

Chloroform-methanol partitioning of *M. arthritidis* indicates that rhamnose is not a component of glycolipid. We have no evidence to support rhamnose as being a residue of polysaccharides produced by *M. pneumoniae* or *M. pulmonis* (Simmons *et al.*, 2013, Daubenspeck *et al.*, 2009). There is evidence for glycoproteins in mycoplasmas (Demina *et al.*, 2009), and the finding of rhamnose at the interphase after extraction with chloroform-methanol suggests that rhamnose may be attached to protein. We are

currently investigating protein glycosylation in *M. arthritidis* to determine whether rhamnose, glucose and mannose are associated with glycoproteins.

M. pneumoniae and *M. pulmonis* are considered to be primarily respiratory pathogens of humans and murine animals, respectively, while *M. arthritidis* is a murine pathogen that persists in joints and causes arthritis. These species are found only in a mammalian host, which provides sterols and other nutrients that are essential for growth. These mycoplasmas have evolved to acquire sugars from host molecules such as glycoproteins and glycosaminoglycans. Bioinformatic analysis indicates that the mycoplasmas produce several glycosidases, which might generate the oligosaccharides to support glycoconjugate synthesis. The bovine pathogen *Mycoplasma dispar* reportedly has a capsule that is induced by coculture with host cells (Almeida and Rosenbusch, 1991), perhaps because the host cells provided glycans that supported polysaccharide synthesis. The strategy of using host oligosaccharides for glycoconjugate synthesis would be favorable from the standpoint of energy conservation and have pathogenic consequences as glycans are stripped from host molecules.

Experimental procedures

Strains and cultures

For initial pilot experiments, GC/MS analysis was performed on *Mycoplasma fermentans* strain PG18 (ATCC 19989), *M. mycoides* subsp. *capri* strain GM9 (Voelker *et al.*, 1995), *M. capricolum* ATCC 27343, *A. laidlawii* strain JA1 (Liss and Maniloff, 1973), *M. gallisepticum* strain PG31 (ATCC 19610), *M. pneumoniae* strain M129 (ATCC 29342), *M. genitalium* strain G37 (ATCC 33530), *M. arginini* ATCC 23838, *M.*

hyorhinis strain GDL (ATCC 23839), *M. arthritidis* strain 158 (Dybvig and Khaled, 1990), and *M. pulmonis* strain CTG (Daubenspeck *et al.*, 2009). The identity of the mycoplasma species was confirmed by amplifying and sequencing the 16S rRNA gene of each strain. For these pilot studies only, the culture medium was mycoplasma broth (MB), prepared as described elsewhere (Dybvig *et al.*, 2010, Dybvig *et al.*, 2008). For growth of the glycolytic species, the MB contained 0.5% dextrose and had a starting pH of 7.8. For growth of the two non-glycolytic species, *M. arginini* and *M. arthritidis*, the only change in the preparation of the MB was that the medium contained 0.5% arginine-HCl in place of dextrose and the pH was adjusted to 7.4.

Other than the pilot experiments, mycoplasmas were propagated in SFM similar to that described by Yus et al. (Yus *et al.*, 2009). The following were mixed in 1 liter of water: 25 ml 20% arginine-HCl for non-glycolytic species of mycoplasma (*M. arthritidis*) or 10 ml 50% dextrose for glycolytic species (*M. pneumoniae* and *M. pulmonis*), 13.5 g Dulbecco's Modified Eagle's Medium (DMEM, Sigma), 6 ml Isovitalex (Becton Dickinson), 1.2 ml 20% DNA (degraded herring sperm, Sigma), 50 mg ampicillin, 2.5 g Select Peptone 140 (Life Technologies), 0.2 mg α-lipoic acid (Sigma), 20 mg uracil (Sigma), 20 mg spermine, 0.5 g glycerol, and 6.8 g amino acid mixture. The amino acid mixture was prepared by mixing 7.1 g L-alanine, 12.5 g L-arginine, 10.6 g L-asparagine, 5.4 g glycine, 11.8 g L-histidine, 8.4 g L-isoleucine, 8.4 g L-leucine, 9.3 g L-lysine, 23.2 g L-methionine, 2.0 g L-phenylalanine, 9.2 g L-proline, 1.3 g L-serine, 7.6 g L-threonine, 2.0 g L-tryptophan, 0.4 g L-tyrosine, and 16.8 g L-valine (all amino acids obtained from Sigma). The SFM mixture was adjusted to pH 7.4 for growth of *M. arthritidis* or pH 7.8 for growth of glycolytic species and sterilized by filtration. SFM was completed by

adding lipid and cholesterol suspensions. The lipid suspension was made by dissolving 11 mg palmitic acid, 11 mg oleic acid, 11 mg linoleic acid, 11 mg glyceryl tripalmitate, 11 mg glyceryl trioleate, and 11 mg glyceryl trilinoleate in 0.5 ml 100% ethanol, heating to 80°C, and injecting it into 1 ml 10 mM HEPES buffer (pH was 7.4) also heated to 80°C. The cholesterol suspension was made by dissolving 20 mg cholesterol in 0.5 ml ethanol, heating to 80°C, and injecting it into 1 ml 10 mM HEPES heated to 80°C. The lipid and cholesterol suspensions were each added to filter-sterilized 20 ml HEPES (pH 7.4) with 1 g BSA. Both of the suspensions were incubated at room temperature for 30 minutes with occasional mixing with a vortex before adding to the SFM mixture. All lipids and cholesterol were obtained from Sigma. Injection is defined as pipetting vigorously at the bottom of the solvent tube. It is important for growth that the lipids and cholesterol be in a suspended state. If the suspensions do not remain in a suspended state after the 30-minute incubation, repeat the process. These suspensions were made fresh each time for optimal growth. For some experiments SFM was supplemented with starch (3.3 mg per ml culture medium) or yeast extract (Invitrogen, 6.6 mg per ml medium).

For labeling with ^{13}C glucose or ^{13}C starch, a low-glucose SFM (LG-SFM) was prepared as described for SFM except that Low Glucose DMEM was used instead of DMEM and a vitamin supplement was devised for use in place of glucose-containing Isovitalex. A 167x stock solution of vitamin supplement was prepared in 10 mM HEPES buffered at pH 7.2 in a total volume of 10 ml with 0.1 mg cobalamine, 0.13 mg para-aminobenzoic acid, 2.5 mg nicotinamide adenine dinucleotide, 10 mg L-cystine, 6.7 mg pyridoxine, 184 mg sodium pyruvate, 0.03 mg thiamine and 1 mg thiamine phosphate (all

obtained from Sigma). The mixture was filter sterilized and stored in 1 ml aliquots at 20°C until use.

Lysate preparation

Cultures were harvested (late-logarithmic to stationary growth phase) and washed three times with phosphate-buffered saline (PBS). The cells were suspended in 1 ml digestion buffer (100 mM Tris-Hcl, 10 mM $MgCl_2$, 5 mM NaN_3, pH 7) and sonicated at full power at 90% duty cycle on a Branson Sonifier 450 for 30 seconds. For experiments employing nuclease and protease digestions, lysates were heated to 80°C for 15 minutes to denature protein, digested overnight with 25 µg RNaseA and 25 µg DNaseI, and then digested overnight with 25 µg proteinase K.

GC/MS

Lysates were subjected to methanolysis to generate methyl glycosides that were analyzed by GC/MS. To accomplish this, lysates were first dialyzed with 5,000 volumes of water (Millipore) in 2,000 molecular-weight cutoff dialysis cassettes (Pierce) and then dessicated. The samples were subjected to methanolysis by treatment with 0.4 ml acidic methanol (1:10 acetyl chloride in methanol) at 80°C for at least 16 hours and dried again. The resulting methyl glycosides were dissolved in 150 µl methanol, transferred to polyspring inserts in glass tubes, evaporated and sealed under argon gas, and injected with 50 µl of reagent from the HMDS + TMCS + Pyridine, 3:1:9 (Sylon^{TM} HTP) Kit (Sigma). The resulting TMS derivatives were analyzed by GC/MS with an Agilent Technologies 6890N Network GC System and a 5973 Network Mass Selective Detector

with MSD Productivity Chemstation Software. The instrument injected 0.20 µl of sample into the 30-meter column holding a temperature of 70°C and after 5 minutes the temperature was increased to 220°C at 20°C/minute, and then increased to 275°C at 10°C/minute where it was maintained for 10 minutes. For GC/MS identification, standards consisted of D-arabinose, D-galactose, D-glucose, D-fucose, D-mannose, D-rhamnose, L-rhamnose, D-ribose, and D-xylose. All standards were obtained from Sigma, except D-rhamnose, which was obtained from Carbosynth Ltd.

Butanolysis

Butanolysis of methyl glycosides was performed as previously described using (R)-(-)-2-butanol (Gerwig *et al.*, 1978). Lysates containing 125-µg protein were subjected to methanolysis as described above. The methyl glycosides were dried in a new ampule and 0.5 ml acidic butanol (1:10 acetyl chloride in butanol) was added and the ampule was sealed and kept at 80° C for 8 hours. The sample was dried again and analyzed by GC/MS as described above. Controls consisted of standards of D- and L-rhamnose.

Isotopes

D-[U-^{13}C]glucose and [U-^{13}C]starch were obtained from Cambridge Isotopes Laboratories, Inc. Six mg D-[U-^{13}C]glucose in 1 ml water was added to 10 ml LG-SFM. In other experiments, 33 mg [U-^{13}C]starch in 1 ml water was added to 10 ml LG-SFM cultures that were also supplemented with 6 mg unlabeled glucose.

Acid hydrolysis of starch

For solubilization, 3.3 mg/ml starch was treated in 1N HCl at 80°C and vortexed every 15 minutes until it remained in solution. The pH was then adjusted to 7.2 with NaOH.

Lipid extraction

Lysates were extracted with chloroform and methanol by the method of Bligh and Dyer (Bligh and Dyer, 1959) to separate lipids, which partition into the organic phase, from hydrophilic material that partitioned into the aqueous phase.

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SUPPLEMENTARY FIGURES

Fig. S1

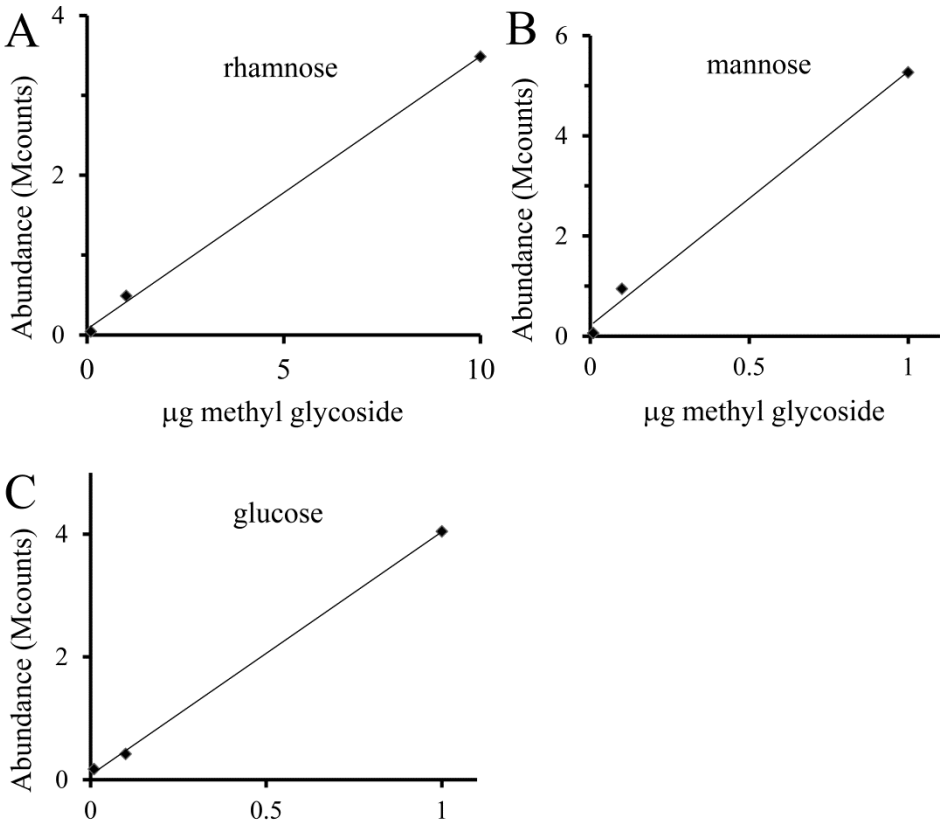

Fig. S1. Standard curves of rhamnose (A), mannose (B) and glucose (C) using known concentrations within the linear range as detected by GC/MS. R^2 of each trend line was greater than 0.99.

Fig. S2

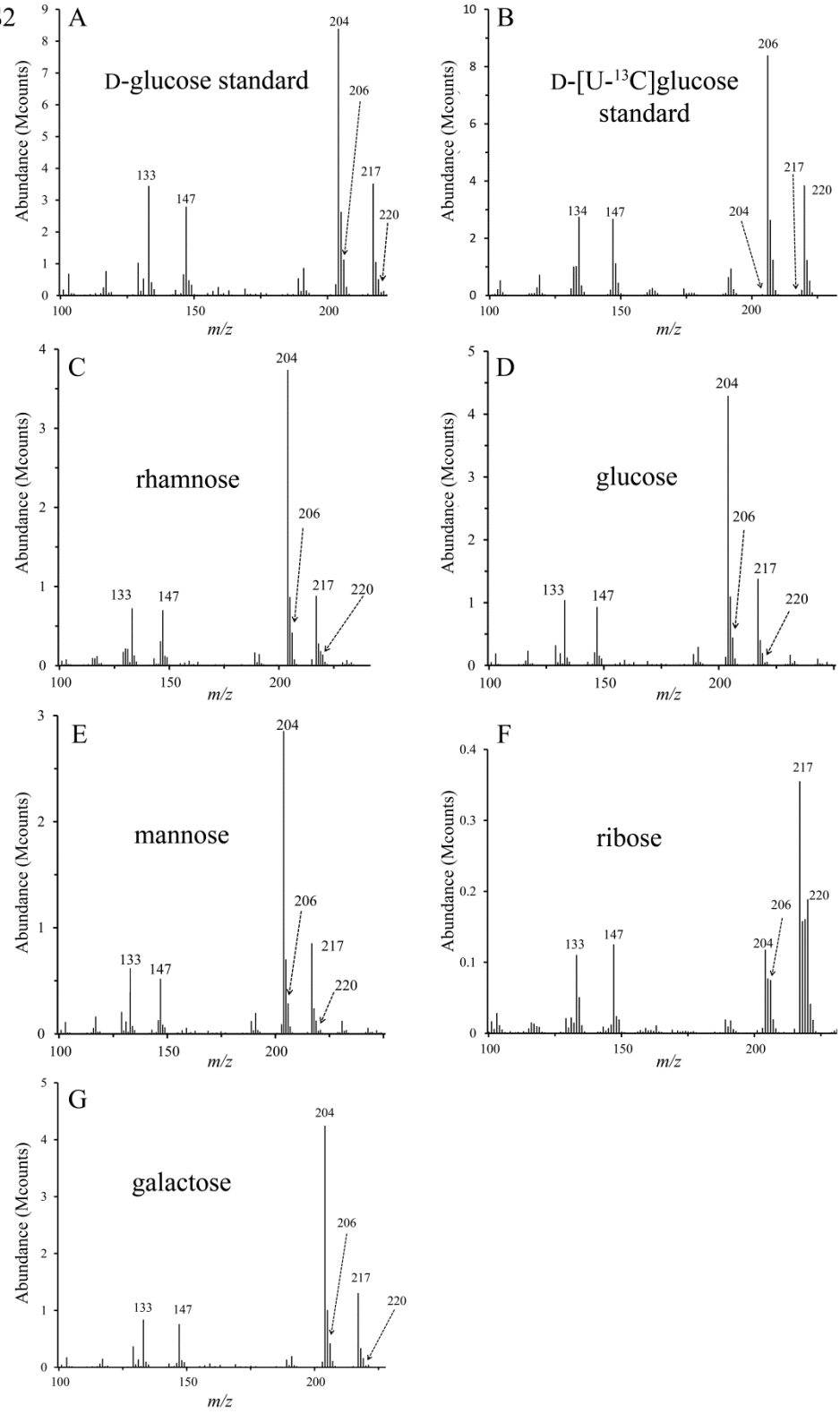

Fig. S2. Mass spectra of TMS derivatives of methyl glycosides from *M. pneumoniae* cells grown in SFM supplemented with D-[U-^{13}C]glucose. (A) ^{12}C glucose standard, (B) D-[U-^{13}C]glucose standard, (C) rhamnose, (D) glucose, (E) mannose, (F) ribose, (G) galactose.

Fig. S3

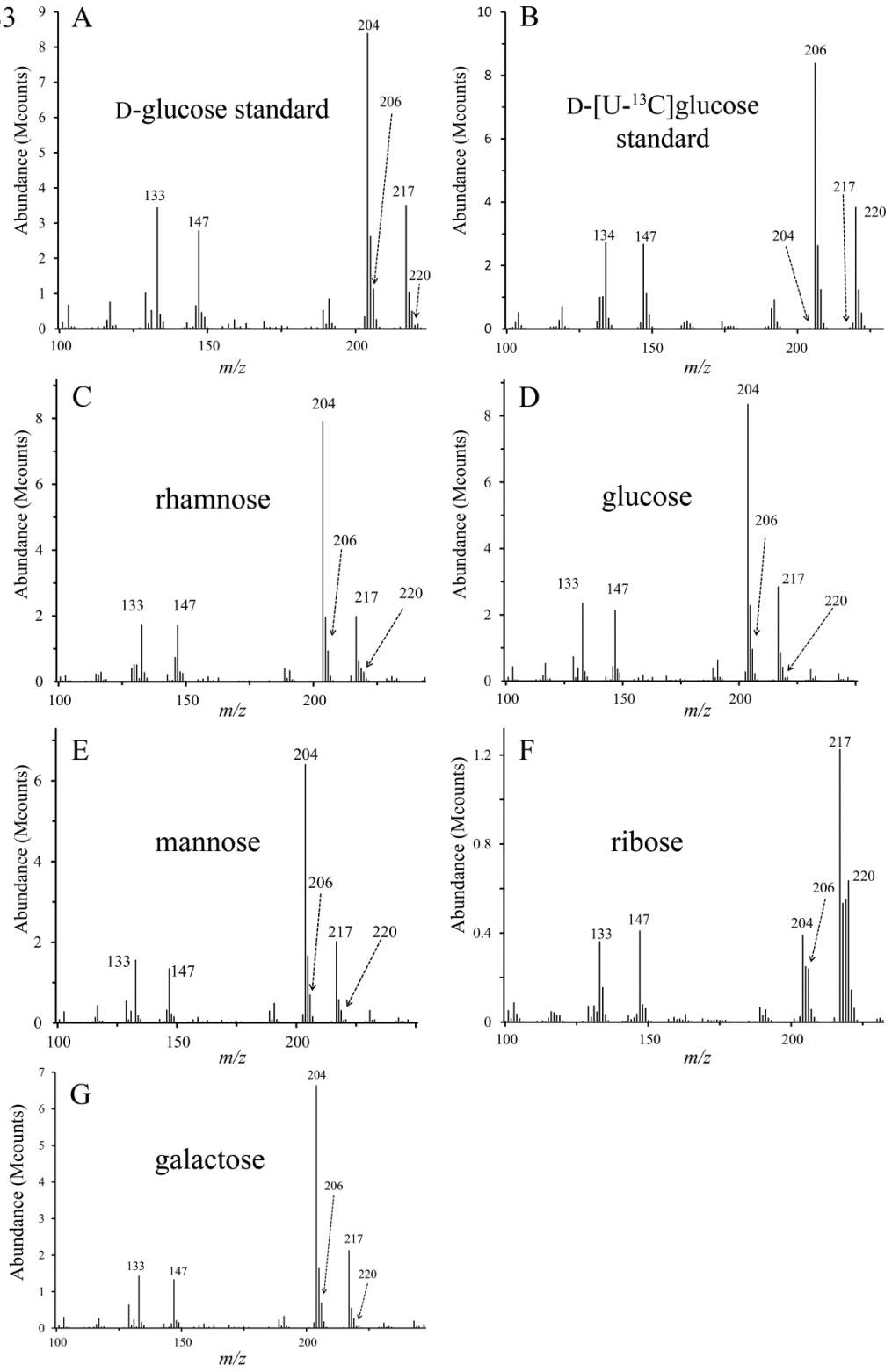

Fig. S3. Mass spectra of TMS derivatives of methyl glycosides from *M. pulmonis* cells grown in SFM supplemented with D-[U-^{13}C]glucose. (A) ^{12}C glucose standard, (B) D-[U-^{13}C]glucose standard, (C) rhamnose, (D) glucose, (E) mannose, (F) ribose, (G) galactose.

Fig. S4

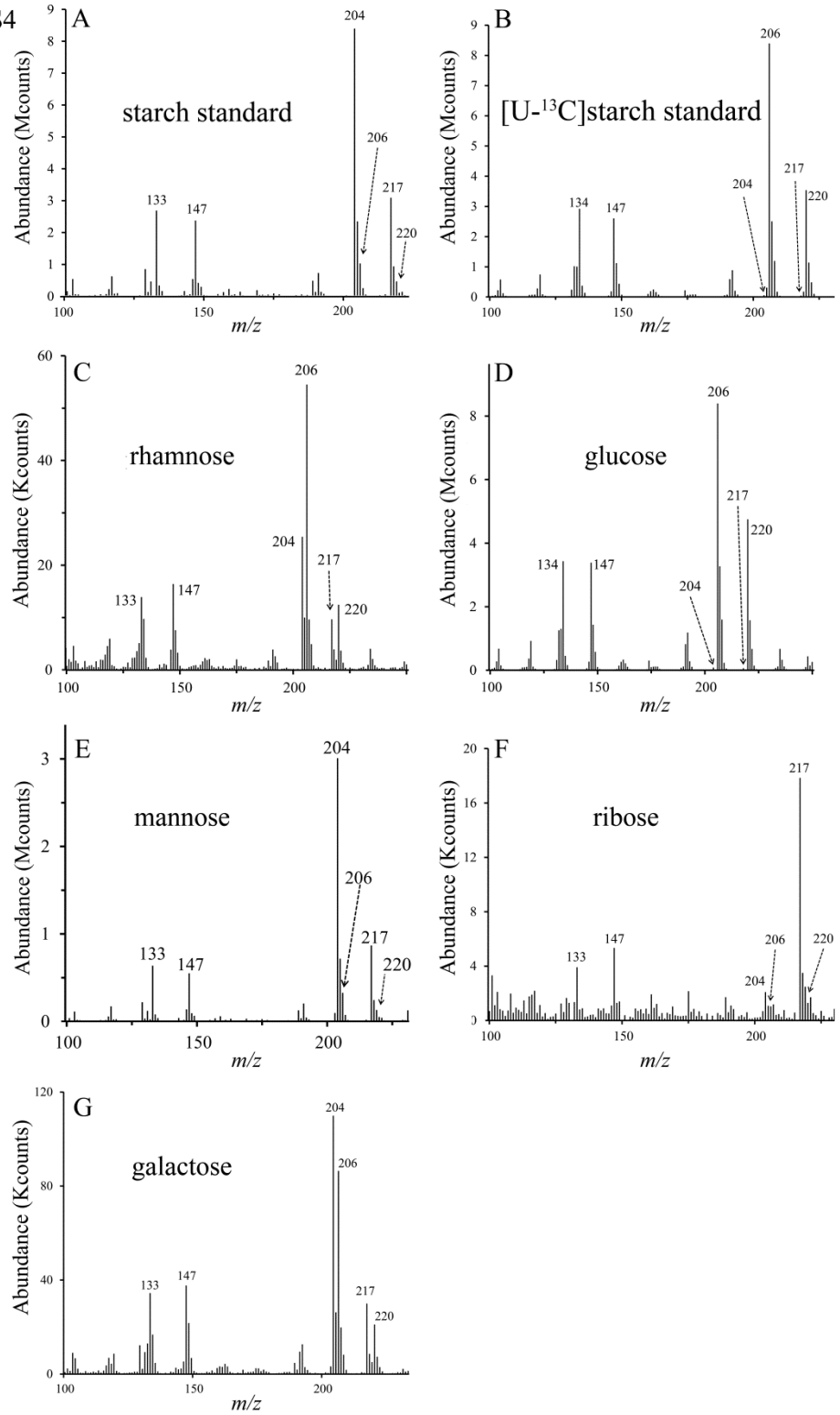

Fig. S4. Mass spectra of TMS derivatives of methyl glycosides from *M. pneumoniae* cells grown in SFM supplemented with [U-^{13}C]starch. (A) ^{12}C starch standard, (B) D-[U-^{13}C]starch standard, (C) rhamnose, (D) glucose, (E) mannose, (F) ribose, (G) galactose.

Fig. S5

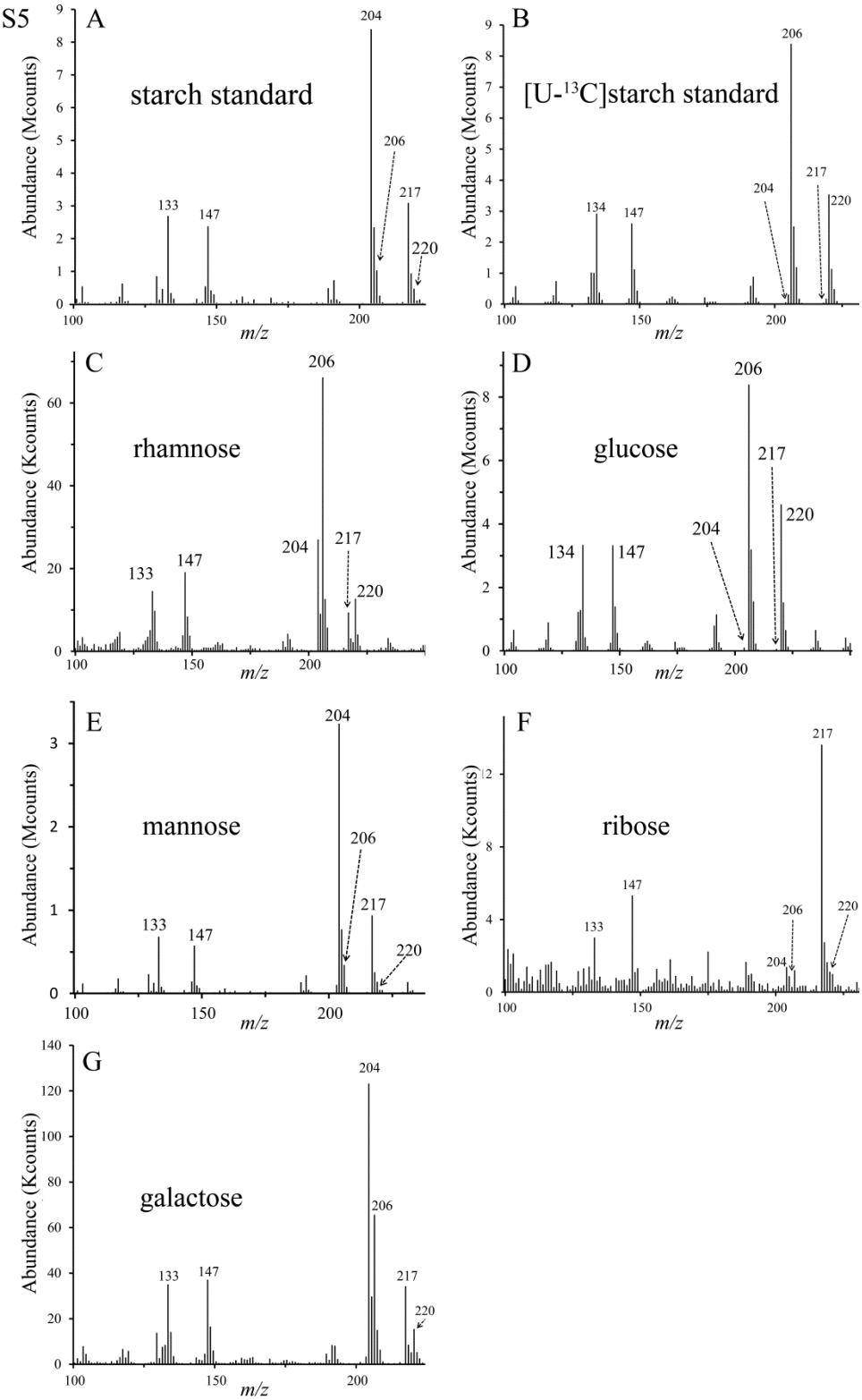

Fig. S5. Mass spectra of TMS derivatives of methyl glycosides from *M. pulmonis* cells grown in SFM supplemented with [U-^{13}C]starch. (A) ^{12}C starch standard, (B) D-[U-^{13}C]starch standard, (C) rhamnose, (D) glucose, (E) mannose, (F) ribose, (G) galactose.

CHAPTER 3: *O*-LINKED PROTEIN GLYCOSYLATION IN MYCOPLASMA

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O-Linked Protein Glycosylation in Mycoplasma

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Running Title: Mycoplasma glycoprotein

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Abstract

Although mycoplasmas have a paucity of glycosyltransferases and nucleotidyltransferases recognizable by bioinformatics, these bacteria are known to produce polysaccharides and glycolipids. We show here that mycoplasmas also produce glycoproteins and hence have glycomes more complex than previously realized. Proteins from several species of *Mycoplasma* reacted with a glycoprotein stain, and the murine pathogen *Mycoplasma arthritidis* was chosen for further study. The presence of *M. arthritidis* glycoproteins was confirmed by high-resolution mass spectrometry. *O*-linked glycosylation was clearly identified at both serine and threonine residues of sequenced *M. arthritidis* proteins. No consensus amino acid sequence was evident for the glycosylation sites of these peptides. A single hexose was identified as the *O*-linked modification, and glucose was confirmed as the hexose at two of the glycosylation sites. This is the first study to conclusively identify sites of protein glycosylation in any of the mollicutes.

INTRODUCTION

Mycoplasmas (Class *Mollicutes*) are noted for causing chronic diseases of the respiratory and genital tracts and joints in many animals including humans. Factors that contribute to disease chronicity are largely unknown but include polysaccharides that protect the mycoplasma from host defenses [1, 2]. Mycoplasmas are related to Gram-positive bacteria but lack a cell wall and have a streamlined genome, usually containing 600-800 protein-coding regions. All species of *Mycoplasma* require cholesterol for growth and hence are only found in nature in an animal host. The fastidious nature of the mycoplasmas is often overcome in the laboratory by the use of rich culture medium containing undefined components such as animal serum, which introduces an enormous variety of glycoconjugates that can adsorb to the surface of the mycoplasma and confound interpretation of carbohydrate assays. We have adapted the serum-free medium described by Yus et al. [3] for growth of several species of mycoplasma, to facilitate studies on glycobiology.

Bioinformatic analysis of the genomes suggests that mycoplasmas have limited machinery for synthesis of polysaccharides and glycoconjugates. There is no evidence that mycoplasmas produce monosaccharides *de novo* and many species lack homologs of epimerase genes for interconversion of hexoses. The murine pathogens *Mycoplasma arthritidis* and *Mycoplasma pulmonis* have only a single annotated gene (Marth_orf849 and MYPU_7700) coding for a glycosyltransferase. One might predict that these mycoplasmas would have simple glycomes due to their lack of a cell wall and their limited biosynthetic machinery, but these bacteria produce a surprising number of

glycomoieties. Glycolipids containing 1 to 3 glucose or galactose residues have been described for several species and in some cases are known to be serologically active [4-6]. Data suggest that neurological manifestations such as Guillain-Barré syndrome following infection with the human pathogen *Mycoplasma pneumoniae* are a result of molecular mimicry with carbohydrate moieties of glycolipids [7-10]. Many species of *Mycoplasma* produce at least one polysaccharide. *M. mycoides* subsp. *mycoides* produces a galactan that has been described as being a component of a slime layer [11, 12]. *M. pulmonis* produces the EPS-1 polysaccharide that consists of equimolar amounts of glucose and galactose [13]. Many mycoplasmal species likely produce an adhesive polysaccharide as a component of the extracellular matrix of a biofilm [14-20].

Glycoproteins are thought to be less common in bacteria than in higher organisms, but evidence is accumulating that bacterial glycosylation is ubiquitous [21]. The data presented here conclusively demonstrate that proteins in *M. arthritidis* are glycosylated. The glycosylation sites identified are *O*-linked at serine or threonine residues. The glycans were only a single monosaccharide, but we predict that larger glycans exist and will be identified in future studies using media of greater complexity.

MATERIALS AND METHODS

Strains and culture conditions. *M. arthritidis* strain TnCtrl [22], derived from strain 158 [23], *Mycoplasma hominis* strain PG21 (ATCC 23114), and *M. pulmonis* strain CTG [13] were grown in serum-free medium similar to that described by Yus et al. [3]. The following were mixed in 1 liter of water: 13.5 g Dulbecco's Modified Eagle's Medium

(DMEM, Sigma), 6 ml Isovitalex (Becton Dickinson), 1.2 ml 20% DNA (degraded herring sperm; Sigma), 50 mg ampicillin, 2.5 g Select Peptone 140 (Life Technologies), 0.2 mg α-lipoic acid (Sigma), 20 mg uracil (Sigma), 20 mg spermine, 0.5 g glycerol, and 6.8 g amino acid mixture. The amino acid mixture was prepared by mixing 7.1 g L-alanine, 12.5 g L-arginine, 10.6 g L-asparagine, 5.4 g glycine, 11.8 g L-histidine, 8.4 g L-isoleucine, 8.4 g L-leucine, 9.3 g L-lysine, 23.2 g L-methionine, 2.0 g L-phenylalanine, 9.2 g L-proline, 1.3 g L-serine, 7.6 g L-threonine, 2.0 g L-tryptophan, 0.4 g L-tyrosine, and 16.8 g L-valine (all amino acids obtained from Sigma). Also added to the broth base was 25 ml 20% arginine-HCl for growth of the non-glycolyic species *M. arthritidis* and *M. hominis* or 10 ml 50% dextrose for growth of the glycolytic species *M. pneumoniae* and *M. pulmonis*. The mixture was adjusted to pH 7.4 for growth of the non-glycolytic species and 7.8 for the glycolytic species. The medium was completed by adding lipid and cholesterol suspensions. The lipid suspension was made by dissolving 11 mg palmitic acid, 11 mg oleic acid, 11 mg linoleic acid, 11 mg glyceryl tripalmitate, 11 mg glyceryl trioleate, and 11 mg glyceryl trilinoleate in 0.5 ml 100% ethanol, heating to 80°C, and injecting it into 1 ml HEPES buffer (10 mM, pH was 7.4) also heated to 80°C. The cholesterol suspension was made by dissolving 20 mg cholesterol in 0.5 ml ethanol, heating to 80°C, and injecting it into 1 ml HEPES buffer heated to 80°C. To each suspension was added to 20 ml HEPES buffer containing 1 g bovine serum albumin. Both of the suspensions were incubated at room temperature for 30 minutes with occasional mixing with a vortex before adding to the 1 liter of broth base. All lipids and cholesterol were obtained from Sigma. Injection is defined as pipetting vigorously at the bottom of the solvent tube. It is important for growth that the lipids and cholesterol be in

a suspended state. If the suspensions did not remain in a suspended state after the 30-minute incubation, the process of heating and cooling was repeated. The medium was made fresh each time for optimal growth.

In indicated experiments mycoplasmas were propagated in mycoplasma broth (MB) This medium is previously described elsewhere [24].

Glycoprotein staining of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Cultures were harvested (late-logarithmic to stationary growth phase) and washed three times with phosphate-buffered saline (PBS). The amount of protein in the samples was determined by using a BCA protein assay kit (Pierce).

Lipoproteins were extracted as previously described [25]. Briefly, 2 mg of protein were suspended in 1 ml solution of cold PBS and 1% TX-114 (Sigma), vortexed and rotated for 2 hours at 4°C. The sample was centrifuged at 12,000 x *g* for 5 minutes at 4°C to pellet insoluble material. The supernatant was incubated for 5 minutes at 37°C and centrifuged at 8,000 x *g* at room temperature. The upper phase was discarded, 900 µl cold PBS was added to the lower detergent phase, and the sample was incubated at 4°C for 5 minutes. This phase separation was repeated three times. The final extract was incubated at 4°C for 5 minutes and centrifuged at 12,000 x *g* to remove remaining insoluble material. The supernatant was collected and stored at -80°C. The extracted proteins were recovered by the addition of 9 volumes of cold methanol followed by precipitation overnight at -80°C. The sample was centrifuged at 12,000 x *g* for 10 minutes to collect the pellets.

Pellets containing 150 μg of total protein or TX-114-extracted protein were suspended in 20 μl SDS loading buffer and boiled for 10 minutes. The samples was loaded on 7.5% SDS-PAGE gels (Biorad) and electrophoresed at 100 V until the dye front reached within 1 cm of the bottom of the gel. Glycoproteins were stained using the Pro-Q Emerald 300 Glycoprotein Gel and Blot Stain Kit (Invitrogen) as described in the package insert protocol and were imaged with ultraviolet light using an AlphaImager EC system. After staining for glycoproteins, the same gel or in some cases parallel gels were stained with Coomassie blue to visualize total proteins in the preparation.

GC/MS. TX-114 was removed from lipoprotein-extracted samples with Pierce® Detergent Removal Spin Columns, dessicated, treated with 0.4 ml acidic methanol at 80°C for at least 16 hours and dried again. The resulting material was dissolved in 150 μl methanol, transferred to polyspring inserts in glass tubes, evaporated and sealed under argon gas, and injected with 50 μl of reagent from the HMDS + TMCS + Pyridine, 3:1:9 (Sylon^{TM} HTP) Kit (Sigma). Samples were analyzed by GC/MS with an Agilent Technologies 6890N Network GC System and a 5973 Network Mass Selective Detector with MSD Productivity Chemstation Software. The instrument injected 0.20 μl of sample into the 30 meter column holding a temperature of 70°C and after 5 minutes the temperature was increased to 220°C at 20°C/minute, and then increased to 275°C at 10°C/minute where it was maintained for 10 minutes. Methyl glycosides were identified by comparison to retention times and mass spectra of known standards.

Labeling of mycoplasma cell cultures. For ^{14}C labeling, ten μCi of either [U-^{14}C]glucose or [1-^{14}C]mannose was added to 1 ml overnight cultures. At times 0, 2, 24 and 48 hours, 100 μl of culture was centrifuged at 16,000 x *g* for 10 minutes and washed three times with PBS and suspended in 100-μl water. The suspensions were added to 4 ml ScintiVerse™ BD Cocktail (Fisher-Scientific) and radioactive counts assessed with a Wallac 1410 liquid scintillation counter.

For ^{13}C labeling, [U-^{13}C]starch was obtained from Cambridge Isotopes Laboratories, Inc. Medium was supplemented with a 1:1 ratio of ^{12}C and ^{13}C starch that had been solubilized by treatment in 1N HCl at 80°C and vortexed every 15 minutes until it remained in solution. The pH was then adjusted to 7.2 with NaOH. Only for the experiments using starch, the culture medium was mycoplasma broth, prepared as described elsewhere [24]. 33 mg starch in 3 ml water was added to 30 ml cultures.

FT-ICR. Individual protein bands were excised from the gel with a razor blade and subjected to in-gel tryptic digestion (with reduction by 10 mm dithiothreitol and alkylation with 50 mm iodoacetamide). Tryptic digests were loaded onto a liquid chromatography-mass spectrometry system composed of a Micro AS autosampler, LC nanopump (Eksigent), and a linear ion trap-Orbitrap Velos hybrid mass spectrometer (Orbitrap Velos, Thermo Fisher Scientific). The analytical column was a 100-μm diameter, 11-cm column pulled tip packed with Jupiter 5-μm C18 reversed-phase beads (Phenomenex). An acetonitrile gradient from 1 to 25% in 0.1% formic acid was run over 50 min at 650 nl/min. Orbitrap parameters were set to normal mass range (MS1, 300 < *m/z* < 1800) with a 50,000 resolution scan followed by five data dependent tandem mass

spectrometry (MS/MS) scans per cycle in profile mode. Dynamic exclusion was set to exclude ions for 2 min after a repeat count of three within a 45-sec duration. *M. arthritidis* peptides were identified by use of TurboSEQUEST v.27 (rev.12, Thermo Fisher Scientific), Byonic (Protein Metrics), algorithms with tryptic cleavage and a parent ion mass accuracy of 10.0 ppm with the *M. arthritidis* database. SEQUEST results were further refined through Scaffold 3.0 (Proteome software) at a 95% confidence at the peptide level and 99% confidence at the protein level [26]. In the algorithm searches, serine and threonine residues were searched for the presence of hexose (162.0528 Da) and deoxyhexose (146.0579 Da) combinations. The *M. arthritidis* proteins identified with glycosylation in the minimal media conditions were identified with at least 10 or more unique peptides for protein assignment. Sites of glycosylation attachment identified by the search algorithms or identified in the MS1 spectra were confirmed by manual interpretation of collision induced dissociation (CID) or electron transfer dissociation (ETD) MS/MS compared to theoretical fragmentation patterns calculated in Protein Prospector MS product tool (UCSF Mass Spectrometry Facility) and by the observance of non-glycosylated and glycosylated precursor ion species in the MS1 spectra with observed mass differences of hexose and/or deoxyhexose residues in different charge states [27].

RESULTS

Glycoprotein staining. As the technology for prokaryotic glycoprotein identification has become more refined, it is becoming clear that these molecules may not be as rare as

once thought [28]. Previous reports suggested the possibility of mycoplasma glycoproteins, but it was not clear if the glycoproteins were produced by the mycoplasma or components of the complex culture medium that had adhered to the mycoplasma surface [29, 30]. In *Mycoplasma gallisepticum* Demina *et al*. identified by periodic-acid based Pro-Q Emerald staining the presence of two putative glycoproteins but did not identify the glycan or the glycosylation site [31]. We stained proteins from several species of *Mycoplasma* with Pro-Q Emerald 300 and found significant levels of staining near the top of the gel and generalized staining throughout the gel approaching a smear (Fig. 1, panel A). *M. arthritidis* proteins reacted with Pro-Q Emerald and was chosen for further study.

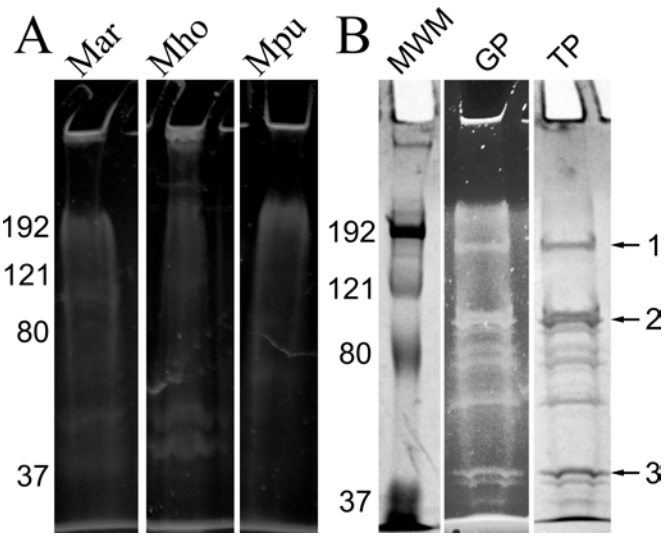

Fig. 1. Glycoprotein staining of SDS-PAGE gels. Panel A, total proteins from *M. arthritidis* (Mar), *M. hominis* (Mho), and *M. pulmonis* (Mpu), stained for glycoproteins with Pro-Q Emerald 300. Panel B: lane MWM, molecular weight markers (Bio-Rad Kaleidoscope); lane GP, glycoproteins extracted from *M. arthritidis* with TX-114 and stained with Pro-Q Emerald 300; lane TP, total proteins identified by subsequent staining with Coomassie of lane GP. Numbers on the left of the gels refer to molecular weight standards in kDa. Arrows on the right refer to bands excised for FT-ICR.

We reasoned that if glycoproteins were produced by mycoplasmas, some of these proteins would be at the cell surface. Many surface proteins in *M. arthritidis* and other species of mycoplasma are lipoproteins [24]. Lipoproteins partition into the detergent

phase when extracted with Triton X-114 (TX-114), whereas most other proteins partition into the aqueous phase [25]. Although an analysis of TX-114-extracted protein would not identify the complete set of glycoproteins of *M. arthritidis*, it was highly likely that at least some of the glycoproteins would be present in this fraction.

TX-114-extracted material was analyzed on SDS-PAGE gels, stained for glycoproteins utilizing the Pro-Q Emerald 300 and then stained again with Coomassie (Fig. 1B). Multiple bands reacted with the glycoprotein stain, suggesting that glycoproteins were abundant in the lipoprotein fraction. When compared to whole cell lysates the lipoprotein fraction concentrated the glycoprotein staining into distinct bands. Three bands (identified by arrows in Fig. 1B) that had the same mobility as the bands that reacted with the glycoprotein stain were excised from the Coomassie-stained gels, digested with trypsin, and the peptides were analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF). The results confirmed that all proteins in the bands originated from *M. arthritidis*. The predominant proteins in bands 1-3 were MARTH_455, MARTH_403, and MARTH_819, respectively. All three of these are annotated in GenBank (accession number CP001047) as lipoproteins.

Monosaccharide analysis of TX-114 extracted material and sugar transport. Gas chromatography showed glucose and mannose to be the major sugars present in the lipoprotein fraction of *M. arthritidis* (Fig. 2). Import of glucose and mannose into the non-glycolytic *M. arthritidis* was investigated in an effort to clarify which of these sugars was potentially associated with glycoproteins. When incubated with ^{14}C glucose or ^{14}C

mannose for periods of time ranging from 0 to 48 hours, no radiolabeling of the mycoplasmas occurred. Using the culture conditions employed in this study, *M. arthritidis* does not import either monosaccharide. Studies are ongoing that investigate oligosaccharide import in mycoplasmas.

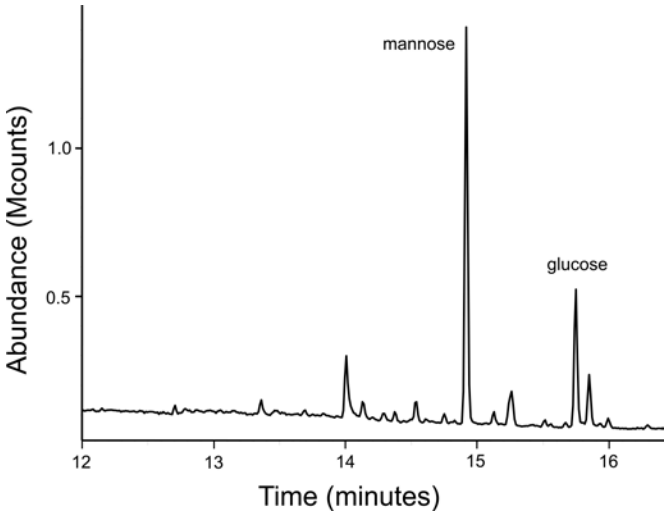

Fig. 2. GC/MS of TX-114 extracted material from *M. arthritidis*.

FT-ICR MS. Peptides were analyzed by liquid chromatography coupled to a hybrid linear quadrupole ion trap velos-Orbitrap Fourier transform mass spectrometer (Orbitrap MS) for glycosylation analysis. Specific glycopeptides were identified from the Orbitrap MS scans that contained a hexose (162.0795 Da). Three glycosylation sites on *M. arthritidis* proteins were conclusively identified. Two of the sites were on MARTH_403, a hypothetical lipoprotein, and the third site was on MARTH_819, an ABC transporter-binding lipoprotein. The glycans were a single *O*-linked hexose attached to either a serine or threonine residue.

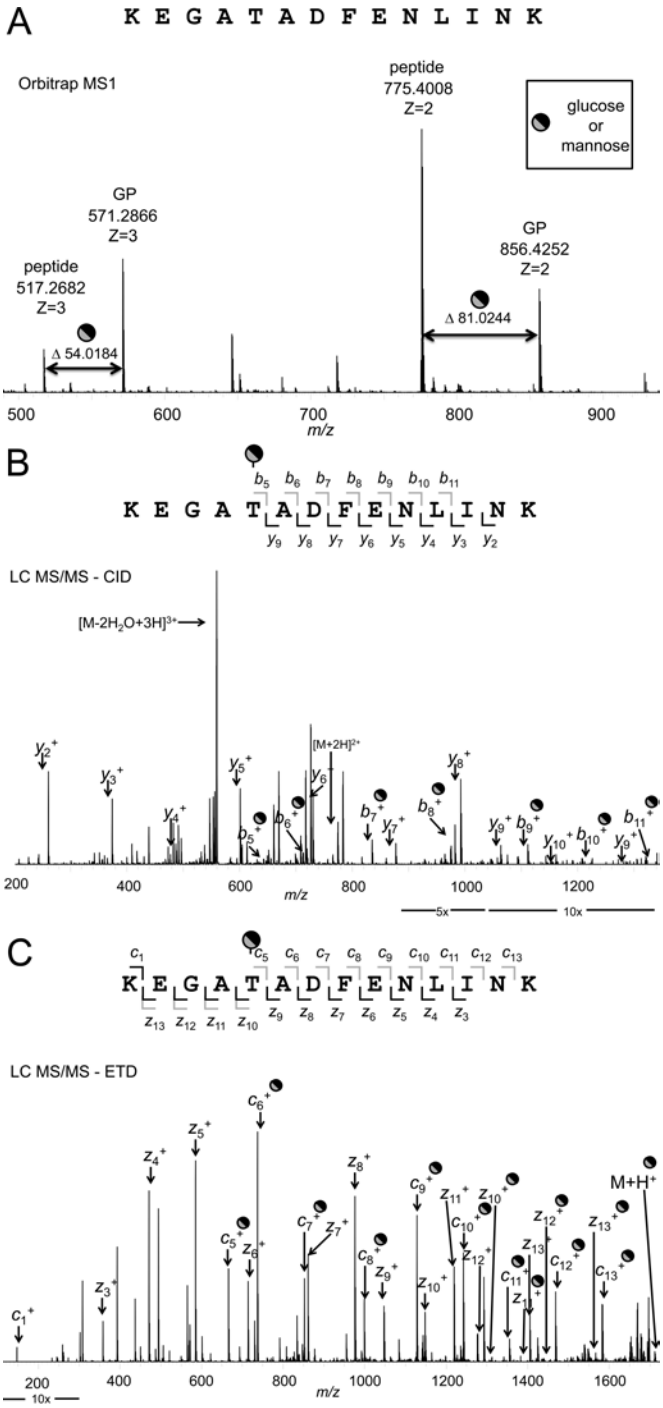

Fig. 3. Glycosylation of Thr[295] in the peptide KEGATADFENLINK of MARTH_403. Panel A, Orbitrap MS1 showing mass shift of the triply- and doubly-charged ions. The 54.0184 shift for z = 3 between the non-glycosylated peptide at *m/z* 517.2682 and the glycosylated peptide (GP) at *m/z* 571.2866 equates to a mass shift of 162.0552 Da, which corresponds to hexose glycosylation with a mass accuracy of 0.007 Da. The 81.0244 shift for z = 2 between the non-glycosylated peptide and the glycosylated peptide equates to a mass shift of 162.0488 Da, which corresponds to a hexose with a mass accuracy of 0.0006 Da. Panel B, LC MS/MS-CID of the glycosylated peptide showing the assigned b and y ions. Panel C, LC MS/MS-ETD of the glycosylated peptide showing the assigned c and z ions. Ion dividers above and below the peptide sequence are gray for glycosylated fragments and black for non-glycosylated fragments.

The first glycosylation site on MARTH_403 is at Thr[295], within the identified peptide sequence of KEGATADFENLINK. The charged species obtained from the Orbitrap MS of the above fragment generated two sets of fragments, the doubly-charged species at *m/z*

856.4252 (glycosylated) and *m/z* 775.8640 (non-glycosylated) and the triply-charged species at m/z 571.2866 (glycosylated) and *m/z* 517.2682 (non-glycosylated) (Fig. 3A). The mass shift between these two sets is determined to be 162.05 Da, the average calculated mass of a hexose linked through an *O*-linkage. The H_2O (18.0153 Da) lost during the glycosidic bond formation is deducted from the mass of monoisotopic hexose (180.0634 Da) for these calculations, not the amino acid. The mass accuracy associated with these techniques is exquisite and strongly indicates that the glycan mass and glycosylation sites are correctly identified.

LC MS/MS-CID was conducted on the triply charged peptide *m/z* 571.2866 captured by the Orbitrap MS, and manual analysis of the spectrum (Fig. 3B) was compared to the theoretical fragmentation patterns calculated in the Protein Prospector MS product tool. The b and y ions corresponded to the predicted pattern with the b series showing the addition of a hexose. The LC MS/MS-ETD showing the c and z ions for this glycosylated peptide fragment is shown in (Fig. 3C). The fragments at z_{10}, z_{11}, z_{12}, and z_{13} were identified in both the glycosylated and non-glycosylated forms adding to the support for glycosylation of the protein.

The second glycosylation site on MARTH_403 is at Ser^{779} within the peptide sequence LSELAEDLAKYEES^{779}HK. The charged species obtained from the Orbitrap MS of the above fragment generated three sets of fragments: the doubly-charged species at *m/z* 1012.493 (glycosylated) and *m/z* 931.466 (non-glycosylated), the triply-charged species at *m/z* 675.330 (glycosylated) and *m/z* 621.314 (non-glycosylated), and the quadruply-charged species at *m/z* 506.750 (glycosylated) and *m/z* 466.238 (non-glycosylated) (Fig. 4A). These three sets show a mass shift determined to be 162.05 Da, once again

indicative of an *O*-linked hexose. The LC MS/MS-CID spectrum (Fig. 4B) showing the b and y ion fragmentation pattern definitively established glycosylation at Ser[779]. The ions y_4, y_6, and y_7 were assigned in the glycosylated and non-glycosylated forms.

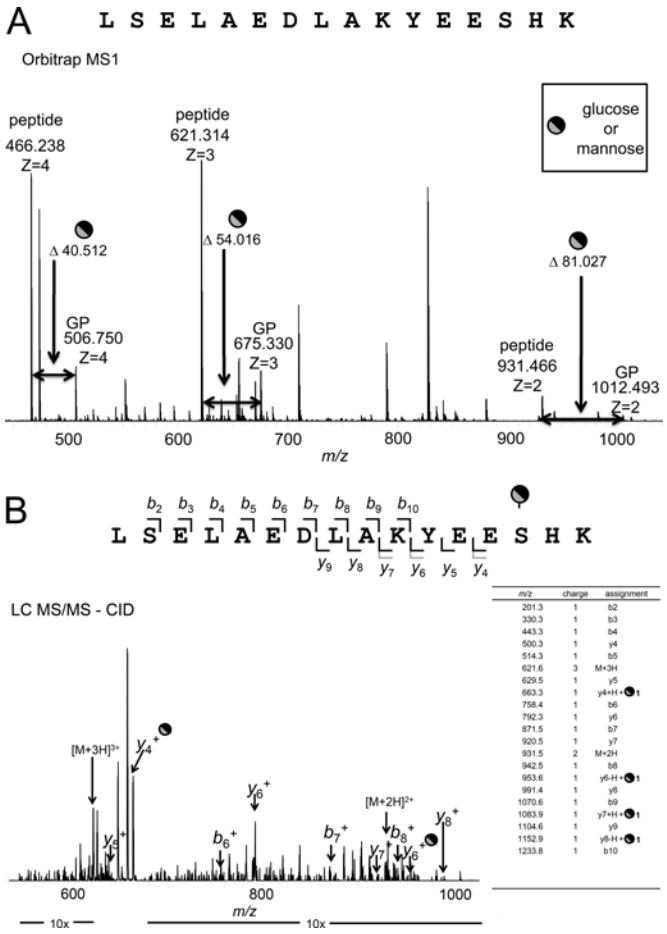

Fig. 4. Glycosylation of Ser[779] in the peptide LSELAEDLAKYEES[779]HK of MARTH_403. Panel A, Orbitrap MS1 showing mass shift of the quadruply-, triply- and doubly-charged ions. The 40.512 shift for z = 4 equates to a mass shift of 162.048 Da. The 54.016 shift for z = 3 equates to a mass shift of 162.048 Da. The 81.027 shift for z = 2 equates to a mass shift of 162.054 Da. These mass shifts correspond to a hexose. Panel B, LC MS/MS-CID of the glycosylated peptide showing the assigned b and y ions.

The glycosylation site on MARTH_819 is at Thr[107] within the identified peptide sequence of VIDGTFQEAYKR. Two sets of fragments were generated by the Orbitrap MS of the above fragment, the doubly-charged species at *m/z* 794.897 (glycosylated) and *m/z* 713.871 (non-glycosylated) and the triply-charged species at *m/z* 530.267 (glycosylated) and *m/z* 476.250 (non-glycosylated) (Fig. 5A). The mass shift was once

again determined to be 162.05 Da. The LC MS/MS-CID spectrum is shown in Fig. 5B. The b and y ions were identified on each side of the glycosylation site at Thr[107].

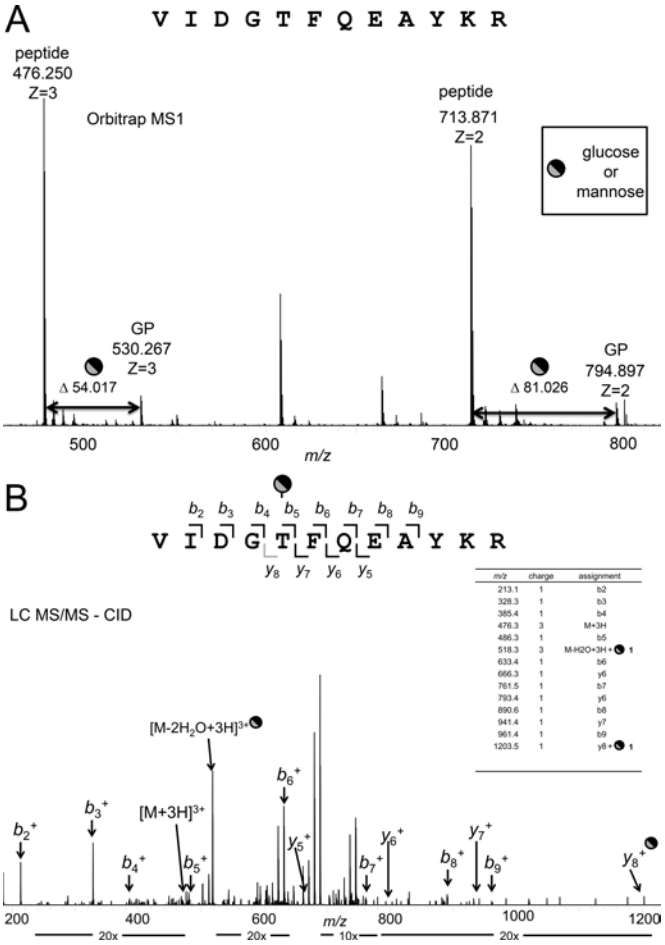

Fig. 5. Glycosylation of Thr[107] in the peptide VIDGTFQEAYKR of MARTH_819. Panel A, Orbitrap MS1 showing mass shift of the triply- and doubly-charged ions. The 54.017 shift for z = 3 equates to a mass shift of 162.051 Da. The 81.026 shift for z = 2 equates to a mass shift of 162.052 Da. These mass shifts correspond to a hexose. Panel B, LC MS/MS-CID of the glycosylated peptide showing the assigned b and y ions.

We have shown that [U-^{13}C]starch isotopically labels glucose, but not mannose, residues of glycoconjugates (manuscript submitted). *M. arthritidis* was grown in the presence of a mixture of ^{13}C-labeled starch with unlabeled (^{12}C) starch to observe any shift in the mass spectra of peptide ions. Both Figs. 6 and Fig. 7 show that grown under these conditions, a doublet of the peptide ions KEGATADFENLINK of MARTH_403

and VIDGTFQEAYKR of MARTH_819 were found in doubly and triply charged states that correspond to the mass of unlabeled and ^{13}C-labeled glucose.

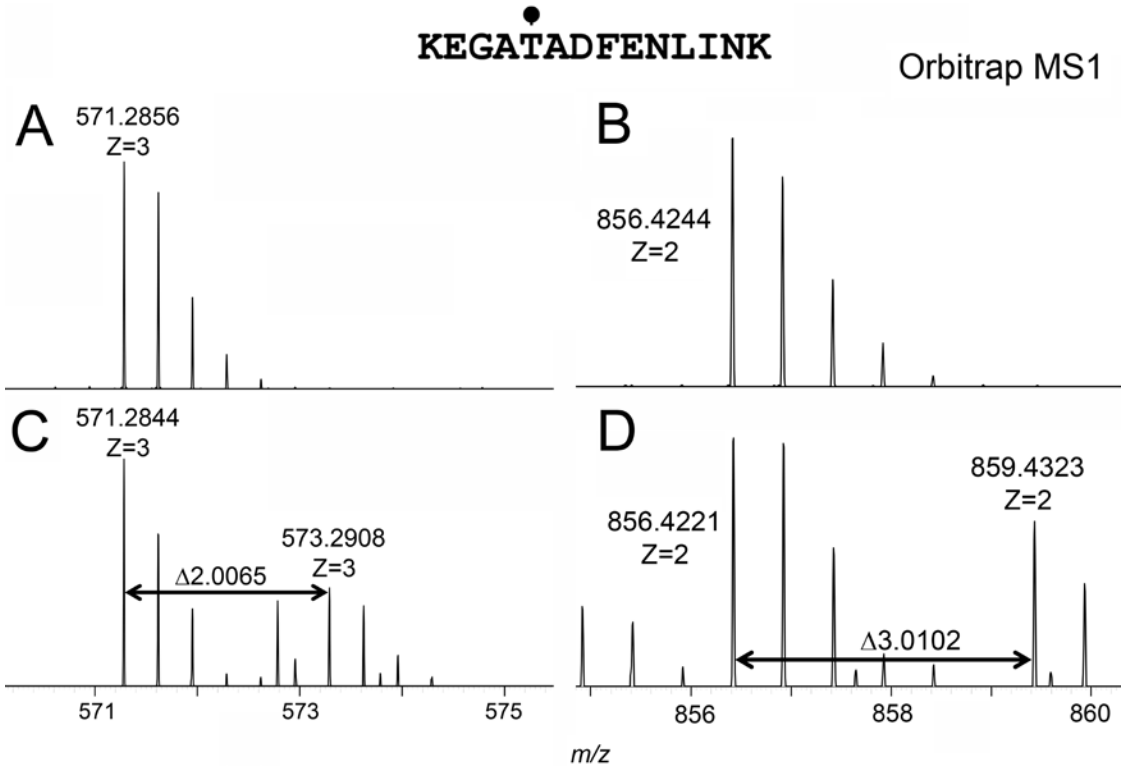

Figure 6. Ions of glycosylated KEGATADFENLINK peptide: (A) triply-charged species grown in SFM, (B) doubly-charged species grown in SFM, (C) triply-charged species grown in MB supplemented with ^{13}C starch, and (D) doubly-charged species grown in MB supplemented with ^{13}C starch.

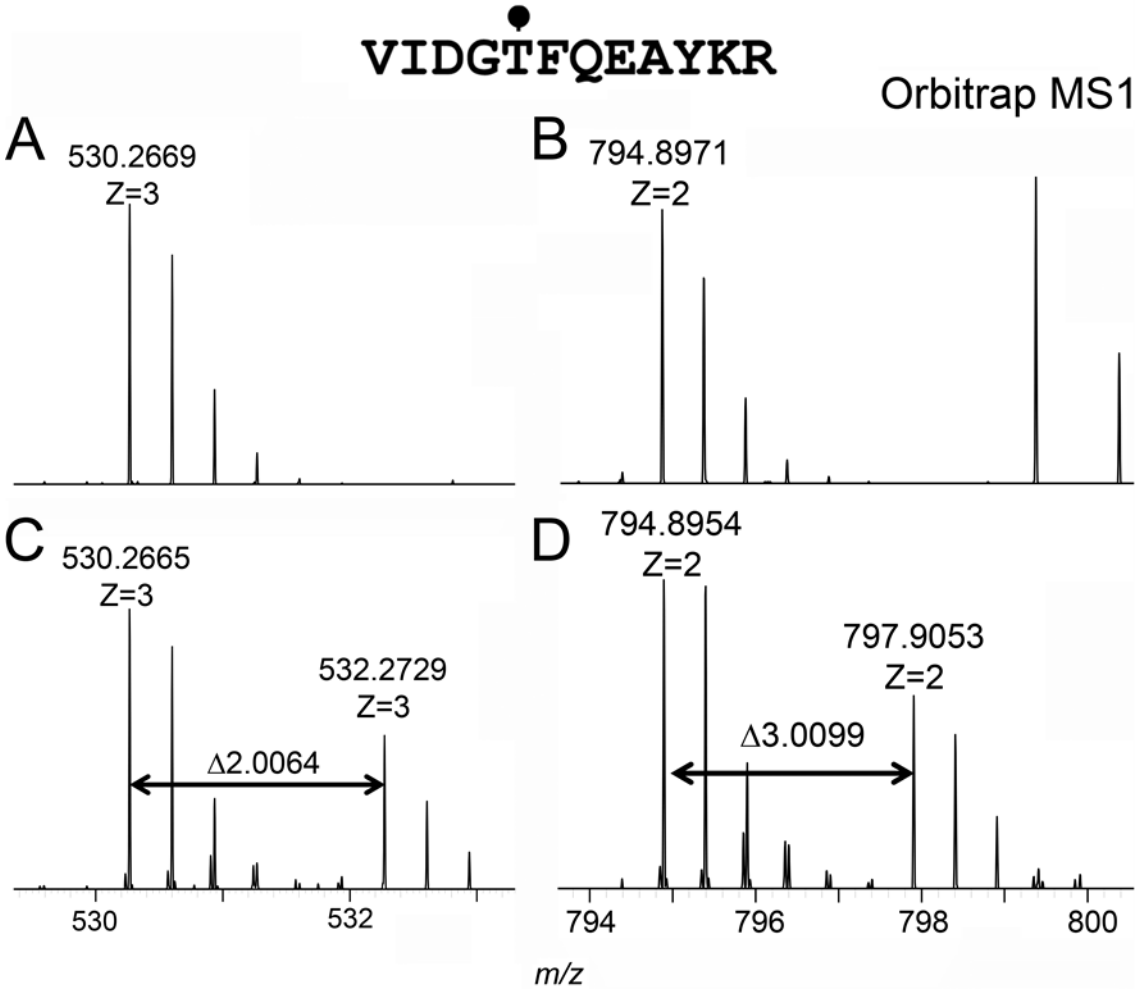

Figure 7. Ions of glycosylated VIDGTFQEAYKR peptide: (A) triply-charged species grown in SFM, (B) Doubly-charged species grown in SFM, (C) triply-charged species grown in MB supplemented with ^{13}C starch, and (D) doubly-charged species grown in MB supplemented with ^{13}C starch.

DISCUSSION

Glycosylation is the most common posttranslational modification to proteins [28]. It has been reported that about two-thirds of eukaryotic proteins are glycosylated [32, 33]. The flood of data concerning bacterial glycosylation that has accumulated over the past

five years leads to the conclusion that the numbers will be similar to those observed for eukaryotic systems. Bacterial examples include the well-characterized general *N*-glycosylation of *Campylobacter jejuni* proteins [34, 35] and *O*-glycosylation of bacterial flagella and pili [36, 37]. Although the function of glycoproteins in bacteria is poorly understood, most of the characterized glycoproteins of pathogens are virulence factors [38]. The function(s) of protein glycosylation in pathogenic obligatory organisms such as mycoplasmas could be to inhibit cleavage by proteases, increase protein stability, disguise the bacterium from host defenses, and contribute to host colonization [7-10, 39-44].

Described in this study are the first protein glycosylation sites identified in any species of mycoplasma. The glycosylation sites are at serine and threonine residues with no obvious consensus amino acid sequence. There is no evidence for *N*-glycosylation in mycoplasmas. *O*-glycosylation may be general and widespread in these organisms. The glycostaining shown in Fig. 1 suggests that multiple proteins are glycosylated in *M. arthritidis*, *M. hominis* and *M. pulmonis*. Although only three definitive glycosylation sites have been identified thus far, ongoing studies suggest that dozens of proteins in *M. arthritidis* are glycosylated. A single hexose would be expected to react weakly with the glycostain but many glycosylation sites might be present on the same protein, enhancing the fluorescent signal. An example might be the MARTH_455 protein found in band 1 of Fig. 1. This protein may be glycosylated at multiple sites as 13% of its predicted amino acids are serine or threonine residues.

The small genomes of the mycoplasmas have limited the conceptual possibilities in regard to the enzymes and the capabilities of these organisms for synthesis of

macromolecules. The shortage of known nucleotidyltransferases, glycosyltransferases, and monosaccharide synthesis enzymes has contributed to the idea that mycoplasmas are simplistic. At the start of our studies on the glycobiology of *M. arthritidis*, we assumed that the single annotated glycosyltransferase in this organism was responsible for the synthesis of the glucolipid that this species is known to produce [45]. The finding of glycoproteins was unexpected because this species did not seem to have the necessary machinery to produce multiple types of glycoconjugates. The *M. arthritidis* genome has over 200 hypothetical proteins of unknown function, and the annotation of many of the proteins with an assigned function may be incorrect. The current study suggests that additional glycosyltransferases exist in *M. arthritidis*. These enzymes should be novel because they were not identified by bioinformatics.

Mycoplasmas are fastidious, host-dependent bacteria that need cholesterol and other complex nutrients for growth. The inability of *M. arthritidis* to import glucose and mannose monosaccharides suggests that oligosaccharides might be required for glycoconjugate synthesis as described for bifidobacteria [46]. Fig. 6 and 7 confirm that glucose is definitely *O*-linked to two peptides, but the possibility yet exists that mannose is *O*-linked to some of the peptides. It is possible that the glycan identified in this study was a single hexose because of the “simple” medium that was used. A culture medium that contained a more complex mixture of oligosaccharides might permit more diversity in the glycans that are attached to the proteins. *M. arthritidis* is a non-glycolytic species that causes dramatic joint disease in murine animals [22]. The concentration of the monosaccharide glucose in the murine joint is low and the major available source for precursors for glycan synthesis would be glycosaminoglycans and heavily glycosylated

proteins such as collagen. Thus, the host environment of *M. arthritidis* would favor a pathway that uses oligosaccharides instead of monosaccharides as precursors for glycoconjugate synthesis. It is possible that the glycans of *M. arthritidis in vivo* would differ significantly from what has been identified *in vitro*.

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CH. 4: DISCUSSION

Experimental Data Validates Bioinformatics

The isotope labeling described in the second chapter corresponds well with the bioinformatic analysis of enzymes available for metabolism of glucose monosaccharide (Fig. 1). As expected for the glycolytic species *Mycoplasma pneumoniae* and *Mycoplasma pulmonis*, glucose can be imported and converted to glucose-6-phosphate (G6P).

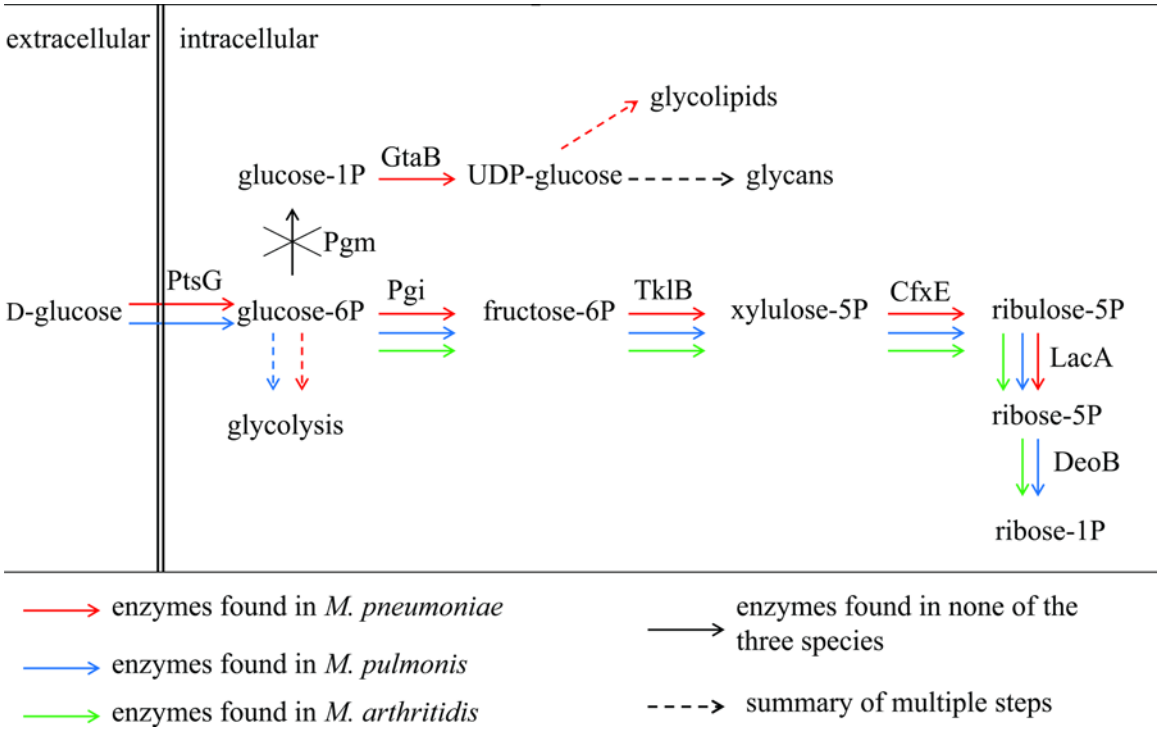

Fig 1. Bioinformatic fate of glucose in *M. pneumoniae*, *M. pulmonis*, and *M. arthritidis*. PtsG, Glucose phophotransferase enzyme; Pgm, phosphoglucomutase; GtaB, UTP-glucose-1-phosphate uridylyltransferase; Pgi, Phosphoglucose isomerase; TklB, transketolase; CfxE, ribulose-5-phosphate 3-epimerase; LacA, Ribose/galactose isomerase; DeoB, Phosphopentomutase.

At this point, G6P can either enter glycolysis or be converted to a phosphorylated form of ribose. The role of phosphorylated ribose in these species is unknown as they lack further genes normally involved in converting this molecule into nucleotides or histidine. This pathway is reversible and the ribose-phosphate can be used in glycolysis should glucose

become limiting. When grown under our conditions with [U-^{13}C]glucose, trimethylsilyl (TMS) derivatives of labeled ribose methyl glycoside could be found by GC/MS. *M. arthritidis* is non-glycolytic but has most of the genes for glycolysis. Most important is the absence of *ptsG* which transports and phosphorylates glucose as the monosaccharide enters the cytoplasm. This bioinformatic prediction was experimentally accurate, as [U-^{13}C]glucose did not label TMS derivatives of ribose methyl glycoside and [U-^{14}C]glucose did label cells. The source of the unlabeled ribose in *M. arthritidis* was the nucleotides provided in the growth medium and incorporated into nucleic acids.

Consistent with bioinformatic analysis of *M.* pneumoniae, Klement et al. previously demonstrated that UDP-glucose is converted to glycolipid. However, it is unknown how the UDP-glucose precursor, glucose-1-phosphate (G1P), could even be available within the cell. There is no known importer or isomerase in mycoplasmas that can convert glucose to G1P. We demonstrated this by showing that [U-^{13}C]glucose did not label any glycoconjugates in any of the mycoplasmas tested. We found that [U-^{13}C]starch could label glycoconjugates, but not ribose. These experiments correspond well with bioinformatic analysis. We also showed that G1P and G6P are generated by distinct pathways. The mycoplasmas also have unique glycoconjugate and rhamnose synthesis machinery that may not utilize nucleotide sugars.

Rhamnose in Mollicutes

Rhamnose synthesis was shown to be common in mycoplasmas. The three species studied were able to make D-rhamnose and *M. arthritidis* made both the D- and L-enantiomers. These species are from two of the main clades of mycoplasmas but do other,

more distant members of the Mollicute class produce rhamnose as well? More intensive scrutiny that leads to finding non-synthesizers, could allow one to eliminate entire sets of genes in common with rhamnose producers. The ultimate goal would be to narrow the set of potential rhamnose synthesis genes.

Why starch is required for rhamnose synthesis is unknown. Starch consists of the linear glucose polymer amylose, which has only α(1→4) linkages, and amylopectin, which consists of multiple amylose polymers branched sporadically via α(1→6) linkages (Bailey & Neish, 1954). Preliminary studies show that some disaccharides such as maltose can be used for rhamnose synthesis. Perhaps the use of disaccharides of different monomer compositions could elucidate which sugar residues are required for each enantiomer as well as the types of linkages that are compatible with synthesis. Preliminary studies show that *M. pulmonis* cannot break glycosidic β linkages. Both starch and yeast extract have the sugar residues and linkages needed to synthesize L-rhamnose but not D-rhamnose. Supplementing the medium with starch or yeast extract favored L-rhamnose production. It is possible that the required composition and linkage for D-rhamnose production is contained in components of the peptone or bovine serum albumin fraction used in the serum-free medium.

We initially screened the *M. arthritidis* transposon mutant library (Dybvig et al., 2008) for mutants deficient in rhamnose synthesis. The screen included mutants within every nonessential gene knocked out, but no mutants deficient in rhamnose synthesis were identified. Two possibilities can account for this. First, if both enantiomers have completely separate synthesis pathways, then a deficiency might not be detected by this screen because there would always be either D- or L-rhamnose. Given the streamlining

and the necessary efficiency of the genome, it seems unlikely to have two synthesis systems through the entire pathway when some of the enzymatic reactions could be shared. The second possibility is that rhamnose is essential. In *Mycobacteria* species, rhamnose is part of a structure linking peptidoglycan to arabinogalactan, and is essential to these bacteria (Ma, Pan, & McNeil, 2002; McNeil, Daffe, & Brennan, 1990).

We were able to rule out that rhamnose was a component of a glycolipid. It mostly partitions with the interphase fraction during methanol-chloroform treatment, but we still were not able to conclusively determine the structural nature of any rhamnose-containing molecules. The interphase of methanol-chloroform treatment of bacterial lysates is known to comprise mostly of protein (Wessel & Flugge, 1984) and it may be that rhamnose is part of a glycoprotein. Rhamnose has been shown to be part of glycoprotein in *Bacillus anthracis* with the sugar residue directly attached to the threonine or serine (Daubenspeck et al., 2004). If not part of a mycoplasmal glycoprotein, then rhamnose might exist in some novel molecular arrangement that precipitates in methanol-chloroform.

If not identified in glycoprotein studies, intense scrutiny of rhamnose glycoconjugates would be exciting to pursue. Resolubilizing this fraction and removing protein could validate that rhamnose is not a component of glycoprotein and might facilitate purification of the rhamnose glycoconjugate. Caputto et al. derived the first known structure of the nucleotide sugar by chemical analyses (Caputto, Leloir, Cardini, & Paladini, 1950). Similar analyses of purified mycoplasmal rhamnose glycoconjugates could provide clues to its molecular structure.

Given the lack of glycoconjugate synthesis machinery recognizable through bioinformatics, it was unexpected to find glycoproteins in *M. arthritidis*. We confirmed three *O*-glycosylation sites and we expect many more to be found within the data set that we have already generated using the FT-ICR technology. The challenge at the moment is developing a reliable system to search peptide masses (MS) as well as peptide sequence (MS-MS) ions for *O*-glycosylation in bacteria. Currently, no software exists that can search MS data because mass accuracy issues that once made this method unreliable have only recently been overcome. Another reason this type of modification is difficult to search is that most MS-MS search software was designed for eukaryotic glycoproteins and the algorithms used are not applicable to prokaryotic glycoproteins due to differences in glycan composition and structure. Developing more specific criteria, search parameters, and validation protocols will be helpful for finding more glycosylation sites in the *M. arthritidis* proteome. We have created a database of FT-ICR mass spectra of *M. arthritidis* lipoproteins as well as for those proteins that were not extracted by TritonX-114, most of which do stain positive for glycosylation. Sifting through gigabytes of data with limited availability to the search software is an ongoing struggle.

Continued data mining as well as refining the search methods should allow us to answer multiple questions about *M. arthritidis* protein glycosylation. In cataloguing the glycosylation sites, we would better understand how widespread protein glycosylation is and whether it is a general system for all exposed threonine and serine residues or whether there is limited glycosylation within certain types of proteins. In the latter case, there

should be some consensus sequence that would enhance the prediction of glycosylation sites. This would be useful for studying glycoproteins in other mycoplasmas as well.

Identifying the glycosylation machinery is also a major goal that would likely have genus-wide implications. Screening candidate mutants in our transposon library with glycoprotein stain could enable identification of genetic loci for further study in determining the role specific genes play in protein glycosylation. It would also allow us to study possible functions of glycosylation by comparing the phenotypes of glycosylated and nonglycosylated strains. However, protein glycosylation in mycoplasmas may be an essential feature. Chapter one describes several bacterial species that glycosylate proteins to form a more stable protein. This function fits well with what we know about mycoplasmas. They cannot synthesize amino acids nor import every amino acid individually. They must import oligopeptides as their sole source of some of the essential amino acids (Yus et al., 2009). It follows that to generate peptides for import, the mycoplasmas must secrete proteases and do so without digesting their own proteins. Perhaps mycoplasma glycoproteins are made so that they are resistant to self-made proteases. If this is the case, then glycoproteins are probably essential and a mutant strain would never be identified. Protection from self-made proteases could still be tested, however by cloning a known glycosylated protein with a purification tag attached and expressing it in a species with no protein glycosylation system. The stability of the purified nonglycosylated protein with that of the purified from the native species could be compared by observing their migration on SDS-PAGE gels. Some treatment with proteases, perhaps from the supernatant of the mycoplasma, could show marked differences if the glycosylated protein is more stable.

As highlighted here, the discovery of mycoplasma glycoproteins opens several new avenues of study. The fact that many mycoplasma species exhibit similar glycoprotein-positive properties on SDS-PAGE gels to that of *M. arthritidis* means that there is reason to believe that protein glycosylation is common throughout this genus or even class of bacteria. Finding these glycoproteins in species of mycoplasma that are pathogenic to humans or commercial livestock could be especially useful in understanding some of the underlying properties of infections with health and economic relevance.

Mycoplasmas' Evolutionary Stage

It is useful to apply Darwin's methods of analyzing lengthy processes with little data to bacterial evolution. Gould wrote that all of Darwin's works described a methodology for analyzing such processes (Gould, 1983):

> Thus, we have three principles for increasing adequacy of data: if you must work with a single object, look for imperfections that record historical decent; if several objects are available, try to render them as stages of a single historical process; if processes can be directly observed, sum up their effects through time.

Using the second principle, consider all bacterial species as stages of a single historical process. It would be expected that one could find species at an earlier stage of evolution and other species closer to an endpoint of the process. Evolutionary endpoints include extinction and total incorporation into other or-

ganisms, such as is the case with mitochondria and chloroplasts. Mycoplasmas are under more evolutionary constraint than the walled bacteria and are closer to an endpoint due to their host-dependence and streamlined genome. They will never reacquire a cell wall or most other lost features.

Mycoplasmas have genomes with minimal redundancies and maximum functions per protein. One study has shown that genome size correlates well with protein-protein interactions of 168 conserved proteins (Kelkar & Ochman, 2013). The smaller the genome, the more protein-protein interactions occur. Perhaps a few of these protein-protein interactions do occur in walled bacteria. Do proteins that drive rhamnose synthesis or glycoprotein production in mycoplasmas have similar functions in walled species? By studying the mycoplasmas, captured in a late evolutionary stage, we may discover many unknown or alternative functions to proteins in other bacteria at earlier stages of the evolutionary process.

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