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Review

Metal utilization in genome-reduced bacteria: Do human mycoplasmas rely on iron?

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ABSTRACT

Mycoplasmas are parasitic bacteria with streamlined genomes and complex nutritional requirements. Although iron is vital for almost all organisms, its utilization by mycoplasmas is controversial. Despite its minimalist nature, mycoplasmas can survive and persist within the host, where iron availability is rigorously restricted through nutritional immunity. In this review, we describe the putative iron-enzymes, transporters, and metalloregulators of four relevant human mycoplasmas. This work brings in light critical differences in the mycoplasma-iron interplay. *Mycoplasma penetrans*, the species with the largest genome (1.36 Mb), shows a more classic repertoire of iron-related proteins, including different enzymes using iron-sulfur clusters as well as iron storage and transport systems. In contrast, the iron requirement is less apparent in the three species with markedly reduced genomes, *Mycoplasma genitalium* (0.58 Mb), *Mycoplasma hominis* (0.67 Mb) and *Mycoplasma pneumoniae* (0.82 Mb), as they exhibit only a few proteins possibly involved in iron homeostasis. The multiple facets of iron metabolism in mycoplasmas illustrate the remarkable evolutive potential of these minimal organisms when facing nutritional immunity and question the dependence of several human-infecting species for iron. Collectively, our data contribute to better understand the unique biology and infective strategies of these successful pathogens.

Contents

Abbreviations: ABC, ATP-binding cassette; ECF, energy-coupling factor; Fur, ferric uptake regulator; Hrl, histidine-rich lipoprotein; *Mge*, *Mycoplasma genitalium*; *Mho*, *Mycoplasma hominis*; *Mpe*, *Mycoplasma penetrans*; *Mpn*, *Mycoplasma pneumonia*; PDB, protein data bank; RNR, ribonucleotide reductase; XRF, X-ray fluorescence; ZIP, zinc-iron permease.

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1. Introduction

Iron is essential for virtually all organisms, which employ its unique properties to perform specific tasks such as to enable electron transfer or as catalytic cofactor of enzymes. It is estimated that bacterial proteomes contain 3.9% non-heme iron-proteins on average [1]. However, iron can also be toxic, *e.g.*, by generating free radicals that can damage the cell [2]. Because of this duality, living organisms possess dedicated mechanisms to maintain cellular iron homeostasis, involving a variety of proteins [3,4]. Remarkably, iron is central in the fight between host and pathogen. One response of the host immune system is to restrict access to essential metals such as iron, a process called nutritional immunity [5]. For example, extracellular iron in the serum is sequestered by the host protein transferrin. In addition, a mechanism used by macrophages to destroy microbes after phagocytosis consists in diminishing the concentration of Mn^{2+} and Fe^{2+} surrounding the pathogen by pumping them out of the phagosome through a specific efflux system [6,7]. Consequently, bacteria have developed effective strategies for the acquisition of iron ions often scarce in their environment, such as to use high-affinity iron-chelating siderophores [8,9]. Interestingly, siderophore-based therapeutics might represent promising new strategies to target human pathogens [10].

Members of the class *Mollicutes*, commonly known as mycoplasmas, are well-known for having no cell wall and a reduced genomic content. Often portrayed as minimal bacteria, these organisms are obligate parasites with complex nutritional requirements and a fastidious growth in cell-free environments. These features are best represented by *M. genitalium*, one of the simplest living cells, whose genomic content barely exceeds the minimum amount of information needed to sustain self-replication (Table 1). Whole-genome design and synthesis established the lower limit for a mycoplasma genome at 0.53 Mb and identified 473 genes as essential for life [11]. Unexpectedly, 149 of these genes were of unknown biological functions, revealing a major gap in our understanding of the biology of these organisms.

To gain access to nutrient-rich environments, mycoplasmas have evolved highly sophisticated genetic systems to escape host defenses, as well as unconventional mechanisms of horizontal gene transfer [12–14]. The remarkable plasticity of these organisms is illustrated by their successful adaptation to a wide range of vertebrate hosts, with the *Mycoplasma* genus encompassing more than 100 well-described species that live in close association with respiratory and urogenital mucosal surfaces, causing chronic, often debilitating infections [15]. Among them are several human-infecting species such as *M. genitalium* (*Mge*), *M. pneumoniae* (*Mpn*), *M. penetrans* (*Mpe*), and the phylogenetically distant *M. hominis* (*Mho*) (Table 1). *Mge* and *Mho* are important sexually transmitted pathogens. Prevalence of *Mge* in the general population is estimated to range between 1.3% and 3.9%, being the higher percentages more usual in countries with lower levels of development [16]. However, the pathogen might be responsible for up to 35% of non-chlamydial non-gonococcal urethritis in men [17]. Furthermore, it has been associated with pelvic inflammatory disease, cervicitis and preterm birth in women [18]. *Mho* seems to increase the risk of female infertility, spontaneous abortion or stillbirth [19]. The pathogenicity of *Mpe* is less clear, but the bacterium was isolated from patients with AIDS and a recent study suggests that it could be associated with non-gonococcal urethritis [20]. Despite its phylogenetic proximity with *Mge* [21], *Mpn* is mainly associated with respiratory infections and responsible for 4 to 8% of community-acquired bacterial pneumonias, leading to an estimated two million cases and 100,000 hospitalizations in the United States annually [22]. In addition to these species, several members of the *Ureaplasma* genus are regularly associated with urogenital infections (Table 1).

Metal utilization in mycoplasmas is poorly understood and the information available on iron homeostasis is puzzling. Indeed, class I ribonucleotide reductase (RNR), an essential enzyme canonically iron- and/or manganese-dependent, has overcome the requirement of metals in mycoplasmas [23]. Furthermore, despite recent studies reporting the existence of a transcriptional regulator of the Fur (ferric uptake regulator) family in *Mge* and *Mpn* [24,25], the specific role of the Fur regulated genes in metal acquisition is unclear. In this review, we aim to discuss the current understanding of iron utilization in four human-infecting mycoplasmas, *Mge*, *Mpn*, *Mho* and *Mpe*, and describe putative iron-dependent enzymes, transporters, and regulatory systems.

2. An iron-free existence in an iron-dependent world?

Iron abstinence, —the elimination of the requirement for iron—, has recently emerged as a novel strategy to overcome host iron limitation in prokaryotes. This unique strategy was adopted by the Lyme disease agent *Borrelia burgdorferi* (class *Spirochaetae*) [26]. Indeed, this obligate parasite can grow in chemically defined media with no available iron, showing identical growth rates in both iron-lacking or iron-supplemented media. The iron-free existence of *B. burgdorferi* is further illustrated by a low intracellular iron content, the paucity of metalloproteins that required iron as a cofactor, and manganese-substitution for iron in these proteins. Whether mycoplasmas may have adopted similar approaches to avoid host iron limitations is still an open question. *Mpn* has been

Table 1
General features of main human-infecting mycoplasmas.

Species	Phylogenetic group	Genome size (Mb)[a]	Associated disease
Mycoplasma pneumoniae (*Mpn*)	Pneumoniae	0.82	Atypical pneumonia
Mycoplasma genitalium (*Mge*)	Pneumoniae	0.58	Non-gonococcal urethritis, pelvic inflammatory disease
Mycoplasma penetrans (*Mpe*)	Pneumoniae	1.36	Unknown (AIDS-associated)
Ureaplasma urealyticum	Pneumoniae	0.87	Urogenital infections
Mycoplasma hominis (*Mho*)	Hominis	0.67	Urogenital infections

found to bind the iron-sequestering lactoferrin through the moonlighting activity of several cytoplasmic proteins [27,28]. However, it remains unclear whether this lactoferrin-binding activity contributes to metal uptake or simply reflects the broad range of host macromolecules that can be found associated with the surface of mycoplasmas [28]. Low passages of *B. burgdorferi* were also found able to bind iron-sequestering proteins, but this phenotype was not essential for bacterial growth under axenic conditions [29].

Both, *Mpn* and *Mge* can be grown in a defined minimal medium with a formulation devoid of iron [30]. This fact suggests that these two pathogenic species have limited or no iron requirement. This hypothesis is consistent with the paucity of iron-dependent proteins in human mycoplasmas (see below) and recent data showing that mycoplasmas can overcome metal requirements in the class I RNR [23]. Although still hypothetical, the iron-free existence of several human-infecting mycoplasmas is further supported by the lack of classical iron-sulfur cluster assembly systems found in more complex bacteria [31–33]. Indeed, only two proteins with sequence similarity to the cysteine desulfurase SufS and the scaffold protein SufU are found conserved across many mycoplasma species, with the notable exception of *Mpe* that encodes a reduced machinery likely associated with iron-sulfur cluster biosynthesis [32]. The *sufS-sufU* locus was identified as an essential component of the minimal bacterial genome [11,34,35] and a potential virulence factor responsible for hemolysis and hydrogen sulfide production in *Mpn* [36]. The biological importance of this locus was further confirmed upon experimental infections with the ruminant pathogen *Mycoplasma agalactiae* that demonstrated its critical role for host-colonization [37]. Although also required for survival of *M. agalactiae* during co-incubation with host cells, this locus has proven to be completely unnecessary under axenic laboratory conditions [38].

In an iron-dependent world, living without iron could be anything but simple, even for mycoplasmas. While iron abstinence may represent a successful strategy for mycoplasma to escape host nutritional defenses, these data also illustrate important differences in the mycoplasma-iron interplay.

3. Iron-dependent enzymes

Iron is the third most common enzyme metal cofactor, estimated to be utilized by around 8% of all enzymes [39]. It can form mononuclear or dinuclear centers as well as be a constituent of hemes or iron-sulfur clusters [40]. Iron is the prevalent metal ion used by oxidoreductases, but it can also serve as a Lewis acid catalyst [41]. At least four possible iron-dependent enzymes are encoded in the genomes of *Mge*, *Mpn*, *Mho* and *Mpe*, but none of them has been experimentally characterized in mycoplasmas. Thus, we briefly assessed whether iron is essential for these enzymes in other bacterial species. Moreover, we performed homology modeling of these enzymes using structural data for the closest homologs available in the Protein Data Bank (PDB). These homology models illustrate that the putative active sites are conserved in mycoplasmas and that their predicted three-dimensional structures are compatible with the binding of metal ions. Such homology modeling approach could represent a first step in the assessment of the druggability of putative metalloenzymes in human mycoplasmas.

The first putative iron-dependent enzyme is TsaD, encoded by MG046, MPN059, MHO3950 and MYPE8610 in *Mge*, *Mpn*, *Mho* and *Mpe*, respectively. This protein is required in the biosynthesis of the tRNA modification N^6-threonylcarbamoyladenosine (t^6A). This modification is almost universally conserved and crucial for translational fidelity [42]. In bacteria, the synthesis of t^6A involves four proteins, TsaB, TsaC, TsaD and TsaE. The active site of the TsaD

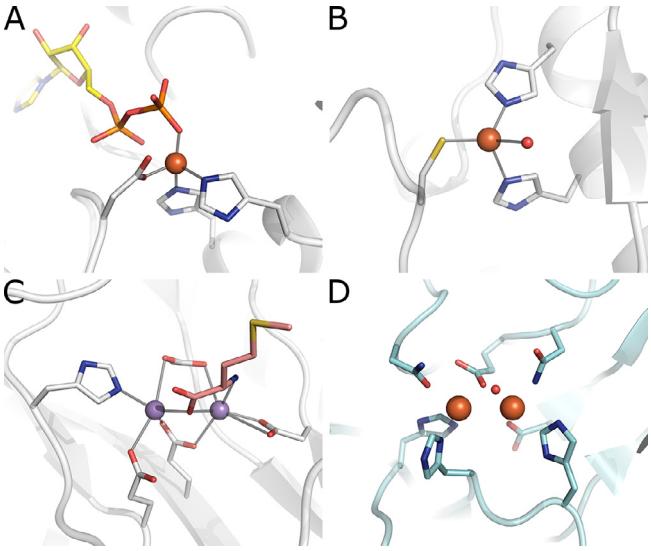

Fig. 1. Models of possible iron-containing enzymes in human mycoplasmas. A. Homology model of TsaD from *Mge* built using *E. coli* TsaD (PDB ID: 4ydu) as template, and harboring an iron ion in interaction with adenosine diphosphate (colored in yellow). B. Homology model of PDF from *Mge* built using *Streptococcus pneumoniae* PDF (PDB ID: 1lm6) as template. C. Homology model of MetAP from *Mge* in complex with methionine (colored in pink) built using *Pseudomonas aeruginosa* MetAP (PDB ID: 4fo8) as template. Note that the metal site was built using a dinuclear manganese form of the enzyme because no MetAP with a diiron cofactor of significant homology with the *Mge* MetAP was found in the PDB. D. Crystal structure of a putative metallophosphoesterase from *Mpn* (PDB ID: 1t71). Iron and manganese ions are represented as orange and purple spheres, respectively. Homology models for *Mge* TsaD, PDF and MetAP were built using SWISS-MODEL [55]. The figure was prepared using the PyMOL Molecular Graphics System, version 2.4 Schrödinger, LLC, and Inkscape 1.0. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

displays a conserved metal center (Fig. 1A) which is proposed to mainly play a structural role by providing a scaffold for binding of the substrate [43]. Several crystal structures from *Escherichia coli* and *Thermotoga maritima* have been reported. The identity of the metal ion was assigned as either iron or zinc based on the electron density and X-ray fluorescence (XRF) measurements [44,45]. Of note, previous spectroscopic and crystallographic data obtained for the archaeal homologue Kae1 suggested that the active site harbors a Fe^{3+} ion [46]. Therefore, the nature of the physiological metal cofactor of TsaD remains unclear.

Besides, two possible iron-enzymes are present in human mycoplasmas to mediate the N-terminal methionine excision of nascent polypeptide chain: a peptide deformylase (PDF) (MG106, MPN245, MHO1100, MYPE5650) and a methionine aminopeptidase (MetAP) (MG172, MPN186, MHO2770, MYPE9960). N-terminal methionine excision is ubiquitous and represents an essential protein modification for most organisms. In bacteria, this process is performed in two successive steps, the initiator methionine of newly synthesized proteins is first deformylated by PDF and then removed by MetAP [47]. PDF was originally characterized carrying a ferrous mononuclear active site in its active form [48,49]. The metal ion is coordinated in a tetrahedral geometry by three conserved residues and a water molecule [50] (Fig. 1B). However later studies showed that PDFs utilize different metal ions in different organisms, *e.g.*, a zinc ion in the iron-deficient bacteria *B. burgdorferi* [51]. Regarding MetAP, the enzyme harbors a dinuclear metal center but the identity of the physiological metal ions is still debated. Indeed, MetAPs have been shown to be activated with several divalent metal ions, including Fe^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} [52] (Fig. 1C).

Finally, *Mge*, *Mpn*, *Mho* and *Mpe* genomes encode a putative metallophosphoesterase which possibly uses an iron cofactor (MG246, MPN349, MHO1200 and MYPE8900). It belongs to a superfamily of metallophosphoesterases exhibiting different active-site motifs that bind different combinations of dinuclear metal ions [53]. The crystal structure of this enzyme from *Mpn* has been solved (PDB ID: 1t71) and the cofactor is modelled as a diiron center (Fig. 1D), but little experimental details are provided. The closest homolog with a known structure is YmdB from *Bacillus subtilis* [54] with 39% sequence identity. When recombinantly produced in *E. coli*, YmdB copurifies with a diiron active site, as identified by inductively coupled plasma mass spectrometry and XRF. The enzyme shows a phosphodiesterase activity against cyclic nucleotides, and a role as a global regulator of late adaptive responses in *B. subtilis*.

Unlike *Mge*, *Mpn* and *Mho*, the genome of *Mpe* encodes a machinery for iron-sulfur cluster biosynthesis. Furthermore, *Mpe* uses at least seven iron-sulfur proteins that are absent in *Mge, Mpn and Mho*: HemW (MYPE5010), MiaB (MYPE7940), TtcA (MYPE5070), RlmN (MYPE5630), IspG (MYPE9400), IspH (MYPE1330) and NrdG (MYPE4970) [32]. HemW is a heme chaperone required for the insertion of heme into hemoproteins which harbors a [4Fe–4S] cluster and one heme per subunit [56]. MiaB, TtcA and RlmN are enzymes involved in the modification of tRNA or rRNA. MiaB is a methylthiotransferase responsible for tRNA modification which binds two [4Fe-4S] clusters [57]. TtcA is a thioltransferase with a [4Fe-4S] cluster which catalyzes thiolation in tRNAs [58]. RlmN is a methyltransferase which seems able to modify both tRNA and rRNA, and containing a single [4Fe-4S] cluster [59,60]. Besides, IspG and IspH are two enzymes involved in the isoprenoid synthesis pathway [32], and both display a [4Fe-4S] cluster [61,62]. Finally, NrdG is the radical-generating subunit of the class III anaerobic RNR which activates the catalytic subunit NrdD (MYPE4960) using a [4Fe-4S] cluster in order to catalyze the synthesis of deoxyribonucleotides from ribonucleotides. This RNR is oxygen-sensitive and can be found in both facultative and obligate anaerobes [63]. Interestingly, the presence of this enzyme in *Mpe* in addition to the oxygen-dependent class I RNR might indicate that this organism can switch between aerobic and anaerobic environments [64].

It seems clear that human mycoplasmas have a different approach to iron when it comes to enzyme chemistry. On the one hand, *Mpe* certainly requires iron for the correct maturation of several enzymes, especially as a component of iron-sulfur clusters. On the other hand, *Mge*, *Mpn* and *Mho* exhibit only a few enzymes that are possibly iron-dependent but further experimental evidence is required.

4. Metal transporters

The number of putative transporters present in the human pathogens *Mpe*, *Mpn*, *Mge* and *Mho* is of 99, 70, 53, and 51 respectively [65]. Among the proteins specifically related to iron transport in *Mpe* and *Mho*, there is at least one system of the Zinc-Iron permease (ZIP) family (MYPE4700 and MHO2490, respectively). In addition, despite two heme transporters (MYPE3570 and MYPE7470) have been annotated for *Mpe* in the TransportDB database, we did not find compelling evidence supporting this role in our *in silico* analyses. However, there are no transporters putatively dedicated to iron acquisition in *Mpn* and *Mge*. Transporters for transition metals such as cobalt, are annotated in the reference genomes of *Mge*, *Mpn*, *Mho* and *Mpe*; they belong to the Energy-coupling factor (ECF) transport family, a recently discovered type of ABC (ATP-binding cassette) importer [66], which constitute potential antimicrobial target candidates [67].

ECF transporters are importers widely spread among prokaryotes, and are specific for vitamins, divalent cations such as nickel and cobalt, and other trace nutrients [68]. Regarding ECF transporters and iron, recent studies have opened the possibility of ECF transporters directly related to heme uptake as a scavenger strategy to overcome iron limitation in *Staphylococcus lugdunensis* [69], but also in species that do not require iron or heme to grow, such as *Lactobacillus sakei* [70]. As ABC transporters, ECF complexes contain two nucleotide binding ATP-hydrolases (EcfA1 and EcfA2) and two transmembrane proteins (EcfT and EcfS). The EcfA and EcfT subunits define the shared module. The EcfT subunit is the "transducer" that connects the ATP-hydrolyzing machinery to the substrate specificity subunit (EcfS) to drive the conformational changes for substrate translocation. ECF-type importers are classified into three-groups, defined by the type of S-subunit. Group I transporters are substrate dedicated, so the ECF complex consists of specific EcfA, EcfT, and EcfS subunits. Genes for all these subunits in group I transporters are normally encoded in the same operon [66]. Examples of these complexes are the divalent cation transport systems for cobalt CbiMNQO and nickel NikMNQO [71,72]. Group II transporters consist of a shared module of EcfA1, EcfA2, EcfT subunits for competing EcfS subunits presenting different substrates. In group II, genes for the shared module are encoded in the same operon and are constitutively expressed, whereas the substrate-specific EcfS subunits are scattered throughout the genome, which expression is regulated by the need of trace nutrients, mostly vitamins [66,68]. Group III are solitary S-subunits independent of the EcfA and EcfT subunits capable of transporting the substrate without any accessory modules [68,73].

In *Mge*, the MG179-MG180-MG181 ECF shared module complex (Fig. 2A and 2B) is encoded in the same operon, coding for the EcfA1 and EcfA2 subunits (MG179, MG180) and a EcfT subunit (MG181). This ECF shared module is represented in all four species, by the MYPE9770-MYPE9760-MYPE9750 operon in *Mpe*, the MHO1970-MHO1980-MHO1990 operon in *Mho* and the MPN193-MPN194-MPN195 operon in *Mpn* (Fig. 2A). This shared module is annotated as a cobalt-related transport system in genome references and in databases such as STRING [74] and UNIPROT [75]. However, mycoplasma species do not possess a group I ECF transport system, such as CbiMNQO and NikMNQO, present in other prokaryotes [66,73]. It has been also described that most mollicutes or other host-associated organisms, such as obligate intracellular parasites and endosymbionts have lost Ni^{2+}/Co^{2+} utilization [76].

According to genome organization, it is likely that the MG179-MG180-MG181 operon encodes a group II ECF transport system with shared modules for specific S-components (such as vitamins) scattered around these mycoplasma genomes. We have identified two putative S-components: MG313 and MG521 (Fig. 2A; B). At least two putative S-components have been previously annotated as folate-specific (FolT; vitamin B_9) in *Mge*, *Mpn* and *Mpe*, and a single one in *Mho* [66]. From the phylogenetic analysis in Fig. 2A, MG521 is likely a riboflavin (vitamin B_2) importer, whereas MG313 falls within the FolT branch as a putative folate transporter. In addition, MG098, MPN236 and MHO0470 could represent potential S-components because the *Mpe* homolog MYPE2080 has been annotated as S-component in the reference strain (accession number: WP_011077035.1).

Beyond the database annotation and the phylogenetic analysis, we assessed further MG098, MG313, and MG521 (and the respective *Mpn*, *Mho* and *Mpe* homologs) as potential EcfS proteins focusing on structural determinants. EcfS have a similar structural fold with six transmembrane helices, but show distinctive features depending on the ECF group [68]. EcfS binding cobalt or nickel

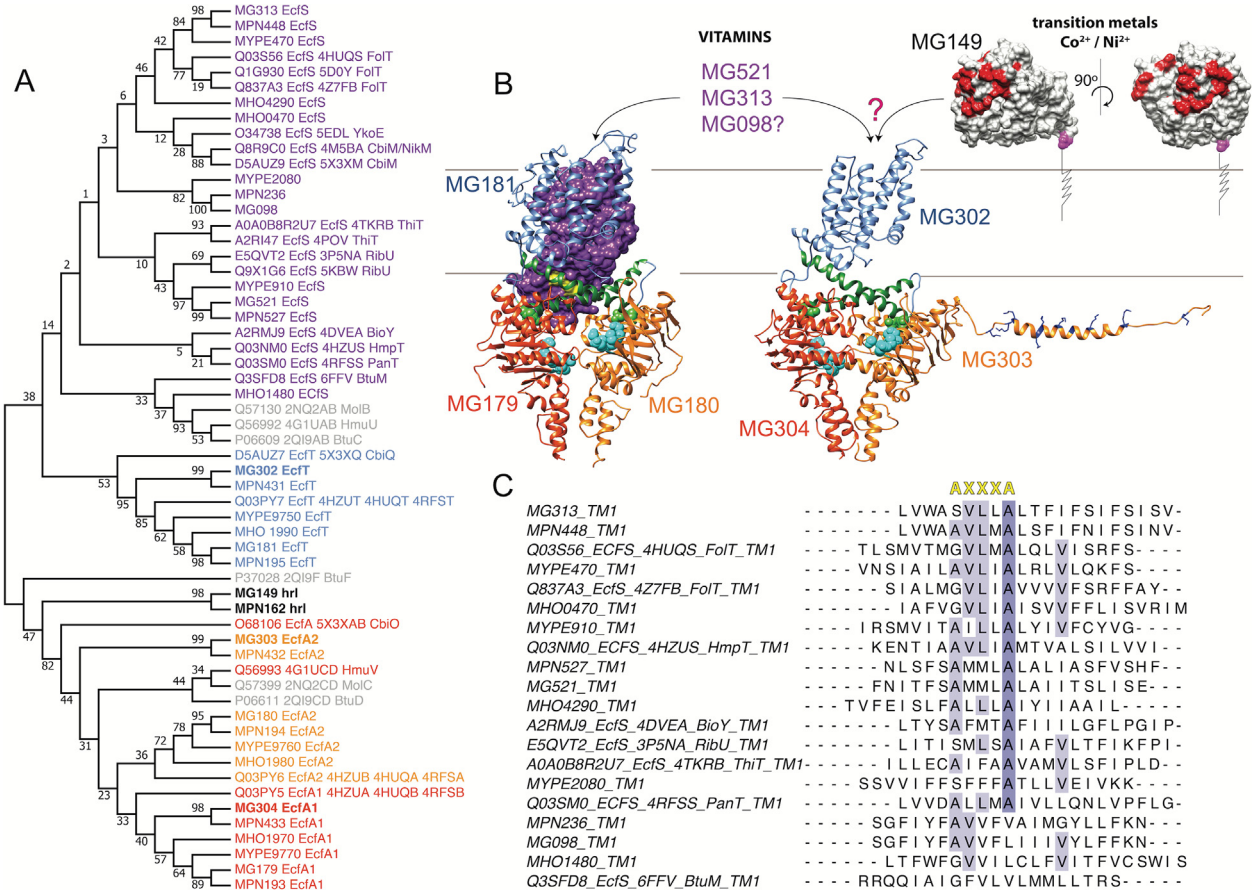

Fig. 2. ECF transporters in human mycoplasmas. A. Phylogenetic tree depicting putative ECF transport proteins in *Mge, Mpn*, *Mho* and *Mpe.* Putative and known EcfS components are represented in purple; EcfT subunits are represented in dark blue; EcfA1 and EcfA2-like subunits are represented in dark and light orange, respectively. The subunits for ABC importers (non-ECF transporters) are indicated in grey. MG149 and MPN162 are in black. The evolutionary history was inferred using the Neighbor-Joining method [77]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [78]. The evolutionary distances were computed using the JTT matrix-based method [79] and are in the units of the number of amino acid substitutions per site. This analysis involved 52 amino acid sequences. All ambiguous positions were removed for each sequence c was a total of 820 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [80,81]. B. Illustrative 3D models for putative ECF transport systems (performed using MODELLER [82] using PDB ID 4huq and 4hzu as templates), following the same color code than in Fig. 2A. Dark green helical ribbons indicate the coupling helices and the green spheres indicate the conserved arginine in the EcfT subunit. The cyan spheres in the EcfA subunits indicate the nucleotide binding site. The lysine-rich (blue residues) N-terminal amphipathic helix in EcfA MG303 is shown. The EcfS is displayed as a purple surface, with the AXXXA motif highlighted in yellow. MG149 without the signal peptide was modelled using I-TASSER [83] and represented as a surface. Red colored residues correspond to histidines, and the magenta residue corresponds to the cysteine harboring the palmitoylation lipid-anchor. The structural models have been represented using UCSF Chimera [84]. C. Alignment of the first transmembrane segments for known and putative S-components to indicate the presence of the AXXXA motif characteristic of group II ECF transporters. The figure was prepared using MEGA X [80,81], MODELLER [82], UCSF Chimera [84] and Adobe Illustrator. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(CbiM or NikM) subunits in group I transporters, exhibit a planar geometry substrate-binding site, which requires an additional N-terminal transmembrane helix, and a strongly conserved N-terminus where a methionine and two histidines are the metal binding residues [72,73]. None of the mycoplasma EcfS subunits show any of these distinctive features. The S-component from group II transporters share low sequence identity, but the first transmembrane segment has a distinctive motif, AXXXA (yellow surface in S-component model in Fig. 2B), which is key for the interaction between the EcfS and EcfT subunits and for the activity of the transporter [68]. Variations of this AXXXA motif have been shown in group II transporters, however the motif is absent in group III transporters. A comparison of the first transmembrane segments of different EcfS subunits is shown in Fig. 2C. Comparing the *Mge, Mpn*, *Mpe* and *Mho* EcfS subunits to known EcfS subunits for group I (CbiM/NikM) and group II (RibU, ThiT, FolT, HmpT, BioY, PanT), the AXXXA motif is present in the MG521/MPN527/MYPE910 homologs, and the MG313/MPN448/MYPE470/MHO4290 homologs (ASXXXA motif variation in MG313), but absent in the MG098/MPN236/MYPE2080/MHO0470 homologs. These observations may indicate that MG313 and MG521 are group II EcfS subunits. However, despite the MYPE2080 annotation, we believe that MG098, MPN236 and MHO0470 do not represent *bona fide* EcfS subunits because the AXXXA motif is absent, the transmembrane topology is diverging from the 6 transmembrane segments fold of S-components, and it shares higher homology with a GatC glutamyl t-RNA amidotransferase (WP_009885655.1, ALA35749.1, BAC43999.1). Besides, we note that *Mho* exhibits an additional protein which resembles S-components (MHO1480) but does not contain the AXXXA motif and is unlikely to constitute a group I/II EcfS protein.

As mentioned above, no iron specific transport systems have been identified in *Mge* and *Mpn*, as opposed to *Mpe* and *Mho*, and the transition metals nickel and cobalt are not utilized in most mollicutes [71,76]. A recent study on *Mge* shows that iron deprivation activates genetic pathways causing an increase of intracellular cobalt and nickel. Among the identified genes involved in these iron-deficiency compensating pathways, the operon MG302-MG303-MG304 encodes an ECF transport system present in *Mge* and *Mpn* (MPN431-MPN432-MPN433) but absent in *Mpe* and

Mho (Fig. 2A). Unlike canonical group II ECF modules which are expressed constitutively [68], the shared ECF module encoded by the MG302-MG303-MG304 operon is overexpressed more than two times under iron deprivation, which is also associated with a nickel (4-fold) and cobalt (2-fold) acquisition increase [24]. This mechanism should be similar in *Mpn*, the only mollicutes that contains this second ECF operon. The putative MG521 S-component is also overexpressed 1.3-fold upon metal depletion [24], but from a basic phylogenetic analysis it is likely a vitamin B_2 importer (Fig. 2A), rather than a transition metal transporter, and it is present in *Mge* (MG521) and *Mpn* (MPN527), but also in *Mpe* (MYPE910) (but not in *Mho*). The MG302-MG303-MG304 (MPN431-MPN432-MPN433) complex seems likely to work together with a protein which is solely present in *Mge* and *Mpn*, but not in *Mpe*. MG149 (MPN162) is a distinctive protein of the minimal genomes of *Mge* and *Mpn*, which shows a 2.1-fold overexpression during iron deprivation [24], and it that has been described as a histidine-rich lipoprotein with a N-terminal signal peptide, which will be anchored to the extracellular side of the membrane through palmitoylation of a cysteine residue (Uniprot entry: P47395). These molecular and structural features resemble the soluble substrate binding protein (SBP) of ABC importers [85], which are not present in ECF transport systems. The histidine-rich feature of MG149, agrees with a Co^{2+}/Ni^{2+} binding protein. As shown in Fig. 2A, MG149 and MPN162 cluster with the SBP subunit BtuF of the *Escherichia coli* vitamin B_{12} (cobalamin) ABC importer BtuCDF.

Animals and rare eukaryotic and prokaryotic microorganisms, such as mycoplasmas, cannot synthesize B-type vitamins and need to obtain them from the environment. It is clear that transporters of B-type vitamins, such as riboflavin, folic acid, and potentially cobalamin are important for mollicutes. The existence of ECF transport complexes, such as the constitutive-type MG179-MG180-MG181 complex together with EcfS subunits (MG313 and MG521) are worth further exploration as potential antimicrobial targets [67]. An important missing link is how the regulated overexpression of the ECF transport complex MG302-303-304 and the histidine-rich lipoprotein MG149 translates into nickel and cobalt intracellular accumulation under iron deprivation.

5. Regulation of metal homeostasis

The fundamental role of metal cations in numerous metabolic pathways dictates the presence of ion-responsive regulatory systems. Studies conducted in the swine pathogen *Mycoplasma hyopneumoniae* [86] and the human pathogen *Mge* [24] have shown that metal starvation triggers a global transcriptional response in mycoplasmas. In these studies, metal deprivation was achieved with the metal chelator 2,2′-bipyridyl (DPP), which binds iron with high affinity. However, likely due to the rich media used to grow these bacteria in axenic culture, iron sequestration in mycoplasmas requires high concentrations of DPP (1 mg/ml) and it can also coordinate other transition metals besides iron. On the other hand, in *Mpn*, changes in gene expression have also been documented upon exposure to the sulfur containing antibiotic thiolutin [25], a potent zinc chelator. Collectively, these studies demonstrate that mycoplasmas avidly respond to fluctuations in metal availability.

When metals are scarce, the expression of several genes coding for predicted ABC transporters and unknown lipoproteins is readily activated in mycoplasmas, which likely increases the uptake capacity of metals and other limiting nutrients [24,86]. Likewise, different genes coding for enzymes implicated in glycerol uptake and metabolism are also upregulated. This fact is remarkable because glycerol metabolism is associated with hydrogen peroxide production, which represents a widespread virulence factor of mycoplasmas [87]. Studies in *Mpn* have demonstrated that the activity of the Glycerophosphodiester phosphodiesterase (GlpQ) is critical for hydrogen peroxide production [88]. Hydrogen peroxide production in this bacterium has been shown to reach maximum levels (9.5 mg/l) when glycerol is available as a carbon source [88]. Of note, the presence of cobalt ions has been shown to increase the activity of cytosolic GlpQ [89]. In this regard, it is tempting to speculate that metal limitation might trigger a virulence response aimed at increasing hydrogen peroxide production and raise the cytotoxic potential of mycoplasmas.

Metal homeostasis is usually facilitated by transcriptional regulators, which operate as intracellular sensors that activate or silence specific sets of genes. The role of a metalloregulator of the Ferric Uptake Regulator (Fur) family (MG236) has been addressed in *Mge* [24]. Regulators of the Fur family are widespread iron-sensing repressors that control the expression of genes implicated in iron transport and storage [90]. The Fur family of metalloregulators include sensors of iron (Fur), zinc (Zur), manganese (Mur), nickel (Nur) and peroxide stress (PerR), among others [91]. Transcriptional analysis of the *Mge fur* mutant revealed the activation of a gene coding for a histidine-rich lipoprotein (Hrl, MG149) and a putative metal transporter annotated as a CbiMNQO uptake system (MG302-MG303-MG304), which are described earlier in this manuscript. Accumulation of nickel ions increases significantly in the *fur* mutant, suggesting a role for the Fur-regulated genes in nickel uptake. In the upstream region of the Fur-regulated genes, a conserved sequence with dyad symmetry implicated in Fur-regulation, was identified [24].

Other mycoplasma species from the pneumoniae group also possess proteins with homology to the *Mge* Fur regulator (Supplementary Fig. S1). The observed homologies vary from that of *Mpn* (MPN329, 75%) to that of *Mpe* (MYPE1200, 50%). Of note, *Mho* seems to be devoid of Fur or other classic metalloregulators. The structure of the Fur proteins from several pathogenic bacteria has revealed the key residues implicated in metal coordination [92–94]. Some of these residues are well-conserved in the *Mge* Fur protein (Supplementary Fig. S1). On the other hand, some Fur proteins contain a zinc structural site important for dimerization [95]. Of note, different lines of evidence indicate that zinc ions regulate Fur activity in *Mge* and *Mpn* [24,25]. The reason why the presence of Fur regulatory proteins seems to be restricted to mycoplasma species of the pneumoniae group is currently unknown. However, other unknown regulatory proteins and riboswitches are likely operating in mycoplasmas, and accordingly, Fur-independent regulation is also prominent [24,86].

In addition to metal transporters and regulatory proteins, ferritins also provide a means to facilitate the homeostasis of iron [96]. Ferritins are iron storage and detoxification proteins that protect the cells by preventing excessive ferrous ions from reacting with hydrogen peroxide, which generates reactive oxygen species. Of note, mycoplasmas are devoid of antioxidant enzymes such as catalases, peroxidases or superoxide dismutases, which likely exacerbates the toxic effect of peroxides. Ferritin-like proteins have been identified in the genomes of *Mpe* [97] and *Mycoplasma iowae* [98], two members of the *Mycoplasma muris* phylogenetic cluster within the pneumoniae group. The presence of iron storage proteins in these two mycoplasma species is puzzling, but suggests that survival of these bacteria within the host rely on the capacity to accumulate iron ions. Perhaps, the presence of pertussis-like toxins in both species [99] allows the release of a large amount of nutrients that function as a reservoir for later use. Frequently, iron storage proteins are under the transcriptional control of regulatory proteins. However, at the present time, it is unknown whether the ferritin of *Mpe* is regulated by the Fur homolog.

Recently, the structure of the *Mpe* ferritin (MYPE2930) has been solved [100]. This ferritin belongs to the canonical ferritin subfamily consisting of 24 subunits, which can coordinate up to 4500 iron ions. Unlike other iron storage proteins, the ferroxidase center of the *Mpe* ferritin is on the inner surface and ferrous ions enter via the B-channels for a slow iron oxidation. The B-channel and the ferroxidase center are negatively charged, creating a fully negatively charged area that allows iron ions to transfer easily. The presence of a ferritin with unique features illustrates the exquisite capacity of mycoplasmas to evolve novel strategies to colonize and persist within the host.

6. Summary and outlook

The capacity to establish persistent infections is a key feature of mycoplasmas. Persistence is supported by multiple factors, including evasion of the immune system, access to the intracellular compartment and effective strategies to obtain essential nutrients within the host. One of the pillars to guarantee the necessary nutrient supply relies on the presence of successful metal acquisition systems. In mycoplasmas, a complete network of dedicated enzymes, transporters and regulators, look out for available metals to scavenge these critical elements from the host. In this report, we present a comprehensive picture of iron utilization and uptake systems in four human mycoplasmas.

Collectively, our analysis reveals two different strategies to deal with iron deprivation in human mycoplasmas. On the one hand, in *Mpe*, the activity of many enzymes relies on iron-sulfur clusters. In consequence, this pathogen displays a putative iron transporter of the ZIP family and a ferritin-like iron storage protein to ensure the supply of this metal. Therefore, a stricter dependence on iron is intimately associated with the presence of effective means to acquire and store iron ions. On the other hand, *Mge*, *Mpn and Mho* seem to be less dependent on iron, as suggested by the promiscuous nature of different metalloenzymes. This notion is further supported by the absence of known high-affinity iron transporters in *Mge* and *Mpn*. Key players in this versatile approach of metal utilization are the putative ECF transport system MG302-303-304 and the histidine-rich lipoprotein Hrl (MG149). Hrl is a protein of unknown function upregulated upon hyperosmotic shock and iron starvation [24,101] and it has been shown to induce a potent pro-inflammatory cytokine response [102,103].

Of note, the identified metal acquisition systems represent potential targets for antimicrobial drug development. This is especially interesting giving the alarming rate of antimicrobial resistance associated to mycoplasma infections, especially because mycoplasmas have no cell wall rendering them naturally resistant to antibiotics targeting cell wall synthesis. Among the first-line therapies for human mycoplasmas are broad-spectrum antibiotics, such as macrolides. Strikingly, macrolide-resistant *Mpn* has become increasingly prevalent worldwide, rising to 100% in Asia [104]. The prevalence of mutations associated with macrolide-resistance in *Mge* is also rapidly increasing globally [105] in line with the observed decrease of macrolide efficacy in the treatment of *Mge* infections [106]. This alarming emergence of antibiotic resistance among mycoplasmas requires that new treatment options are investigated. In this regard, targeting proteins involved in iron acquisition in human mycoplasmas could represent an appealing therapeutical strategy. First, it is anticipated that compounds capable to inhibit the function of metalloenzymes will exhibit antimicrobial activity. Indeed, many patented compounds have been shown to have antibacterial activity because they interact with the metal ion within the active site of the enzymes [107]. For example, PDF has been identified as a promising drug target against several human pathogens including *Mpn* [108]. Although no PDF inhibitor is currently on the market [109], this metalloenzyme is the subject of numerous active investigations [110,111]. In these efforts, homology modeling, combined with additional *in silico* methods, could provide valuable information in the structure-based druggability assessment of a specific target [112]. Another route to be explored in the development of new antimicrobials concerns iron-sulfur proteins. Many of them contribute to bacterial pathogenesis [113] and disrupting the iron-sulfur cluster biogenesis has been shown to affect the survival of several human pathogens [114,115]. Besides, ECF transporters for trace elements, such as vitamins and transition metals, represent attractive antimicrobial targets [67]. In this sense, compounds inhibiting the activity of the histidine-rich lipoprotein Hrl may also prevent *Mge* and *Mpn* growth *in vivo*. Similarly, immunotherapy with antibodies targeting mycoplasmal metal uptake systems could provide alternative therapeutic strategies to combat multiresistant strains.

CRediT authorship contribution statement

Alex Perálvarez-Marín: Writing – original draft, Writing – review & editing. **Eric Baranowski:** Writing – original draft, Writing – review & editing. **Paula Bierge:** Writing – original draft, Writing – review & editing. **Oscar Q. Pich:** Writing – original draft, Writing – review & editing. **Hugo Lebrette:** Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.10.022.

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