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Formylated N-terminal methionine is absent from the *Mycoplasma hyopneumoniae* proteome: Implications for translation initiation

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ABSTRACT

N-terminal methionine excision (NME) is a proteolytic pathway that cleaves the N-termini of proteins, a process that influences where proteins localise in the cell and their turnover rates. In bacteria, protein biosynthesis is initiated by formylated methionine start tRNA (fMet-tRNA$_f^{Met}$). The formyl group is attached by formyltransferase (FMT) and is subsequently removed by peptide deformylase (PDF) in most but not all proteins. Methionine aminopeptidase then cleaves deformylated methionine to complete the process. Components of NME, particularly PDF, are promising therapeutic targets for bacterial pathogens. In *Mycoplasma hyopneumoniae*, a genome-reduced, major respiratory pathogen of swine, *pdf* and *fmt* are absent from its genome. Our bioinformatic analysis uncovered additional enzymes involved in formylated N-terminal methionine (fnMet) processing missing in fourteen mycoplasma species, including *M. hyopneumoniae* but not in *Mycoplasma pneumoniae*, a major respiratory pathogen of humans. Consistent with our bioinformatic studies, an analysis of in-house tryptic peptide libraries confirmed the absence of fnMet in *M. hyopneumoniae* proteins but, as expected fnMet peptides were detected in the proteome of *M. pneumoniae*. Additionally, computational molecular modelling of *M. hyopneumoniae* translation initiation factors reveal structural and sequence differences in areas known to interact with fMet-tRNA$_f^{Met}$. Our data suggests that some mycoplasmas have evolved a translation process that does not require fnMet.

1. Introduction

Genome reduced bacteria often evolve novel mechanisms to execute essential cellular functions. The most intensively genome-reduced organisms tend to be obligate symbionts or obligate pathogens, existing in stable, nutrient-rich host environments (Kelkar and Ochman, 2013). Genes associated with biosynthetic pathways are the most frequently lost, leading to a reliance on the host for survival (McCutcheon and Moran, 2011). However, studies have also correlated the evolutionary loss of metabolic pathways to the onset of bacterial virulence (Lawrence, 2005), and some of the most lethal pathogens have undergone reductive genome evolution (Cole et al., 2001; Weinert and Welch, 2017). Moreover, pathogenic species such as *Rickettsia prowazekii*, *Yersinia pestis* and *Mycobacterium leprae* all have smaller genomes than their closely related, less virulent, counterparts (Diop et al., 2017; Merhej et al., 2009). Genome reduction, rather than genome complexification, is also the dominant mode of evolution in most bacterial lineages (Wolf and Koonin, 2013), and it is notable that genome-reduced bacteria tend to exhibit levels of transcriptional and structural complexity not seen in larger bacterial genomes (Kelkar and Ochman, 2013). The reader is therefore invited to put aside any notions of "smaller is simpler" and instead adopt a "do more with less" view on genome reduction.

In most organisms, the first ubiquitous proteolytic process occurs co-translationally with the removal of N-terminal methionine (nMet). This process is called N-terminal methionine excision (NME) (Giglione et al., 2003). Methionine initiates protein synthesis in eukaryotic and prokaryotic proteins and is present at the N-terminus of all newly synthesised proteins (Bonissone et al., 2013). However, during translation nMet is removed from 50 to 70% of proteins via NME (Giglione et al., 2004; Narayanan and Nampoothiri, 2012). NME directs intracellular localisation and influences post-translational modifications (PTMs), protein stability, folding, and activity (Calcagno and Klein, 2016; Giglione et al., 2015).

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However, NME differs depending on the type of organism and cellular compartment. In bacteria, mitochondria, and chloroplasts, protein synthesis begins with a nMet loaded onto $tRNA_f^{Met}$, then modified to create fMet-$tRNA_f^{Met}$. The addition of a formyl group to nMet is catalysed by methionyl-tRNA formyltransferase (FMT) using the substrate 10-formyltetrahydrofolate (10-fTHF). 10-fTHF is previously synthesised from folate through the actions of methylenetetrahydrofolate dehydrogenase (FolD) and dihydrofolate reductase (FolA) (Laursen et al., 2005; Piatkov et al., 2015). To initiate translation, fMet-$tRNA_f^{Met}$ along with mRNA and three initiation factors; IF1, IF2 and IF3, bind to the ribosomal 30S subunit and work in concert to ensure correct start codon selection for elongation to proceed. As a consequence, fnMet becomes the first residue of a polypeptide to emerge from a bacterial ribosome (Kramer et al., 2009).

If the emerging protein possesses an N-terminal signal peptide, it is recognised and bound by signal recognition particle (SRP) and subsequently directed for membrane translocation via the Sec secretion pathway (Green and Mecsas, 2016). If no signal peptide is detected, the formyl group is removed in approximately 95% of proteins by peptide deformylase (PDF) (Bienvenut et al., 2015). The de-formylated nMet can then be cleaved by methionine aminopeptidase (MAP), thus completing NME (Varland et al., 2015).

The NME process is more straightforward within the cytoplasm of eukaryotes as translation is initiated with an unformylated methionine (Giglione et al., 2015). Therefore, cytoplasmic NME does not require FMT, FolA, FolD or PDF, and does not produce formylated signal peptides.

The fMet moiety of bacterial fMet-$tRNA_f^{Met}$ increases translation initiation efficiency by binding IF2 and facilitating its loading onto the ribosome (Cai et al., 2017). However, the production of formylated peptides has drawbacks. Formylated peptides display pathogen-associated molecular patterns (PAMPs) and are the primary ligands for formylated peptide receptors (FPRs) expressed on mammalian white blood cells (Bufe et al., 2015). The likely primary source of formylated peptides is secreted bacterial signal peptides (Bufe et al., 2015). Once bound to FPRs, formylated peptides trigger an immune response including chemotaxis, reactive oxygen species and hydrogen peroxide production, degranulation, and phagocytosis (Boulay et al., 1990; Dorward et al., 2015; Mader et al., 2010). The production of specific FPR inhibitors by some bacteria underscores the importance of FPRs during infection (Mader et al., 2013). It is notable in this regard that formylated peptides from *Listeria monocytogenes* are being touted as promising vaccine targets (Winther et al., 2016).

The production of formylated peptides appears detrimental to bacterial fitness prompting speculation as to why the evolutionary process has not lost strict fnMet usage in bacteria. One theory that strives to address this question is that fnMet may serve as a degradation signal for protein quality control, similar to the N-terminal acetylated-degron signal used in eukaryotes (Piatkov et al., 2015). Moreover, fnMet is not strictly essential. Null mutations in *fmt* (the gene encoding FMT, the enzyme that adds a formyl group onto nMet) produce viable, albeit growth-impaired, cells in several bacteria species, including *Pseudomonas* aeruginosa (Newton et al., 1999), *Staphylococcus aureus* (Margolis et al., 2000) and *Mycobacterium bovis* (Vanunu et al., 2017). While FMT may not be essential, inhibition of PDF (the enzyme that removes the formyl group from nMet) causes cell death (Margolis et al., 2001). PDF is currently a target for new antimicrobial development, yet resistance is already being heralded by the appearance of suppressor strains with inactivated fmt genes, a process known as the FMT bypass (Guay, 2007).

Intriguingly, there are bacterial species described as missing both *fmt* and *pdf* genes. A genomic study comparing metabolism and infection mechanisms between eight mycoplasmas found that only *M. hyopneumoniae* was missing the *pdf* gene (Arraes et al., 2007). A more recent genomic study comparing 33 mycoplasma genomes found that both *pdf* and *fmt* genes were missing in six mycoplasmas including *M. hyopneumoniae*, and three phytoplasmas (Grosjean et al., 2014).

M. hyopneumoniae is a genome-reduced pathogen that destroys mucociliary function in the upper respiratory tract of the domestic pig, *Sus scrofa*. *M. hyopneumoniae* is the aetiological agent of porcine enzootic pneumonia, a chronic, widespread disease responsible for substantial economic losses (DeBey and Ross, 1994). Mycoplasmas are the smallest known self-replicating organisms to be considered true eubacteria (Razin, 1997) and are distinguishable by an absent cell wall, instead being surrounded by a single plasma membrane (Razin, 1997). Mycoplasmas are derived from low G + C content Firmicutes and are the product of a reductive evolutionary process that resulted in the loss of genes required for metabolic processes including the TCA cycle, gluconeogenesis, and the pentose phosphate pathway (Arraes et al., 2007; Razin, 1997). Consequently, mycoplasmas are host-dependent parasitic bacteria unable to synthesise amino acids, phospholipids, glycolipids, cholesterol, vitamins, and nucleotides (Razin, 1997). Despite these metabolic limitations, mycoplasmas thrive in vertebrates, reptiles, insects, plants, and a plethora of other non-vertebrate hosts (and an estimated 5–30% of the world's cell lines (Nikfarjam and Farzaneh, 2012)) and often cause chronic disease (Razin, 1997).

Here, through a bioinformatic analysis of 54 mycoplasma proteomes, we complement, and expand on previous work (Arraes et al., 2007; Grosjean et al., 2014) by demonstrating that *M. hyopneumoniae* and 13 other mycoplasma species are missing FolA, FolD, FMT and PDF, thus removing all components needed to form, attach, and subsequently remove the formyl group of nMet. We undertook a comprehensive search of mass spectrometry (MS) datasets for the presence of PTMs with the specific aim of identifying tryptic peptides containing evidence of fnMet in cytosolic, and surface proteins of respiratory pathogens *M. hyopneumoniae* and *M. pneumoniae*. Additionally, amino acid sequence analysis and computational molecular modelling of *M. hyopneumoniae* initiation factors IF1, IF2 and IF3 uncovered irregularities, including areas pertaining to fMet-$tRNA_f^{Met}$ interactions.

2. Materials and methods

2.1. Strains, culturing, and harvesting

M. hyopneumoniae strain J was provided by the Iowa State University Veterinary Research Medical School, USA. *M. hyopneumoniae* cultures were grown in modified Friis media (Scarman et al., 1997) inoculated 1: 50 with previously grown culture or cells from −80 °C storage, to mid-log phase for 48 h at 37 °C while rolling. Cells were pelleted by centrifugation at 12,000 x g for 15 min and washed three times in PBS.

M. pneumoniae (M129 strain, ATCC 29342) cells were grown in modified Hayflick's medium (Hayflick, 1965) in tissue culture flasks at 37 °C. *M. pneumoniae* cells were grown to confluency and harvested by first washing the cells with PBS and then lysed with sonication in 7 M urea, 2 M thiourea, 40 mM Tris-HCl, 1% (w/v) C7BzO detergent. Reduction and alkylation were performed to a final concentration of 5 mM tributylphosphine and 20 mM acrylamide monomers followed by precipitation with acetone. The insoluble pellet was resuspended in 7 M urea, 2 M thiourea, 40 mM Tris-HCl, 1% (w/v) C7BzO for 1D- and 2D-SDS PAGE.

2.2. M. hyopneumoniae and M. pneumoniae enzymatic cell surface shaving

Enzymatic cell surface shaving experiments for both mycoplasmas were performed as previously described (Jarocki et al., 2015; Widjaja et al., 2015). Briefly, freshly harvested cells were washed in PBS and pelleted by centrifugation. Cells were resuspended in PBS, and enzymatic cell shaving using trypsin was performed at 37 °C for 5 min. Intact cells were pelleted by centrifugation and the supernatant containing liberated surface proteins collected on ice to cease trypsin activity. Surface proteins were analysed by 1D gel electrophoresis or further

digested to peptides with trypsin before analysis by LC–MS/MS (Tacchi et al., 2016).

2.3. Cell surface biotinylation of M. hyopneumoniae and M. pneumoniae proteins

Capturing surface proteins via biotinylation was performed on both mycoplasmas as previously described (Jarocki et al., 2015; Widjaja et al., 2017). Briefly, freshly harvested cells were washed in PBS and pelleted by centrifugation. Cells were resuspended in PBS and biotinylated with Sulfo-NHS-LC-Biotin [Thermo Scientific, USA] for 30 s on ice. The reaction was then quenched with the addition of a final concentration of 50 mM Tris-HCl (pH 7.4) and incubated for 15 min. Cells were washed and pelleted by centrifugation. Cells were lysed and precipitated protein was pelleted and resuspended in buffers appropriate for downstream application as previously described (Jarocki et al., 2015; Widjaja et al., 2017). Approximately 1 mg of protein was subjected to avidin affinity chromatography in a column packed with 1 mL of immobilised monomeric avidin [Thermo Scientific, USA], separated by 1D-SDS-PAGE and analysed by MS.

2.4. LC-MS/MS/ms

The presence of fnMet in *M. hyopneumoniae* and *M. pneumoniae* proteins were searched using data obtained from several mass spectrometers (MS).

For unpublished data, a QSTAR Elite Quadrupole-TOF-MS (Sciex, USA) was used in conjunction with an Eksigent AS-1 autosampler connected to a Tempo nanoLC system (Eksigent, USA). A gel digest of less than 5 µg was injected in a volume of 10 µL. Samples were first loaded on to a C8 trap (Michrom, USA) in solvent A; 2% Acetonitrile and 0.2% Trifluoracetic Acid. The sample was injected and ran at 300 nL min-1 through a 75 µm × 150 mm PicoFrit column packed with C18AQ resin (Michrom Biosciences, USA) with the voltage across the nano-column at 2300 V for ionisation. The gradient stepping as follows 5–50% solvent B (98%Acetonitrile and 0.2% Trifluoracetic Acid) over 8 min, 50–80% over 5 min, held at 80% B for 2 min and dropped to 5% over three minutes. The mass spectrometer was operated in data-dependent analysis mode where charge states of $^{+}2$-$^{+}5$ ions were selected for fragmentation inside of the 375–1500 m/z mass range. All ions selected for fragmentation were above 30 counts and once fragmented the ion was dynamically excluded for 15 s.

For published data, information on the *M. hyopneumoniae* proteome sample preparation and LC–MS/MS can be found on the pride database using the identifier PXD000118 (Pendarvis et al., 2014).

2.5. M. hyopneumoniae and M. pneumoniae proteome analysis

All Data Searches were performed utilising the PEAKS Studio Platform 8.5 (Ma et al., 2003). Search tolerance parameters by each machine; the in-house QSTAR Elite Quadrupole-TOF-MS data; Parent mass error tolerance was set to 100.0 ppm with a fragment mass error tolerance of 0.2 Da, missed cleavages; 3, Non-specific cleavage at both ends. Published *M hyopneumoniae* Proteome (PXD000118) data tolerances were taken from the quoted values briefly; LTQ FT ULTRA parent mass tolerance of 10 ppm and a fragment mass tolerance of 0.4 Da, with missed cleavage set to two. Non-specific cleavage at both ends. LTQ Velos Pro parent mass and fragment mass tolerance of 0.4 Da, max missed cleavages of 2 and non-specific cleavage at both ends.

Peaks DB search was performed against the mycoplasmas proteomes from UniProt (Downloaded:24/10/2014) with all taxons and contaminates. Variable modifications preferentially searched were Deamidation (NQ): 0.98, Oxidation (M): 15.99, Propionamide: 71.04, Formylation: 27.99 (K, X, and at N-termini). Subsequently a PTM search was performed against Deamidation (NQ): 0.98, Oxidation (M): 15.99, Propionamide: 71.04, Formylation: 27.99 and 309 inbuilt PTMs. False Discovery threshold was set at 1% which was estimated by the PEAKS studio estimation method which utilises a decoy fusion database, this database was of the same size across the searches and allowed for a comparable FDR for each unique machine and dataset (Zhang et al., 2012).

2.6. Bioinformatic analysis and computational modelling

To investigate NME machinery required to synthesise and attach formyl groups to the N-termini of proteins during biosynthesis, an example strain from each mycoplasma species (from the 189 Mycoplasma proteomes available on UniProt (UniProt, 2017)) was selected for analysis, using reference proteomes where available. First the gene names for FolA (*dhfr/folA/cd*), FolD (*folD*), FMT (*fmt*), PDF (*pdf*) and MAP (*map*) were searched in each species. Then the protein names and synonyms available from the BRENDA database (Placzek et al., 2017) were searched: FolA synonyms: dihydrofolate reductase 7,8-dihydrofolate reductase, dihydrofolate reductase, dihydrofolate reductase-thymidylate synthase, dihydrofolate reductase, thymidylate synthase, NADPH-dihydrofolate reductase, pteridine reductase dihydrofolate reductase, R-plasmid-encoded dihydrofolate reductase, R67 dihydrofolate reductase, thymidylate synthase-dihydrofolate reductase, and thymidylate synthetase-dihydrofolate reductase. FolD synonyms: methylenetetrahydrofolate dehydrogenase, methylenetetrahydrofolate dehydrogenase cyclohydrolase, methylenetetrahydrofolate dehydrogenase methenyltetrahydrofolate cyclohydrolase, and Mg^{2+}/NAD-dependent methylenetetrahydrofolate dehydrogenase. If a query protein was not found in a certain species after these searches, a BlastP search, using default NCBI and UniProt parameters, of a corresponding protein from a mycoplasma of the closest phylogenetic relationship was performed. If the protein was still not found, it was deemed absent from that species. For the 14 mycoplasmas missing FolA, FolD, PDF and MAP, BLAST searches for each corresponding gene where performed to confirm their absence in the genomes. The phylogenetic relationship between searched mycoplasma species was executed using the RAxML-VI-HPC pipeline via PATRIC (Wattam et al., 2017) and visualised using Interactive Tree Of Life (iTOL) v3 (Letunic and Bork, 2016).

Where solved structures for IF1, IF2 and IF3 were unavailable from the Protein Databank, comparative molecular modelling of was performed using Phyre2 (Kelley et al., 2015). The structure of *M. hyopneumoniae* IF1 (MHJ_0168) was modelled in the intensive mode with 100% residues modelled at > 90% confidence. The structure *M. hyopneumoniae* IF2 (MHJ_0585) was modelled in the intensive mode with 93% residues modelled at > 90% accuracy. *M. hyopneumoniae* IF3 (MHJ_0121) was modelled in intense mode at 75% residues at > 90% confidence, and *E. coli* IF3 was modelled in the same mode at 91% residues at > 90% accuracy (all template details in Supplementary File 1). Final structures were rendered using the Chimera molecular modelling system v. 1.12 (Pettersen et al., 2004). rRNA binding sites were deduced from sequence searches by NCBI SEQUENCE VIEWER v. 3.25.2 and added manually onto structures. Amino acid sequence alignments were performed using EMBL-EBI CLUSTALOMEGA. ISIS predicted Protein-protein interaction sites via PredictProtein (Ofran and Rost, 2007; Yachdav et al., 2014).

3. Results

3.1. fnMet processing enzymes are missing in 14 mycoplasma species

Typical prokaryotic NME requires the actions of FolA, FolD, FMT, PDF, and MAP. However, BLAST searches of mycoplasma proteomes available through UniProt failed to identify many of these enzymes. Of the 54 Mycoplasma species searched (Supplementary File 2), the only universally conserved NME enzyme was MAP (Fig. 1). Most striking was the absence of FolA, FolD, FMT and PDF in 14 mycoplasmas, including *M. hyopneumoniae*, and all assessed haemoplasmas (Fig. 1). A BLAST

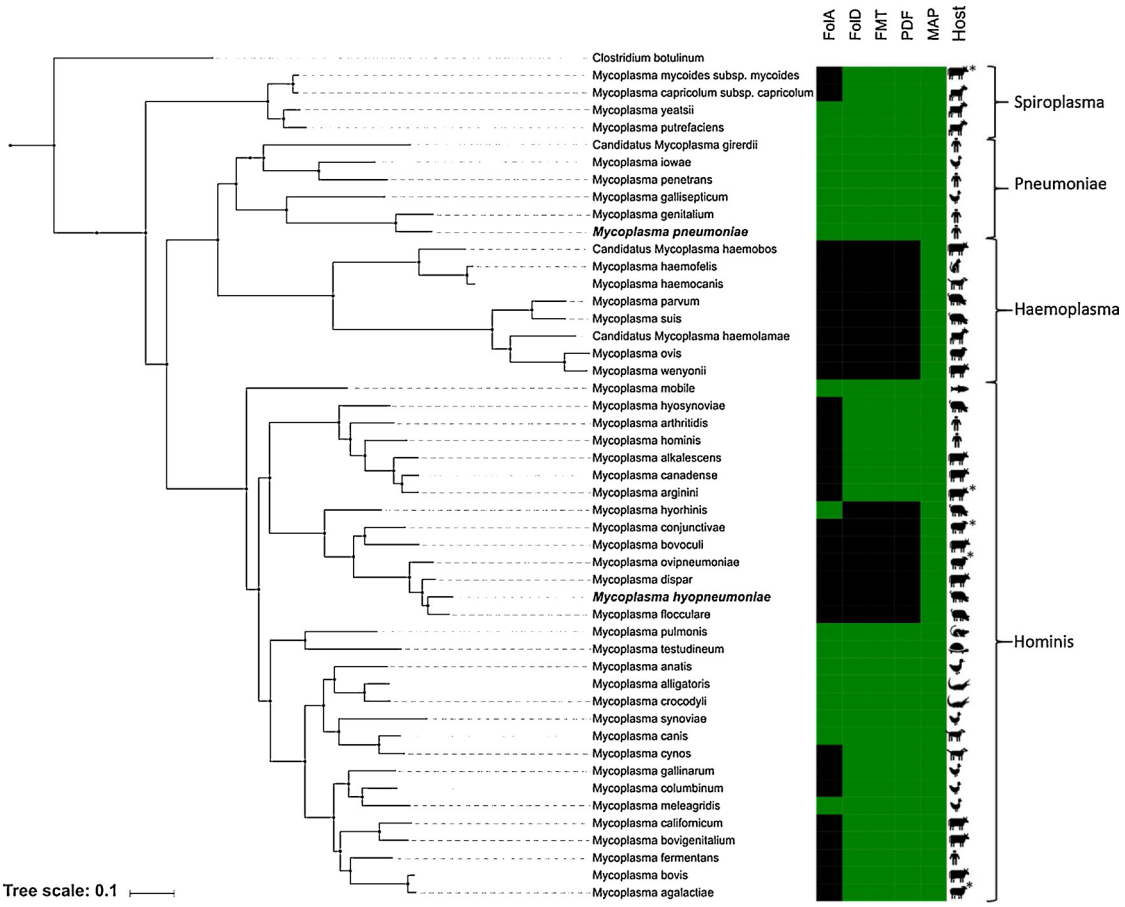

Fig. 1. Phylogenetic tree and heat map showing the evolutionary relationships of analysed mycoplasma species and the presence or absence of NME machinery. In the heat map, genes present are illustrated in green and genes absent are in black. Mycoplasmas that have lost FolA, FolD, FMT and PDF proteins consisted of all assessed haemoplasmas and a branch of the clade Hominis, which includes *M. hyopneumoniae* (shown in bold). All of the clade Pneumoniae retained NME machinery, including *M. pneumoniae* (shown in bold). MAP was universally conserved in all mycoplasmas. The nodes next to each mycoplasma are colour coordinated to their primary host. * Multiple hosts (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

search of the 14 mycoplasmas missing these key enzymes also found the corresponding genes absent from their genomes. Nineteen mycoplasmas retained all NME machinery, including *M. pneumoniae*, and 21 mycoplasmas were missing FolA only (Fig. 1) *Mycoplasma hyorhinis* was the only mycoplasma that retained FolA and MAP only. Notably, the only swine Mycoplasma missing FMT and PDF was *Mycoplasma hyosynoviae*, and similarly, all other mycoplasmas associated with arthritis retained FMT and PDF (*M. synoviae*, *M. fermentans*, *M. arthritidis*, and *M. hominis*). No correlation between proteome size and missing NME machinery was observed, as evidenced by one of the largest (*M. haemofelis*: 1524 proteins) and smallest (*M. bovoculi*: 579 proteins) mycoplasmas both lacking all four enzymes.

A bootstrapped (100 trials) maximum likelihood-based phylogenetic analysis of 54 mycoplasma genomes was performed using the RAxML-VI-HPC pipeline available at PATRIC (Wattam et al., 2017) (Fig. 1). The analysed mycoplasmas fall into four clades – Hominis, Haemoplasma, Pneumoniae and Spiroplasma (Fig. 1). Of the clade Hominis, 27% (8) retained all NME machinery, 52% (16) were missing FolA only, and 19% (6) were missing FolA, FolD, PDF and FMT. The mycoplasma species in clade Hominis lacking the four enzymes, consisting of respiratory and ocular species found in swine and ruminants (*M. hyopneumoniae*, *M. flocculare*, *M. dispar*, *M. ovipneumoniae*, *M. bovoculi* and *M. conjunctivae*), were clustered together suggesting an evolutionary basis to lost fnMet processing enzymes. *M. hyorhinis* is further down the branch containing these respiratory and ocular mycoplasmas and has uniquely lost FolD, FMT and PDF, but retains FolA. All members of the clade Haemoplasma have lost FolA, FolD, PDF and FMT, and conversely all members the clade Pneumoniae have retained NME machinery. Of the mycoplasma spp. belonging to the Spiroplasma cluster *M. putrefaciens* and *M. yeatsii* have retained NME machinery, and *M. mycoides* and *M. capricolum*, have lost FolA (Fig. 1).

3.2. No N-terminal formylation of methionines in *M. hyopneumoniae* proteins

Tryptic peptide datasets sourced in-house and online, consisting of multiple biological replicates of both *M. hyopneumoniae* and *M. pneumoniae,* were analysed for the presence of fnMet. While fnMet peptides were identified in *M. pneumoniae,* an intensive investigation of peptide datasets found none in *M. hyopneumoniae* (Fig. 2). The experiments were analysed utilising PEAKS studio 8.5 (Zhang et al., 2012) with the post-translational modification (PTM) search. This analysis matches more PTMs with greater confidence and less computational overhead.

fnMet is removed from most (> 94%) proteins, so the rate of identification of fnMet was expected to be statistically low. One tryptic peptide dataset derived from a gel fractionated whole cell lysate of *M. pneumoniae* identified fnMet occurring in 5 out of 394 identified proteins including 30S ribosomal protein S9 (MPN_446), transcription elongation factor GreA (MPN_401), 10 kDa chaperonin (MPN_574), glycerol uptake facilitator protein (MPN_043), and DNA-directed RNA polymerase subunit delta (MPN_024) with multiple spectral hits. In the first instance, a dataset generated from an in-house gel fractionated whole cell lysate of *M. hyopneumoniae* did not, unlike *M. pneumoniae*, show evidence of fnMet. Nor did an in-house gel fractionated

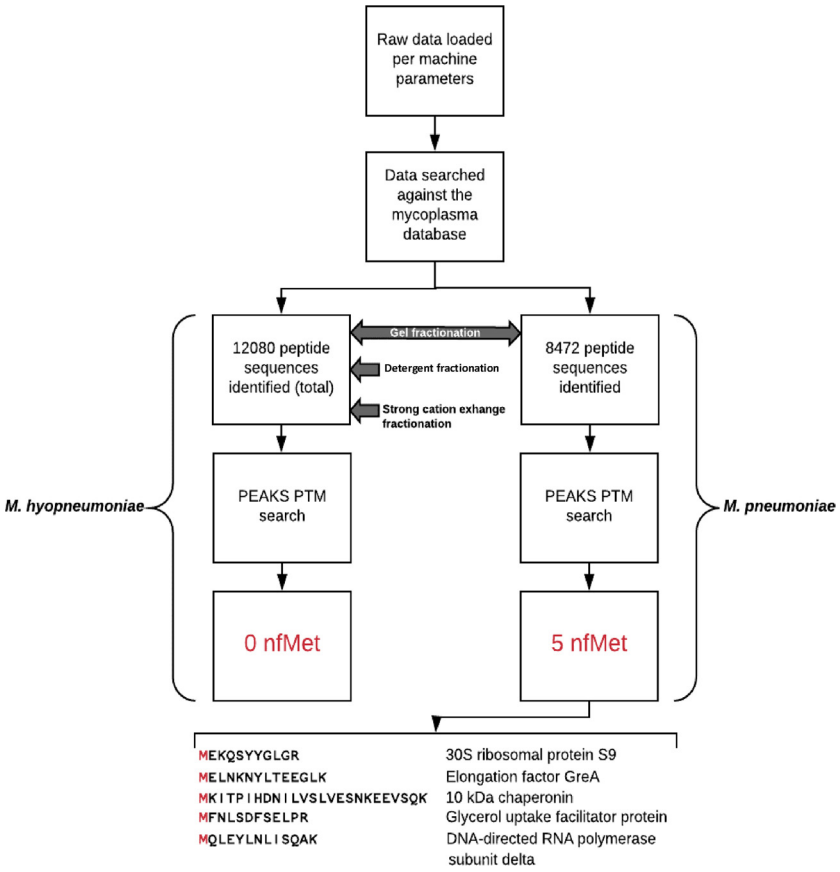

Fig. 2. Flow diagram of fnMet searches. 12,080 tryptic peptides from trypsin digestion of i) SDS-PAGE fractionated whole cell lysates; ii) a TX-114 detergent fraction; iii) strong cation exchange MS data for *M. hyopneumoniae* were searched for PTMs through PEAKS Studio. No fnMet peptides were identified. Five fnMet peptides, mapping to five proteins were identified when 8472 tryptic peptides derived from trypsin digestion of SDS-PAGE fractions of *M. pneumoniae* whole cell lysate was searched through PEAKS using the same parameters used for the analysis of *M. hyopneumoniae*.

surfaceome data of *M. hyopneumoniae*. To further investigate the *M. hyopneumoniae* proteome, an in-depth analysis dataset using tryptic peptides generated from whole cell lysates and insoluble proteins from *M. hyopneumoniae* captured using strong cation exchange and detergent fractionation was download from PRIDE DB (PXD000118) (Pendarvis et al., 2014) was searched, and also showed no evidence of peptides containing fnMet (PEAKS search results for both mycoplasmas in Supplementary File 3).

3.3. *M. hyopneumoniae translation initiation factors have structural and sequence irregularities*

Initiation of bacterial translation requires six components; the ribosome, fMet-tRNA$_f^{Met}$, mRNA, and three initiation factors IF1, IF2 and IF3. mRNA binds to ribosomal 30S subunit through the aid of fMet-tRNA$_f^{Met}$ and initiating factors. The purpose is to select the correct start codon for elongation to proceed, achieved through an initiation factor-mediated codon-anticodon interaction of mRNA with fMet-tRNA$_f^{Met}$ (Simonetti et al., 2009). Here we show that *M. hyopneumoniae* initiation factors deviate in structure and amino acid sequence compared to other eubacteria.

3.3.1. *IF1 lacks conserved rRNA binding sites*

IF1 binds to the ribosome where it interacts with rRNA to induce significant conformational changes in the 30S subunit (Gualerzi et al., 2001). The structure of *M. hyopneumoniae* IF1 (MHJ_0168) was modelled using Phyre2. *M. hyopneumoniae* IF1 takes on a similar fold to solved NMR and crystal IF1 structures (Fig. 3A). The fold includes the five beta strand barrel, typical of the S1 family of OB-fold proteins (Bycroft et al., 1997). However, *M. hyopneumoniae* has only two (E^{26} and H^{38}) of fourteen conserved rRNA binding sites (examples provided in Fig. 3B). Additionally, *M. hyopneumoniae* IF1 is predicted to have two unique rRNA binding sites at T^{17} and N^{36} (Fig. 3B).

3.3.2. *IF2 lacks N-terminal domains and fMet-tRNA$_f^{Met}$ binding residues*

IF2 was of particular interest to us as this protein binds fMet-tRNA$_f^{Met}$. *M. hyopneumoniae* IF2 (MHJ_0585) is smaller at 599 residues compared to ˜900 residues in other bacteria. *M. hyopneumoniae* IF2 was modelled using Phyre2. A combination of solved structures for *Geobacillus stearothermophilus* were used as a comparison because amino acids both directly implicated and selectively affected in binding fMet-tRNA$_f^{Met}$ were identified in this bacteria by NMR and mutagenesis (Guenneugues et al., 2000).

M. hyopneumoniae IF2, like other mycoplasmas, is missing N-terminal domains (Fig. 4A, N-term domain), however it is also missing fMet-tRNA$_f^{Met}$ binding residues in the C-terminus (Fig. 4B). The N-terminal domain of IF2 has a high affinity for the ribosome but is not present in IF2s of Eukaryotes (Wienk et al., 2012). The IF2 C-terminus (Fig. 4A, boxed Domain VI-2) contains all the molecular determinants necessary for the recognition and binding of fMet-tRNA$_f^{Met}$ (Meunier et al., 2000). The C-terminal Domain IV-2 of *M. hyopneumoniae* differs considerably in shape to that of *G. stearothermophilus*, and the fMet-tRNA$_f^{Met}$ binding residues are fewer in number and appear less surface exposed (Fig. 4A, boxed, shown in red). *M. hyopneumoniae* IF2 has the mutation K/R→Q^{558}. A positive residue in this position is critical for fMet-tRNA$_f^{Met}$ binding (Guenneugues et al., 2000). Additional mutations are seen at Y/F→D^{560} and C→F^{573}. Substitutions in these positions greatly lower binding affinity to fMet-tRNA$_f^{Met}$ (Guenneugues et al., 2000) (Fig. 4B). Also of note is that the IF2 signature motif [GLES]-x-[LIVM]-x(2)-L-[KR]-[KRHNS]-x-K-x(5)-[LIVM]-x(2)-[GNKADS]-x-[DEN]-[CRG]-[GI] (underlined in Fig. 4B) is present in all the example bacteria sequences but not in *M. hyopneumoniae* (deviations marked in red).

3.3.3. *IF3 contains an additional unique amino acid insert and C-terminal sequence*

Initiation factor IF3 consists of an N-terminal and C-terminal domain separated by a flexible linker. The C-terminal domain performs all

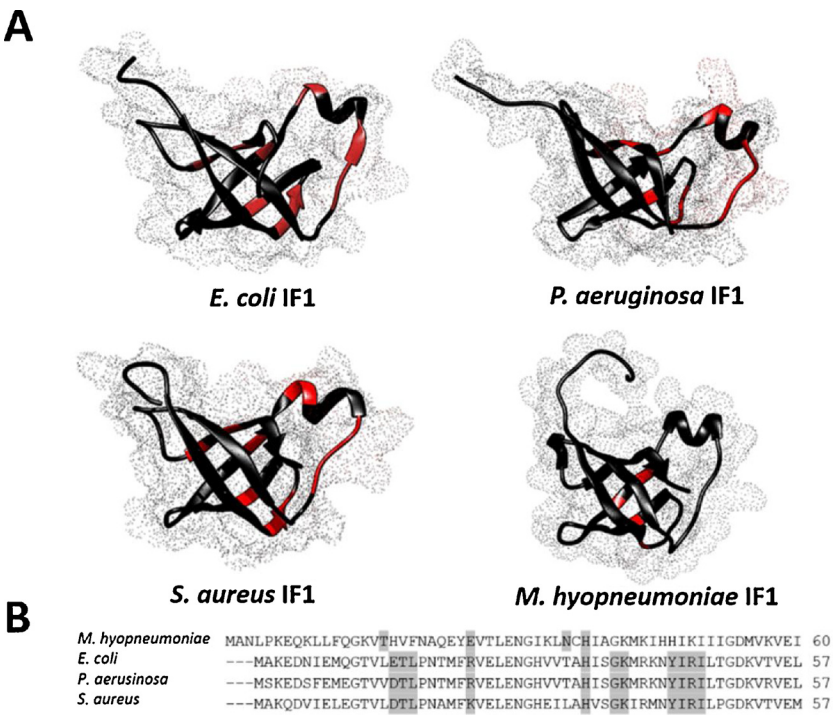

Fig. 3. *M. hyopneumoniae* IF1. A. Solved IF1 structures from *E. coli*, *P. aeruginosa* and *S. aureus* (PDB: 1AH9, 2N78 and 2N8N, respectively) were used to compare the predicted *M. hyopneumoniae* IF1 ribbon and surface structure. B. Amino acid sequence showing distribution of rRNA binding residues (in grey) among *M. hyopneumoniae* and other bacteria.

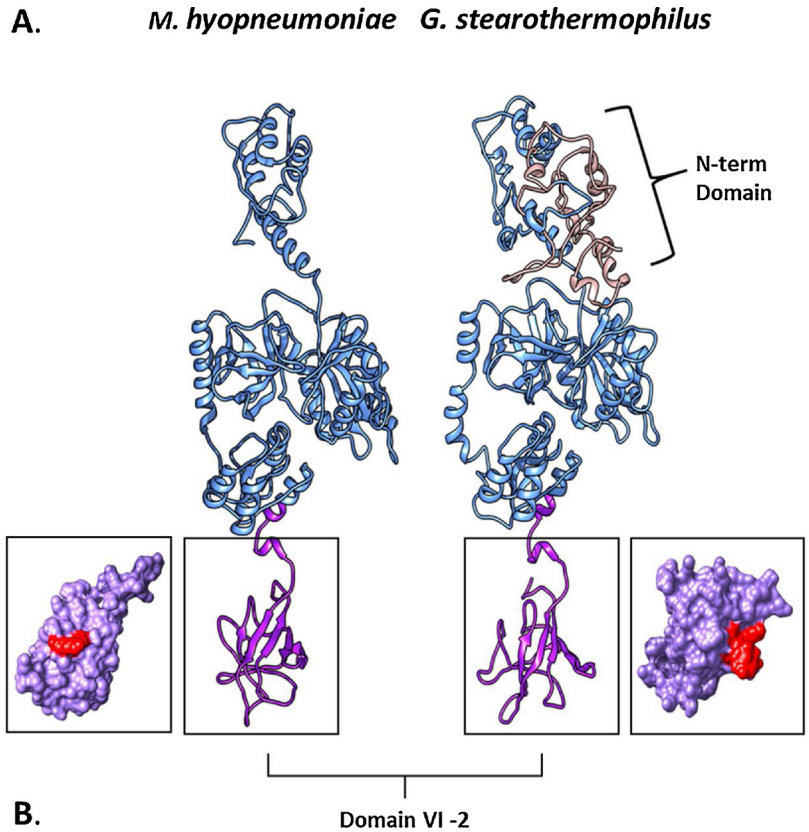

Fig. 4. *M. hyopneumoniae* IF2. A. Left: ribbon structure of *M. hyopneumoniae* IF2. The C-terminal domain VI-2 is boxed, and the surface structure is presented adjacently. rRNA binding residues are shown in red. Right: Ribbon structure of *G. stearothermophilus* IF2. An N-terminal domain is labelled, which is missing in *M. hyopneumoniae* IF2. Both the ribbon and surface structure of C-terminal domain VI-2 are boxed, and rRNA binding residues are shown in red. B. A partial amino acid sequence of domain IV-2 illustrating the location of rRNA binding residues. The IF2 signature motif is underlined ([GLES]-x-[LIVM]-x(2)-L-[KR]-[KRHNS]-x-K-x(5)-[LIVM]-x (2)-[GNKADS]-x-[DEN]-[CRG]-[GI]), and the residues that deviate from the motif in *M. hyopneumoniae* are shown in red (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

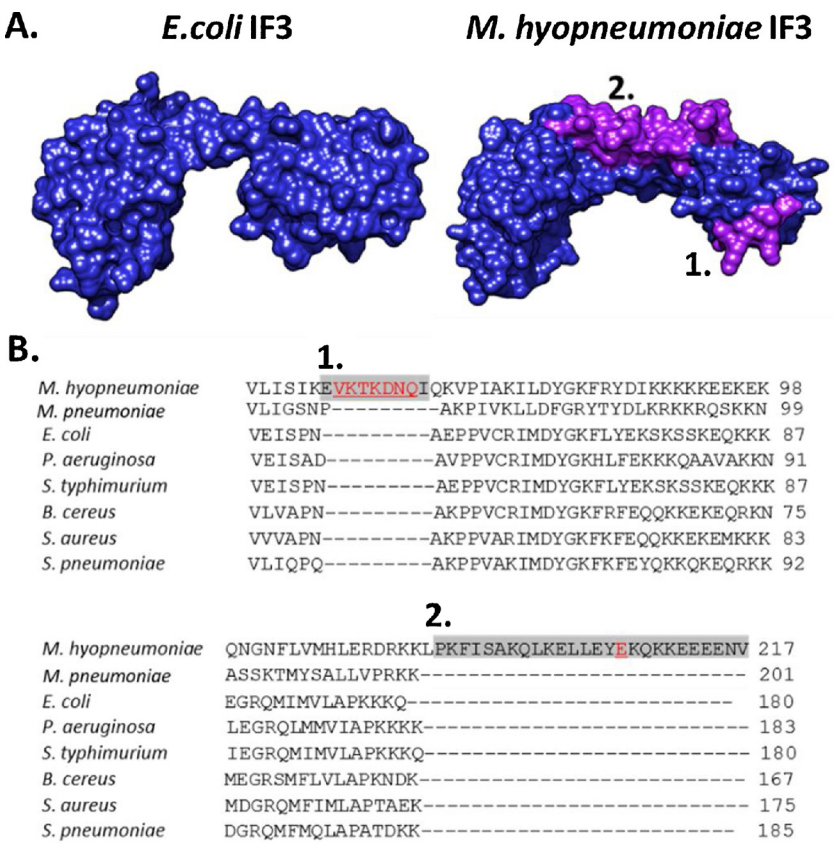

Fig. 5. *M. hyopneumoniae* IF3. A. Left: predicted surface structure of *E. coli* IF3. Right: predicted surface structure of *M. hyopneumoniae* IF3. An insert "1." and additional C-terminal sequence "2." are shown in purple and illustrated in the amino acid sequence alignments in B. Predicted protein: protein interaction sites are underlined and in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the known IF3 activities, including activating 30S-bound mRNA, dissociation of fMet-tRNA$_f^{Met}$, and stimulating mRNA translation regardless of its start codon (Petrelli et al., 2001).

Phyre2 was used to model *M. hyopneumoniae* IF3 (MHJ_0121) and *E. coli* IF3. IF3 amino acid sequence alignments from both Gram-positive (including *M. pneumoniae)* and Gram-negative bacteria, as well as human mitochondrial IF3, demonstrate that *M. hyopneumoniae* IF3 contains an additional insert at ^{62}EVKTKDNQI^{70}. *M. hyopneumoniae* also possess a 26 amino acid long additional C-terminal sequence (Fig. 5). A similar insert and C-terminal extension is seen in other Mycoplasma members missing fMet processing machinery (Supplementary file 4). Notably, a C-terminal extension of similar length is present in human mitochondrial IF3 (Fig. 5B). Approximately half of the residues in these C-terminal extensions are charged (7 glutamic acids and 6 lysines for *M. hyopneumoniae*; 9 glutamic acids and 5 lysines for *H. sapiens*). Interestingly, 7 of the 9 residues within the *M. hyopneumoniae* insert are predicted to be protein-protein interaction sites (^{62}EVKTKDNQI^{70}, protein: protein interaction sites underlined), and a protein: protein interaction site is predicted at E^{207} (Fig. 5B) within the additional C-terminal sequence.

4. Discussion

N-terminal methionine excision (NME) is an essential process that occurs in all organisms. However, the pathways leading to NME differ markedly between bacteria and eukaryotes (Bloes et al., 2015). Specifically, bacteria use fMet-tRNA$_f^{Met}$ for translational initiation, whereas eukaryotes (in cytoplasmic NME) and archaea use unmodified Met-tRNA^{Met} (Bloes et al., 2015). The reason behind strict bacterial reliance on fMet-tRNA$_f^{Met}$ is unclear. There appears to be an evolutionary requirement to retain formylation of methionine in bacteria despite its use producing formylated peptides which are secreted by bacteria and detected by formyl peptide receptors expressed on leukocytes and other host cells.

Enzymes involved in bacterial NME include FolA, FolD, FMT, PDF and MAP (their activities are summarised in Fig. 6A below). Individually, *pdf* and *map* gene knockouts are lethal (Margolis et al., 2001; Yuan et al., 2011), while null mutations in *fmt* cause growth defects to varying degrees. The *pdf* gene has been previously reported as absent in *M. hyopneumoniae* (Arraes et al., 2007), and the authors speculated that the loss of PDF might provide *M. hyopneumoniae* with a mechanism to evade detection by the host immune system. Through a bioinformatic analysis of 54 mycoplasma proteomes, we demonstrated that *M. hyopneumoniae* along with 13 other mycoplasma species are missing FolA, FolD, FMT and PDF, thus removing all components needed to form, attach and subsequently remove the methionine start tRNA formyl group.

With the four missing enzymes, the resulting NME process in these mycoplasmas is reminiscent of eukaryotes rather than bacteria (Fig. 6B). Notably, all proteomes available for Haemoplasmas had missing fnMet processing enzymes. Haemoplamsas, such as *M. suis*, reside and replicate on the surface of red blood cells (Hoelzle et al., 2007), and as formyl peptide receptors are most predominantly found on white blood cells, the loss of formylated peptide production is undoubtedly advantageous to their survival. *M. hyopneumoniae* infections are known to elicit numerous immune responses, such as increasing levels of proinflammatory cytokines, TNF-α (Choi et al., 2006; Rodríguez et al., 2016) mucins (Kim et al., 2012), plasmin (Woolley et al., 2013), as well as activating NF-κB and several mitogen-activated protein kinase pathways (Damte et al., 2011). Not producing formylated peptides could aid immune evasion by this pathogen in order to establish chronic infection.

The only universally conserved NME enzyme across the analysed mycoplasmas was MAP, meaning that the ability to excise N-terminal methionine is conserved across mycoplasma species. Within the Hominis and Spiroplasma clades, 21 mycoplasmas were only missing FolA (also known as dihydrofolate reductase or DHFR). FolA has a role in the One-Carbon pathway which is important for DNA, RNA and

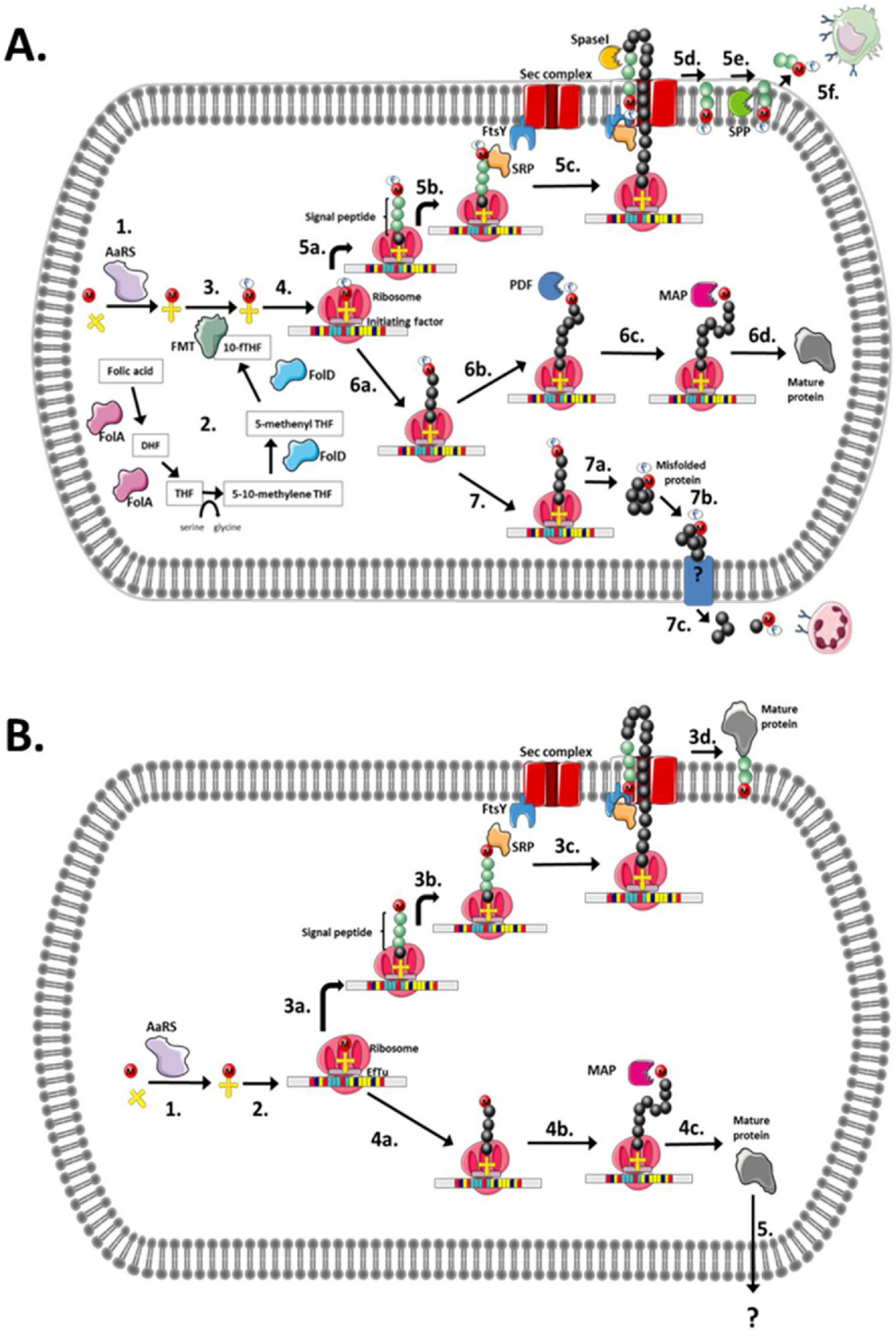

Fig. 6. Bacterial and Proposed *M. hyopneumoniae* NME processes. A) Bacterial NME 1: Aminoacyl-tRNA synthetase (AaRS) attaches Met to $tRNA_f^{Met}$. 2: Folic acid is reduced to dihydrofolate (DHF) and then tetrahydrofolate (THF) by FolA. THF is converted to 5–10 methylene THF and reduced to 5-methenyl THF and then 10-fTHF by FolD. 10-fTHF is used by FMT to attach a formyl group (f) to Met-$tRNA_f^{Met}$ shown in 3. 4: fMet-$tRNA_f^{Met}$ preferentially binds to IF2 on the bacterial ribosome, initiating translation. 5a: Proteins with signal peptides are bound by signal recognition particle (SRP) and moved to SRP's receptor FtsY (5b). 5c: The signal peptide is anchored into the membrane and translation continues. Once translation is completed the signal peptide is cleaved by signal peptidase I (SpaseI). 5e: the embedded signal peptide is removed by signal peptide peptidase (SPP). 5f: formyl peptide receptors present on WBCs recognise dislodged formylated signal peptides. 6a: If no signal peptide is present, PDF removes N-terminal f (6b), and Met is subsequently cleaved by MAP (6c) to ensure proper folding of the mature protein (6d). 7: This pathway represents the f-degradon theory (Piatkov et al., 2015). 7a: if PDF does not cleave f, then MAP cannot cleave nMet leading to protein misfolding (7a). 7b: the misfolded protein is degraded, possibly by membrane protease FtsH. 7c: peptides including formylated ones, are released which attract WBCs. B) Proposed *M. hyopneumoniae* NME 1: AaRS attaches Met to tRNAMet. 2: M. hyopneumoniae is missing FolA, FolD and FMT so f cannot be synthesised or attached to Met-tRNAMet. Thus unformylated Met-tRNAMet binds to IF2 or Ef-Tu within the bacterial ribosome, and protein synthesis begins. Signal peptide recognition and translocation (3a, 3b, 3c) are the same as in other bacteria only M. hyopneumoniae does not have an annotated SSP nor a functional SpaseI, so unformylated signal peptides remain embedded in the membrane and may serve as anchors for mature proteins (3d). 4a: An emerging protein without a signal peptide lacks a formyl group, removing the need for PDF. 4b: MAP cleaves nMet ensuring correct folding. 5: Some cytosolic proteins are moved to the cell surface by an unknown mechanism.

protein biosynthesis. It was once thought essential and became the target of antimicrobial agents, such as Trimethoprim (Huovinen et al., 1995). However, an absence of FolA has been described in other bacteria, including *R. prowazekii*, *Helicobacter pylori*, and *Treponema pallidum* (Myllykallio et al., 2003), all genome-reduced pathogens.

The 14 mycoplasmas identified as missing NME machinery are a mixture of pathogenic and commensal species. However, a maximum likelihood-based phylogenetic analysis showed that they are all clustered, suggesting an evolutionary basis for the lost proteins. Perhaps the most infamous of the 14 mycoplasmas is *M. hyopneumoniae*, a highly contagious, economically significant, and globally distributed agent of swine respiratory disease.

To strengthen our bioinformatic analysis, we sought to gather experimental LC-MS/MS data to support the hypothesis that fnMet is indeed absent from the *M. hyopneumoniae* and present in *M. pneumoniae*, a human respiratory pathogen that retains all NME machinery. This aim was ambitious for two reasons. Firstly, due to the removal of fnMet from > 94% of all proteins, the retention of N-formyl group was expected to be limited to a few polypeptide chains (Bienvenut et al., 2015). Secondly, N-terminal enrichment strategies we had used previously to examine *M. hyopneumoniae* NME (Berry et al., 2017), could not be implemented as the N-dimethyl label used has a monoisotopic mass difference of 0.0365 daltons from the mass of the N-formyl modification, making the two PTMs arguably indistinguishable from

each other. Nevertheless, the *M. pneumoniae* proteome was determined through gel fractionation and LC-MS/MS. Of a possible 694 ORFs, 392 were identified, 5 of which had fnMet present. These five proteins are likely to have true fnMets (and not identified prior to removal) as the in vivo fnMet lifespan, from becoming the first residue of a protein to the removal of the formyl group, is approximately 60 s (Piatkov et al., 2015).

Conversely, gel fractionation coupled with LC-MS/MS (using the same mass spectrometer used for *M. pneumoniae*) to determine the *M. hyopneuominae* proteome did not identify any fnMets. Additionally, published *M. hyopneumoniae* proteome data, which utilised strong ion exchange and detergent fractionation methods and two different high-resolution mass spectrometers, was downloaded, searched for fnMets, and subsequently not found. Due to the extensive fractionation by three methods on six biological replicates as well as the utilisation of three types of mass-spectrometers; this data provides the best representation of the *M. hyopneumoniae* proteome, which in turn has provided no identification for fnMet in any proteins.

Our data suggest that *M. hyopneumoniae* does not use fMet-tRNA$_f^{Met}$ for translation initiation. Null and suppressor mutations in the *fmt* gene have previously proven that other bacteria are also capable of initiating translation with Met-tRNA^{Met} rather than fMet-tRNA$_f^{Met}$ through a pseudo 70S initiation complex pathway (Ghanti et al., 2018). However, this pathway is more time consuming and results in growth impairments as well as other detriments including reduced antimicrobial resistance and biofilm formation (Cai et al., 2017).

fMet-tRNA$_f^{Met}$ is known to interact with three initiation factors, IF1, IF2, and IF3. These three initiation factors assist in the formation of the first codon-anticodon interactions between fMet-tRNA$_f^{Met}$ and mRNA (Duval et al., 2015). We, therefore, sought to determine whether *M. hyopneumoniae* IF1, IF2 and IF3 possessed structural and sequence deviations to suggest lowered fMet-tRNA$_f^{Met}$ affinity. Using computational molecular modelling and amino acid sequence alignments we found that while *M. hyopneumoniae* IF1 had less rRNA binding residues compared to other bacterial IF1s, both IF2 and IF3 had significant structural differences.

IF2 binds directly to the 30S ribosome and provides a docking site for fMet-tRNA$_f^{Met}$ (Yamamoto et al., 2016), located at the C-terminus (Meunier et al., 2000). When *M. hyopneumoniae* IF2 was compared to *G. stearothermophilus* IF2, two observations were made. Firstly, an N-terminal domain spanning 136 amino acids was missing *in M. hyopneumoniae* IF2. The N-terminus of IF2 has a high affinity for the ribosome; however, it is expendable in vivo and not present in higher organisms (Wienk et al., 2012). It is also important to note that *G. stearothermophilus* IF2 is already missing an N-terminal domain of approximately 150 amino acids, present in some other bacteria (Gualerzi and Pon, 2015). The two missing N-terminal domains in *M. hyopneumoniae* IF2 explain its relatively small size of 599 amino acids, compared to approximately 900 amino acids in other bacteria. The second observation was that *M. hyopneumoniae* IF2 C-terminal domain is missing critical fMet-tRNA$_f^{Met}$ binding residues. *M. hyopneumoniae* IF2 has the substitution K/R→Q^{558}; however, a positive residue in this position is crucial for fMet-tRNA$_f^{Met}$ binding (Meunier et al., 2000). Additional mutations are seen at Y/F→D^{560} and C→F^{573}. These substitutions are known to significantly lower binding affinity to fMet-tRNA$_f^{Met}$ (Meunier et al., 2000). These data suggest that *M. hyopneumoniae* IF2 ability to bind fMet-tRNA$_f^{Met}$ is diminished. Additionally, the IF2 C-terminus is highly conserved among bacteria and contains the signature IF2 motif. We show through amino acid sequence alignments that *M. hyopneumoniae* IF2 does not possess this motif due to three amino acid substitutions.

Both IF2 and IF3 are responsible for the fidelity of decoding the initiation start codon by fMet-tRNA$_f^{Met}$. While *M. hyopneumoniae* IF2 has lost sequence information, IF3 was found to have additional structural and sequence information compared to most eubacteria. Amino acid sequence alignments from Gram positive, Gram negative, and human mitochondrial IF3 demonstrated that *M. hyopneumoniae* IF3 has a unique insert at ^{62}EVKTKDNQI^{70}, which is almost entirely made up of putative protein: protein interaction sites. A similar insert was also observed in members of the clade hominis that were also missing fnMet processing enzymes. Furthermore, these clade members and human mitochondrial IF3s possess a C-terminal extension of approximately 30 amino acids and consisting largely of charged residues. Importantly, deletion of this C-terminal extension in mitochondrial IF3 increased fMet-tRNA$_f^{Met}$ binding affinity (Bhargava and Spremulli, 2005). A C-terminal extension is also present in chloroplastic IF3s, and similarly, deletion of the extension causes increased fMet-tRNA$_f^{Met}$ binding (Bhargava and Spremulli, 2005).

5. Conclusion

Our PTM search of comprehensive tryptic peptide datasets found no evidence of fnMets in *M. hyopneumoniae*, and our bioinformatic analysis suggests that FolA, FolD, FMT and PDF are missing in *M. hyopneumoniae* and 13 other mycoplasma species. These data allow us to propose that these organisms have evolved to utilise nMet to initiate protein synthesis, rather than fnMet. Moreover, structural and sequence irregularities in *M. hyopneumoniae* initiation factors suggest a reduced affinity with fMet-RNA$_f^{Met}$. These irregularities may prove to be adaptations required to initiate protein synthesis with nMet, without compromising growth or virulence.

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Competing interests statement

The authors have no competing financial interests, or other interests that might be perceived to influence the results and/or discussion reported in this article.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2019.03.005.

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