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Hydrogen peroxide production and myo-inositol metabolism as important traits for virulence of *Mycoplasma hyopneumoniae*

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Mycoplasma hyopneumoniae is the causative agent of enzootic pneumonia. In our previous work, we reconstructed the metabolic models of this species along with two other mycoplasmas from the respiratory tract of swine: *Mycoplasma hyorhinis*, considered less pathogenic but which nonetheless causes disease and *Mycoplasma flocculare*, a commensal bacterium. We identified metabolic differences that partially explained their different levels of pathogenicity. One important trait was the production of hydrogen peroxide from the glycerol metabolism only in the pathogenic species. Another important feature was a pathway for the metabolism of myo-inositol in *M. hyopneumoniae*. Here, we tested these traits to understand their relation to the different levels of

pathogenicity, comparing not only the species but also pathogenic and attenuated strains of *M. hyopneumoniae*. Regarding the myo-inositol metabolism, we show that only *M. hyopneumoniae* assimilated this carbohydrate and remained viable when myo-inositol was the primary energy source. Strikingly, only the two pathogenic strains of *M. hyopneumoniae* produced hydrogen peroxide in complex medium. We also show that this production was dependent on the presence of glycerol. Although further functional tests are needed, we present in this work two interesting metabolic traits of *M. hyopneumoniae* that might be directly related to its enhanced virulence.

Abbreviated Summary

Virulence and pathogenicity of *Mycoplasma hyopneumoniae*, the causative agent of enzootic pneumonia, has never been fully understood. We present in this work two interesting metabolic traits of *M. hyopneumoniae* that might be directly related to its enhanced virulence, especially regarding its ability to produce hydrogen peroxide.

Keywords

Mycoplasma — Pathogenicity — Glycerol Metabolism — Hydrogen Peroxide Production — Myo-inositol Metabolism — Swine Mycoplasmas — Virulence

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Introduction

The notion that the lungs are sterile is frequently stated in textbooks; however, no modern studies have provided evidence for the absence of microorganisms in this environment [1]. Several bacteria colonize the respiratory tract of swine. *Mycoplasma hyopneumoniae*, *Mycoplasma flocculare*, and *Mycoplasma hyorhinis* are some of the most important species identified so far [2, 3, 4, 5].

M. hyopneumoniae is widespread in pig populations and is the causative agent of enzootic pneumonia [6]; *M. hyorhinis*, although not as pathogenic as *M. hyopneumoniae*, has already been found as the sole causative agent of pneumonia, polyserositis and arthritis in pigs [7, 8, 9, 10]. *M. flocculare*, on the other hand, has high prevalence in swine herds worldwide, but up to now, is still considered a commensal bacterium [7].

Because of the genomic resemblance of these three *Mycoplasma* species [11, 12], it remains unclear why *M. hyopneumoniae* can become highly virulent if compared with the other two. It is also essential to understand that the simple presence or absence of each species is not in itself a determinant factor in the development of enzootic pneumonia: most piglets are thought to be vertically infected with *M. hyopneumoniae* at birth [6, 13, 14] and many can become carriers of the pathogen throughout their entire life without developing acute pneumonia. Moreover, *M. hyopneumoniae* also persists longer in the respiratory tract, either in healthy animals or even after successful treatment of the disease [15, 16, 17, 18].

To make it even more complex, different strains of each species bear different levels (or even lack) of pathogenicity. For instance, *M. hyopneumoniae* has six sequenced strains, two of which are known to be attenuated by culture passages [19, 20]. These strains cannot cause the clinical symptoms of pneumonia *in vivo* and up to now it is not clear why.

In contrast to other pathogenic bacteria, and as revealed by the analysis of the sequenced genomes from several mycoplasmas [21, 22, 23, 24, 12], pathogenic *Mycoplasma* species seem to lack typical primary virulence factors such as toxins, invasins, and cytolysins [25, 26]. For this reason, classical concepts of virulence genes are usually problematic and a broader concept for virulence is used for these species. In this way, a virulence gene in mycoplasmas is described as any non essential gene for *in vitro* conventional growth, which is essential for the optimal survival (colonization, persistence or pathology) inside the host [27].

There have been many different types of virulence factors described so far in several *Mycoplasma* species, most of them related to adhesion [28], invasion [29], cytotoxicity [30, 31], host-evasion [32] and host-immunomodulation [33, 34]. As for *M. hyopneumoniae* and *M. hyorhinitis*, adhesion factors such as antigen

surface proteins and the ability of these organisms to produce a capsular polysaccharide have already been described in the literature [9, 35, 36, 37, 38].

However, while the diseases caused by these swine mycoplasmas have been extensively studied, only recently their metabolism has been explored from a mathematical and computational point of view by our group [39]. We are well aware that metabolism does not fully explain the pathologies caused by either of them. However, adhesion proteins, classically related to virulence in mycoplasmas cannot be associated with the different levels of pathogenicity between *M. hyopneumoniae* and *M. flocculare*. Both species harbor similar sets of adhesion proteins [40] and have been shown to adhere to cilia in a similar way [41]. Thus, it remains unclear what prevents *M. flocculare* to cause disease in this context.

In our previous work [39], we compared the reconstructed metabolic models of these three *Mycoplasma* species, and pointed out important metabolic differences that could partly explain the different levels of pathogenicity between the three species. The most important trait was related to the glycerol metabolism, more specifically the turnover of glycerol-3-phosphate into dihydroxyacetone-phosphate (DHAP) by the action of glycerol-3-phosphate oxidase (GlpO, EC 1.1.3.21), which was only present in the genomes of *M. hyorhinis* and *M. hyopneumoniae*. This would allow the usage of glycerol as a primary energy source, with the production of highly toxic hydrogen peroxide in the presence of molecular oxygen. The metabolism of glycerol and the subsequent production of hydrogen peroxide by the action of GlpO are essential for the cytotoxicity of lung pathogens *Mycoplasma pneumoniae* [31] and *Mycoplasma mycoides* subsp. *mycoides* [30]. Moreover, the *Mycoplasma hominis* group is not the only one where hydrogen peroxide production via *glpO* has been reported. In some *Spiroplasma* species (specifically *Spiroplasma taiwanense*) and within the *pneumoniae* group (for instance in *Mycoplasma*

penetrans), the presence of this enzyme was also associated with virulence [42, 43].

Another major difference between our previous models was related to the presence of a complete transcriptional unit (TU) encoding proteins for the uptake and metabolism of myo-inositol in *M. hyopneumoniae* (with the exception of one enzyme). This could be another important trait for the enhanced virulence of this species if compared with the other two. Here, we studied this pathway in more detail to try to find this missing enzyme and the possible reasons as to why natural selection kept these genes only in this *Mycoplasma* species.

In a recent review, Maes and collaborators [26] emphasize the need for the further investigation of the role of glycerol and myo-inositol metabolism and their contribution to virulence in *M. hyopneumoniae*. Here, we experimentally tested these two traits to show how they might be related to the different levels of pathogenicity, by comparing not only the species themselves but different strains of *M. hyopneumoniae*. Contrary to what we anticipated, only the two pathogenic strains of *M. hyopneumoniae* were able to produce hydrogen peroxide in complex medium, and we confirmed that this production was dependent on the presence of glycerol. The myo-inositol metabolism, in turn, was tested with the aid of deuterated myo-inositol in Friis medium. We were able to detect by mass spectrometry (MS) a slight decrease in the marked myo-inositol concentration throughout time, indicating the ability of *M. hyopneumoniae* to uptake such carbohydrate. We also show here that only the *M. hyopneumoniae* strains remained viable when myo-inositol was the primary energy source.

We present here two metabolic traits specific to *M. hyopneumoniae* that might be directly related to its enhanced virulence, specially in its ability to successfully overgrow the other two *Mycoplasma* species in the respiratory tract of swine, persist longer in this environment and possibly cause disease.

1 Results

1.1 Comparative genomics of *glpO* from glycerol metabolism

Highly conserved homolog genes to *glpO* from *M. mycoides* subsp. *mycoides* (EC 1.1.3.21) were found only in the genomes of *M. hyopneumoniae* and *M. hyorhinis*. Despite the annotation as a dehydrogenase in both *M. hyopneumoniae* and *M. hyorhinis*, we propose this enzyme to act as glycerol-3-phosphate oxidase (GlpO), using molecular oxygen as the final electron acceptor and producing DHAP and hydrogen peroxide. We therefore refer to the encoded protein in *M. hyopneumoniae* and *M. hyorhinis* as GlpO, rather than GlpD. The high similarity between these predicted proteins (Supplementary Figure S1A) may be an indication that this trait might be essential for the pathogenicity of these *Mycoplasma* species.

Particularly, the cytotoxicity of *M. mycoides* subsp. *mycoides* is considered to be related to the translocation of the hydrogen peroxide into the host cells [44]. This is presumably possible because of the close proximity to the host cells along with the integral membrane GlpO [25, 45]. Different transmembrane prediction softwares [46, 47, 48, 49] identified putative transmembrane portions in the GlpO proteins from *M. hyopneumoniae* and *M. hyorhinis* (Supplementary Figure S1B). Similar results were reported for the homolog enzyme in *M. mycoides* subsp. *mycoides* [25], and a recent proteomic study has detected GlpO from *M. hyopneumoniae* in surface-enriched extracts through LC-MS/MS (Personal communication from H. B. Ferreira, [50]).

1.2 Pathogenic *M. hyopneumoniae* strains produce hydrogen peroxide from glycerol

Contrary to what we had anticipated, we were only able to detect the production of hydrogen peroxide from the two pathogenic strains of *M. hyopneumoniae* (7448 and 7422) in Friis medium, as can be seen in Figure 1A. The attenuated strain from the same species (*M. hyopneumoniae* strain J), along with *M. hyorhinis* and *M. flocculare* did not produce detectable quantities of this toxic

product. In order to verify if the amount of hydrogen peroxide produced was comparable between strains, we also counted the number of cells for each replicate. In this way, the two pathogenic strains produced approximately the same amount of hydrogen peroxide and had cell counts of the same order of magnitude (available in Supplementary Table S1).

We also show (Figure 1B) that the hydrogen peroxide produced by the *M. hyopneumoniae* strains 7448 and 7422 was dependent on the presence of glycerol in the incubation buffer.

1.3 Levels of *glpO* transcripts do not differ from pathogenic to attenuated strains of *M. hyopneumoniae*

We tested the three *M. hyopneumoniae* strains (7448, 7422 and J) in order to compare the mRNA expression levels of *glpO* gene by RT-qPCR. Since the transcript levels of normalizer genes were not comparable between strains, we used relative quantification normalized against unit mass; in our case, the initial amount of RNA. We chose one of the replicates from strain 7448 as the calibrator, and we were able to show (Figure 2 and Supplementary Table S2) that there was no significant difference in the transcript levels of *glpO* in all tested strains from *M. hyopneumoniae*.

1.4 Enzymes for the uptake and catabolism of myo-inositol are specific to *M. hyopneumoniae* strains

M. hyopneumoniae is the only reported species among the Mollicutes that contains genes involved in the catabolism of myo-inositol. Since *Mycoplasma* species seem to maintain a minimum set of essential metabolic capabilities, we decided to further investigate this pathway and the influence of its presence on the metabolism and pathogenicity of *M. hyopneumoniae*. The degradation of inositol can feed glycolysis with DHAP and also produces an acetyl coenzyme-A (AcCoA) (Figure 3). A TU for the myo-inositol catabolism is present in all *M. hyopneumoniae* strains, with the exception of the gene that codes for the enzyme

6-phospho-5-dehydro-2-deoxy-D-gluconate aldolase (lolJ, EC 4.1.2.29), responsible for the turnover of 6-phospho-5-dehydro-2-deoxy-D-gluconate (DKGP) into malonate semialdehyde (MSA).

The gene encoding lolJ in other organisms is similar to the one coding for enzyme fructose-bisphosphate aldolase (Fba) from glycolysis (EC 4.1.2.13). There are two annotated copies of the gene *fba* in *M. hyopneumoniae* (*fba* and *fba-1*, Supplementary Table S3). We performed homology and gene context analyses, 3D comparative modeling and protein-ligand interaction analysis to check if either of them would be a suitable candidate for this activity.

The gene context and protein sequence alignment for 15 selected Fba homologs in Mollicutes can be seen in Supplementary Figures S2 and S3. Comparative models for both copies of Fba from *M. hyopneumoniae* and previously characterized lolJ and Fba from *Bacillus subtilis* [51] were constructed based on available structures of Fba in PDB [52] (Figure 4 and Supplementary Table S4). Fba structures from *Escherichia coli* and *Giardia intestinalis* were used to gather more information about substrate binding (Supplementary Figure S3). The alignment shows a highly conserved zinc binding site (residues marked as ‘*’), essential for substrate binding and catalysis. Positions ‘a’, ‘b’, ‘c’, ‘d’ and ‘e’ surround the substrate cavity. The structural analysis suggests that the interaction mode of DKGP (substrate of lolJ) with the zinc ion of the active site is similar to that observed for FBP (fructose-1,6-bisphosphate, substrate of Fba). Nevertheless the substrate specificity is strongly dependent on the residues that form the substrate cavity.

While there seems to be several common features between Fba and lolJ (residues ‘c’, ‘d’, ‘e’ and ‘*’), residue ‘a’ appears to be essential for the substrate interaction with lolJ. This residue is generally occupied by an arginine (R52) in several putative lolJs from other organisms (Supplementary Figure S4), and absent in all predicted Fbas analysed in this study. From the predicted structures,

the presence of this positively charged arginine in lolJ seems to disfavour the interaction with the phosphate group of FBP whilst it is complementary to the carboxyl group from DKGP.

In this way, the predicted structure of Fba-1 from *M. hyopneumoniae* resembles more the Fba structures from the experimentally solved Fbas in *B. subtilis*, *E. coli* and *G. intestinalis*. The annotated Fba from *M. hyopneumoniae*, on the other hand, seems to be more similar to the lolJ structure from *B. subtilis*. Although functional studies are needed to test this hypothesis, we propose that all enzymes needed for the myo-inositol catabolism are present in *M. hyopneumoniae*.

1.5 *M. hyopneumoniae* is able to uptake myo-inositol from the culture medium

In order to ascertain the ability of different bacteria to uptake myo-inositol, we used two different approaches. The first was the use of marked myo-inositol in complex medium and analysis by MS, and the second was to check the viability of cells (through ATP production) whenever myo-inositol was used as primary energy source.

When we tested if cells were able to uptake the marked myo-inositol, over the course of 48 h, we found no significant difference in *M. flocculare* and *M. hyorhinis* when compared to the control medium (CTRL), as observed in Figure 5A. As expected, the concentrations of myo-inositol for both strains of *M. hyopneumoniae* after 48 h of growth were lower than the control medium. We also collected two extra time points for *M. hyopneumoniae* strain 7448 and CTRL: 8 h and 24 h of growth (Figure 5B). In all time points, there is significant difference between the residual marked myo-inositol and the control medium, which implies that *M. hyopneumoniae* is able to uptake such carbohydrate from the medium. MS peak data is available in Supplementary Table S5.

Since we had glucose and glycerol present in this complex medium analysed by MS, we also wanted to check if the viability of the different strains and species altered when myo-inositol was the primary energy source. For this, we incubated cells in myo-inositol defined medium (depleted with glucose and glycerol) for 8 hours and measured the amount of ATP these cells were able to produce. This in turn was directly related to the amount of viable cells after cultivation in the specific medium tested. Considering we do not know the energetic yield and efficiency of each strain and species, we could not directly compare the amount of ATP produced between different organisms. For this reason, growth in regular defined medium (with glucose) for each strain was used as a normalization control and the ratio of ATP production in both media was used to compare the viable cells between strains. Since there was no other energy source available in the medium and in accordance with our previous predictions and results, only *M. hyopneumoniae* cells remained viable (ranging from 75% to 280%) when compared to their control growth in the regular defined medium (Figure 6 and Supplementary Table S6). The viability of the other species in this medium was 11.5% for *M. hyorhinis* and 0.2% for *M. flocculare*. We also achieved similar results when comparing the growth in myo-inositol defined medium versus Friis medium (Supplementary Figure S5).

2 Discussion

In this study, we wanted to find possible differences between pathogenic and attenuated strains of *M. hyopneumoniae* and also compare them with *M. hyorhinis* and *M. flocculare* and assess possible links to the enhanced virulence of *M. hyopneumoniae*. While *M. hyopneumoniae* strains 7422 and 7448 are considered pathogenic, strain J became attenuated after serial passages of *in vitro* culture; *M. hyorhinis* strain ATCC 17981 was isolated from swine but, to our knowledge, its level of pathogenicity has not been tested *in vivo*; and even though *M. flocculare* is not considered pathogenic, strain ATCC 27399 was isolated from a case of swine pneumonia (strain ATCC 27716 is derived from this

strain). In our previous study [39], through mathematical modeling, we predicted two traits of *M. hyopneumoniae in silico* that could be associated with its enhanced virulence: the myo-inositol catabolism and the link between the glycerol and the glycolysis metabolism, with the production of highly toxic hydrogen peroxide (by the activity of the GlpO enzyme). In this work, we tested whether these species indeed differed from each other regarding their ability (i) to produce hydrogen peroxide *in vitro* and whether this was related to the availability of glycerol, (ii) to uptake myo-inositol, and (iii) to remain viable in a defined medium with myo-inositol as the primary energy source. While the uptake of myo-inositol might be a general feature of *M. hyopneumoniae*, the production of hydrogen peroxide in complex medium seems to be specific to pathogenic strains of this species.

Glycerol metabolism and hydrogen peroxide production

Even though the GlpO enzyme was previously detected in proteomes from both pathogenic and attenuated strains of *M. hyopneumoniae* (232 and J) [53, 54], only the pathogenic strains tested in our study (7448 and 7422) were able to produce detectable amounts of hydrogen peroxide in Friis medium (Figure 1). To our knowledge, no other study up to now was able to show that *M. hyopneumoniae* strains were able to produce this toxic product *in vitro* [26]. We also show here that the production of hydrogen peroxide in the pathogenic strains of *M. hyopneumoniae* is dependent on the presence of glycerol (Figure 1B).

The metabolism of glycerol and the formation of hydrogen peroxide were described as essential for the cytotoxicity of lung pathogens *M. mycoides* subsp. *mycoides* [30] and *M. pneumoniae* [31]. Moreover, although both *M. hyopneumoniae* and *M. flocculare* can adhere to the cilia of tracheal epithelial cells in a similar way, only the adhesion of *M. hyopneumoniae* causes tissue damage [41].

We showed that the difference in enzyme activity was not related to the expression levels of *glpO* gene from the strains tested (Figure 2). We did not find any extreme differences in their aminoacid sequences either (Supplementary Figure S3). This could be an indication that either this enzyme undergoes post-translational modifications in order to be active and/or the availability of the substrate (glycerol) intracellularly might be a limiting step for its activity. Post-translational modifications have been extensively reported experimentally in several proteins of *M. hyopneumoniae* [37, 55, 56, 57, 58]. From transcriptomic and proteomic literature data, we were not able to find any enlightening differences in this pathway between strains or species (Supplementary Table S7).

As for the availability of intracellular glycerol, in our previous metabolic models, we predicted differences in the metabolism of glycerol among the three *Mycoplasma* species (Supplementary Figure S6). While *M. hyopneumoniae* has five different ways of uptaking glycerol (dehydrogenation of glyceraldehyde, ABC transport of glycerol and glycerol-phosphate, import of glycerophosphoglycerol, and glycerophosphocholine), the other two species lack at least two reactions. This might also limit the rate of production of hydrogen peroxide in each species. In this way, the enhanced pathogenicity of *M. hyopneumoniae* over *M. hyorhinis* and *M. flocculare* may therefore also be due to hydrogen peroxide formation resulting from a higher uptake of glycerol as an energy source. Similarly, one reason that could partially explain why *M. mycoides* subsp. *mycoides* is highly pathogenic in comparison with the less pathogenic *M. pneumoniae* might be the greater intracellular availability of glycerol due to the presence of a specific and very efficient ABC transporter in *M. mycoides* subsp. *mycoides*.

Since the production of hydrogen peroxide was not reported as essential to the *in vivo* virulence of *Mycoplasma gallisepticum* [59], more studies are needed to better understand the importance of this metabolism in *M. hyopneumoniae*. Moreover, future biochemical and functional studies are needed to prove that

GlpO is indeed responsible for the activity proposed here and to check if the enzyme in attenuated strains/species is functional.

Myo-inositol uptake and catabolism

M. hyopneumoniae is the only *Mycoplasma* species with sequenced genome that has the genes for the catabolism of myo-inositol. Myo-inositol is an essential precursor for the production of inositol phosphates and inositol phospholipids in all eukaryotes [60]. Myo-inositol is also widespread in the bloodstream of mammals [61], which would make it a suitable energy source for bacteria in the extremely vascularized respiratory system. Previously, *Mycoplasma iguanae* was described to produce acid from inositol [62], but the methods used in that paper are not clear and there is no complete genome from this organism for us to draw any conclusions. Based on sequence homology, orthology, synteny and tridimensional analyses, we proposed a possible candidate for the missing enzyme lolJ in *M. hyopneumoniae*, namely a duplication of the *fba* gene from glycolysis. This functional divergence after duplication is particularly interesting in bacteria for which evolution was mostly driven by genome reduction. Another reported example of this event is the duplication of the *trmFO* gene in *Mycoplasma capricolum* and more recently in *Mycoplasma bovis*. The duplicated TrmFO in *M. capricolum* was reported to catalyze the methylation of 23S rRNA [63] while the duplicated copy in *M. bovis* has been described to act as a fibronectin-binding adhesin [64].

We showed here that *M. hyopneumoniae* was able to uptake marked myo-inositol from a complex culture medium (Figure 5); in addition this was the only species that remained viable whenever myo-inositol was used as the primary energy source (Figure 6). From our metabolic model predictions [39], the use of myo-inositol would be much more costly than the uptake and metabolism of glucose, which corroborates the small uptake of myo-inositol in Friis medium (glucose-rich) (Figure 5). This basal uptake of myo-inositol could also be an indication that this pathway is important not only for energetic yield. Supporting

this idea, microarray studies on strain 232 showed that several genes (if not all) from the myo-inositol catabolism were differentially expressed during stress treatments: heat shock (downregulated) [65], iron depletion (upregulated) [66], and norepinephrine (downregulated) [67]. Moreover, a previous transcriptome profiling of *M. hyopneumoniae* [40] showed that all genes from the myo-inositol catabolism were transcribed under normal culture conditions. Furthermore, three genes from the pathway (*iolB*, *iolC* and *iolA*) belonged to the list of the 20 genes with the highest number of transcript reads. Besides the transcription of these genes, proteomic studies of *M. hyopneumoniae* strains 232 [54], 7422, 7448 and J [53, 68] (Supplementary Table S7) showed that several enzymes from this pathway were present in normal culture conditions.

Indeed, myo-inositol has been extensively reported in several organisms as a signaling molecule [69, 70]. Moreover, the myo-inositol catabolism has been experimentally described as a key pathway for competitive host nodulation in the plant symbiont and nitrogen-fixing bacterium *Sinorhizobium meliloti* [71]. Host nodulation is a specific symbiotic event between a host plant and a bacterium. Kohler and collaborators (2010) showed that whenever inositol catabolism is disrupted (by single gene knockouts from the inositol operon), the mutants are outcompeted by the wild type for nodule occupancy. This means that genes for the catabolism of inositol are required for a successful competition in this particular symbiosis. Moreover, the authors were not able to find a suitable candidate for the lolJ activity. In our case, we proposed that the activity of the missing enzyme lolJ is taken over by a duplication of *fbA*. We were able to find a similar duplication (also not inside the myo-inositol cluster) in the genome of *S. meliloti* 1021 (SM_b21192 and SM_b20199, both annotated as fructose-bisphosphate-aldolase, EC 4.1.2.13). This means that in at least one other symbiont that has the myo-inositol catabolism genes, there could exist a putative lolJ not close to the myo-inositol cluster, just as we proposed here.

Whether this entire pathway is functional in *M. hyopneumoniae* is yet to be tested and further experiments should take place to support this hypothesis. However, the ability of *M. hyopneumoniae* to persist longer in the swine lung if compared to the other two mycoplasmas might come from the fact that this species is able to uptake and process myo-inositol. Furthermore, the ability of *M. hyopneumoniae* to grow in diverse sites [72] if compared to *M. flocculare* might also be due to this specific trait.

Concluding remarks

It is important to remember that even though *M. hyopneumoniae* is considered highly pathogenic, the three *Mycoplasma* species studied here are widespread in pig populations and can easily be found in healthy hosts [17, 73]. However, the main question permeating this fact is: what causes the switch from a non-pathogenic *Mycoplasma* community to a pathogenic one? And what makes some strains pathogenic while others inflict no harm to the host cells?

Some strains of *M. hyopneumoniae* become less pathogenic in broth culture and, after serial passages, they lose their ability to produce gross pneumonia in pigs [9]. In a proteomic study comparing strains 232 and J, researchers have described that the attenuated strain J switches its focus to metabolism and therefore has developed better capabilities to profit from the rich culture medium while the ability to infect host cells becomes less important so that adhesion-related genes are downregulated [74]. This might be related to the fact that here we detected a higher production of ATP in this attenuated strain when compared to the pathogenic strains 7448 and 7422. Liu and collaborators [20] have investigated genetic variations between *M. hyopneumoniae* strains 168 and attenuated 168-L and found out that almost all reported *Mycoplasma* adhesins were affected by mutations. Tajima and Yagihashi [35] reported that capsular polysaccharides from *M. hyopneumoniae* play a key role in the interaction between pathogen and host. Indeed in several bacterial species it has been reported that the amount of capsular polysaccharide is a major factor in their

virulence [75] and it decreases significantly with *in vitro* passages [76]. In this way, it is likely that the difference in pathogenicity between strains in *M. hyopneumoniae* does not solely depend on their metabolism, but also on their ability to adhere to the host.

A recent metagenomic analysis of community composition [5] has described that *M. hyopneumoniae* is by far the most prevalent species in both healthy and diseased hosts. The difficult isolation of *Mycoplasma* species from diseased lung extracts is due to the fact that, in culture, fast-growing bacteria will overcome the slow-growth of mycoplasmas [77]. This means that, *in vitro*, the competition for an energy source between fast and slow-growing bacteria usually ends with an overpopulation of the fast growing ones. Given the fact that mycoplasmas survive for longer periods inside the host even in competition with other bacteria [17, 18], we must assume that other factors exist and are usually not mimicked in cell culture.

While *M. hyopneumoniae* might cause no harm, depending mostly on the environment, the characteristics of the host, and the composition of this dynamic lung microbiome, any unbalance in this system is probably capable of turning a non-pathogenic community into a pathogenic one. The final conclusion is that the disease is a multifactorial process depending on several elements that include intra-species mechanisms, community composition, host susceptibility and environmental factors. One possibility is that the competition with fast-growing species could result in a lower carbohydrate concentration and that *M. hyopneumoniae* might have to overcome this environmental starvation with the uptake of glycerol or myo-inositol. Since the uptake of myo-inositol does not lead to the production of any toxic metabolite, it is more interesting for its persistence in the long run. Other bacteria will strongly compete for glucose and other related carbohydrates, while *M. hyopneumoniae* will have the entire supply of myo-inositol for itself. The uptake of glycerol as an energy source, on the other hand, will probably lead to the production of toxic hydrogen peroxide as reported in

other *Mycoplasma* species. This toxic product combined with other toxins from the external bacteria in the system would most probably recruit immune system effectors. Since *M. hyopneumoniae* has efficient mechanisms of host evasion [17, 26], the newly introduced and fast-growing bacteria might be eliminated faster and *M. hyopneumoniae*, in this way, would be able to persist longer than other species inside the host (as it is reported *in vivo*).

As mentioned before, virulence factors in *Mycoplasma* species cover a broader concept if compared to other species: they are genes not essential for *in vitro* conventional growth that are instead essential for optimal survival *in vivo*. From our *M. hyopneumoniae* metabolic models, neither the GlpO activity nor the uptake and metabolism of myo-inositol seem to be essential features for *in vitro* growth. However, we were able to show that they might be two metabolic traits important for the enhanced virulence of *M. hyopneumoniae* when compared to *M. hyorhinis* and *M. flocculare* and could be essential for its survival *in vivo* and directly affect its pathogenicity.

3 Experimental Procedures

3.1 *Mycoplasma* cultivation

We used the following strains for experimental validation: *M. hyopneumoniae* strains 7448, 7422 (field isolates) and J (ATCC 25934), *M. hyorhinis* ATCC 17981 and *M. flocculare* ATCC 27716. Cells were cultivated in Friis medium [78] at 37 °C for varying periods of time with gentle agitation in a roller drum.

3.2 Hydrogen peroxide detection

Hydrogen peroxide was detected in culture medium by the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen Cat. No A22188), according to the manufacturer's manual. *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* were cultivated for 48 h in modified Friis medium (with no Phenol Red) and thereafter centrifuged. The supernatant was used for the hydrogen peroxide

readings compared to a standard curve (Supplementary Figure S7). The medium without bacterial growth was used as negative control. We used biological and technical triplicates to infer the average amount of hydrogen peroxide produced, and the concentration was standardized based on the average number of cells from each culture. Statistical analyses were performed using GraphPad Prism 6 software by one-way ANOVA followed by Dunnett's multiple comparison test considering *M. flocculare* as a control ($p < 0.05$).

In order to determine if the hydrogen peroxide production was dependent on the glycerol metabolism, we used the Merckoquant Peroxide Test (Merck Cat. No 110011) with detection range of 0.5 to 25 μg of peroxide per mL of solution (as described in [31]). Fifteen mL of *M. hyopneumoniae* 7448 and 7422 strains were grown for 48 h in Friis medium, harvested by centrifugation at 3360 g and washed twice in the incubation buffer (67.7 mM HEPES pH 7.3, 140 mM NaCl, 7 mM MgCl_2). Cells were resuspended in 4 mL of incubation buffer and aliquots of 1 mL were incubated for 1 h at 37 °C. To induce hydrogen peroxide production, either glycerol or glucose (final concentration 100 μM or 1 mM) was added to the cell suspension and samples were incubated at 37 °C for additional 2 h. Hydrogen peroxide levels were measured using colorimetric strips according to the manufacturer's instructions. Aliquots without any added carbon source served as an incubation control. The statistical significance of the results was calculated using one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$). The results represent four biological replicates with at least two technical replicates each.

3.3 Mycoplasma cell count with flow cytometry

Mycoplasma cells cultivated for hydrogen peroxide detection were sedimented at 3360 g for 20 min at 4 °C and washed three times with NaCl 0,9% (1x 3360 g for 20 min and 2x 3360 g for 4 min). Cells were resuspended in 1 mL of NaCl 0,9% and diluted 1:30 for flow cytometry readings in a Guava EasyCyte cytometer (Millipore, USA). Cells were characterized by side-angle scatter (SSC) and

forward-angle scatter (FSC) in a four-decade logarithmic scale. Absolute cell counting was performed up to 5000 events and the samples were diluted until the cell concentration was below 500 cells/ μL . The number of counts obtained was then converted to cells/mL.

3.4 Transcript levels of *glpO* with the use of real-time quantitative RT-PCR

Total RNA was isolated from 20 mL culture of *M. hyopneumoniae* strains 7448, 7422 and J grown at 37 °C for 24 h. Cells were harvested by centrifugation at 3360 *g* for 15 min, resuspended in 1mL of TRizol (Invitrogen, USA) and processed according to the manufacturer's instructions followed by DNA digestion with 50 U of DNaseI (Fermentas, USA). Absence of DNA in the RNA preparations was monitored by PCR assays. The extracted RNA was analysed by gel electrophoresis and quantified with the Qubit™ system (Invitrogen, USA). A first-strand cDNA synthesis reaction was conducted by adding 500 ng of total RNA to 500 ng of pd(N)6 random hexamer (Promega, USA) and 10 mM deoxynucleotide triphosphates. The mixture was heated for 65 °C for 5 min and then incubated on ice for 5 min. First-strand buffer (Invitrogen, USA), 0.1 M dithiothreitol and 200 U M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase – Invitrogen, USA) were then added to a total volume of 20 μL . The reaction was incubated at 25 °C for 10 min and at 37 °C for 50 min followed by 15 min at 70 °C for enzyme inactivation. A negative control was prepared in parallel, differing only by the absence of the RT enzyme. Quantitative PCR (qPCR) assay was performed using 1:2.5 cDNA as template and Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen, USA) with specific primers for *glpO* (5'GGTCGGGAACCTGCTAAAGC3' and 5'CCAGACGGAAACATCTTAGTTGG3') on StepOne Real-Time PCR Systems (Applied Biosystems, USA). The qPCR reactions were carried out at 90 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A melting curve analysis was done to verify the specificity of the synthesized products and the absence of primer dimers. The amplification efficiency was

calculated with the LinRegPCR software application [79]. A relative quantification normalized against unit mass (500 ng of total RNA) was used to analyse the expression data with the equation: $Ratio(test / calibrator) = 2^{\Delta CT}$, where $\Delta CT = CT_{test} - CT_{calibrator}$ [80] and MHP_7448 (Replicate 2) was chosen as calibrator. Statistical analyses were performed using GraphPad Prism 6 software by one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

3.5 Comparative modeling and protein-ligand interaction analysis of Fba and lolJ

The SWISS-MODEL server [81, 82] was used for template search and the comparative modeling for all Fba and lolJ proteins in this study. The best homology models were selected according to coverage, sequence identity, Global Model Quality Estimation (GMQE) and QMEAN statistical parameters [83, 84]. The Fba from *M. hyopneumoniae* along with lolJ and Fba from *B. subtilis* were modeled using the crystal structure of fructose 1,6-bisphosphate aldolase from *Bacillus anthracis* in complex with 1,3-dihydroxyacetonephosphate (PDB 3Q94) while Fba-1 from *M. hyopneumoniae* was modeled using the fructose-1,6-bisphosphate aldolase from *Helicobacter pylori* in complex with phosphoglycolohydroxamic acid (PDB 3C52). Both selected templates have the same resolution range (2.30Å). Fba structures experimentally solved from *E. coli* [85] and *G. intestinalis* [86] were used to include information about substrate binding in the active site. The DKGP and FBP ligands were drawn in the Avogadro version 1.1.1 [87] by editing the tagatose-1,6-bisphosphate (TBP) molecule complexed with the Fba structure of *G. intestinalis* (PDB 3GAY). Each model was submitted to 500 steps of an energy minimization protocol using the universal force field (UFF). The DKGP and FBP molecules were inserted into the substrate binding sites of the acquisition models obtained by superposition of the models with the Fba structure of *G. intestinalis*.

3.6 Detection of marked myo-inositol through mass spectrometry

Solvents and Reagents

Acetonitrile and formic acid (Optima LC/MS Grade) were purchased from Fisher Scientific (Loughborough, UK). MilliQ water was obtained from a Direct-Q 5UV system (Merck Millipore, Billerica, Massachusetts, USA). Deuterated myo-inositol-1,2,3,4,5,6- d_6 was purchased from CIL (C/D/N Isotopes Inc. Cat No. D-3019, Canada).

Cultivation in the presence of marked myo-inositol

Cells were cultivated in Friis medium supplemented with 0.25 g/L of deuterated myo-inositol-1,2,3,4,5,6- d_6 (C/D/N Isotopes Inc. Cat No. D-3019). Cultures were interrupted after 8 h, 24 h and 48 h of cultivation for mass spectrometry analysis.

Sample preparation

All samples were filtered and concentrated with the use of Amicon Ultra 3 kDa (Merck Millipore Cat. No. UFC200324). After this step, samples were dried in a miVac sample concentrator (Genevac, Ipswich, UK) for approximately 45 min at 50 °C. All samples were resuspended in ultra pure water to a final concentration of 10 g/L and were subsequently submitted to mass spectrometry.

Mass spectrometry

Aqueous extracts of *Mycoplasma* sp. and commercial deuterated myo-inositol-1,2,3,4,5,6- d_6 were analysed using an Accurate-Mass Q-TOF LCMS 6530 with LC 1290 Infinity system and Poroshell 120 Hilic column (3x100 mm, 2.7 μ m) (Agilent Technologies, Santa Clara, USA). The extracts were dissolved in water (10 g/L) and injection volume was 3 μ L. A binary mobile phase system (A: 0.4% formic acid in milliQ-water and B: acetonitrile) was pumped at a flow rate of 0.9 mL/min at the following gradient: 0-3.5 min, 90% B; 3.5-7 min, 90% to 0% B; 7-9.5 min, 0% B; 9.5-10 min 0% to 90% B; 10-15 min, 90% B (total run: 15 min). MS and MS/MS spectra were obtained in negative mode, with the following conditions: nebulization gas (nitrogen) at 310 °C, at a flow of 10 L/min and 40 psg

pressure. The capillary tension was 3600 V and gave ionisation energy of 100 eV. In targeted MS/MS mode, collision energy was set at 18 eV. Acquisition range was m/z 50-500. MassHunter Qualitative Analysis Software (version B.07.00) was used for data analysis.

Data analysis

Deuterated myo-inositol-1,2,3,4,5,6- d_6 was quantified in all aqueous extracts by HPLC-MS. For that, a calibration curve (based on peak area) of a commercial myo-inositol was performed from 0.001 g/L to 0.05 g/L in replicate (4 times during the batch analysis). Statistical analyses were performed using GraphPad Prism 6 software. One-way ANOVA followed by Dunnett's multiple comparison test was used to test for differences in residual marked myo-inositol in culture after bacterial growth of all tested strains for 48 h ($p < 0.05$). A two-tailed unpaired t-test was used to compare the residual marked myo-inositol between *M. hyopneumoniae* 7448 and the control medium with two extra timepoints: 8 and 24 h ($p < 0.05$).

3.7 Determination of cell viability of *M. hyopneumoniae* in myo-inositol defined medium

All available strains were grown in Friis medium at 37 °C for 48 h, sedimented by centrifugation at 3360 *g* for 20 min at 4 °C, washed twice with ice cold PBS and inoculated in glucose regular defined medium (described in [39], supplemented with 5 g/L of succinate) or myo-inositol defined medium (regular defined medium depleted with glucose and glycerol and supplemented with 0.5 g/L of myo-inositol). Viability of cells was measured by ATP production with live cells recovered after 8 h of growth in either media with a BacTiter-Glo™ Microbial Cell Viability Assay Kit (Promega, USA) according to the manufacturer's manual. Luminescence was recorded in a SpectraMax MiniMax 300 Imaging Cytometer (Molecular Devices, USA) with an integration time of 0.5 s in an opaque-walled multiwell plate. Average ATP production was calculated with biological duplicates and technical triplicates. The ATP production of each strain was compared

between regular defined medium and myo-inositol defined medium to determine the ratio of viable cells and to allow a comparison between strains. A 10-fold serial dilution of ATP was used as a standard curve (Supplementary Figure S8). Statistical analyses were performed using GraphPad Prism 6 software by one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

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Authors' contributions

MGF, SGM, MFS and AZ conceived and designed the work. MGF and SGM performed most of the experimental work. DP, GM and GC collaborated in the mass spectrometry experiments and analysis. JFB performed tridimensional analysis of proteins. All authors collaborated in the analysis of all data. MGF and SGM wrote the manuscript with inputs from the other authors. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Supplementary material

Figure S1: A. Comparative genomics of GlpO. B. Transmembrane prediction of GlpO from *M. hyopneumoniae* 7448 and *M. hyorhinis* HUB-1. **Figure S2:** Gene context from MGcV viewer [88] for 15 *fba* homologs from Mollicutes. **Figure S3:** Protein multiple alignment for 15 *fba* homologs from Mollicutes. **Figure S4:** Alignments of lolJ proteins described in the literature. **Figure S5:** Viability of cells in myo-inositol defined medium in comparison with Friis medium. **Figure S6:** Schematic representation of glycerol metabolism in *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare*. **Figure S7:** Hydrogen peroxide standard curve and average hydrogen peroxide production for *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare*. **Figure S8:** ATP standard curve.

Table S1: Cytometry cell counts and replicate readings for the calculation of hydrogen peroxide production. **Table S2:** Replicate readings of real-time RT qPCR for *glpO* transcript relative expression. **Table S3:** Gene locus tag for the genes from the uptake and metabolism of myo-inositol in *M. hyopneumoniae* strains. **Table S4:** Comparative modeling summary. **Table S5:** Average peak surface for marked myo-inositol from mass spectrometry experiments. **Table S6:** ATP production replicates and average for each sample. **Table S7:** Literature experimental data available for genes important for the glycerol and myo-inositol metabolism.

File S1: Statistical analyses results.

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Fig. 1 Hydrogen peroxide production by swine mycoplasmas. **A. In Friis medium after bacterial growth:** Hydrogen peroxide was only detected in growth media from pathogenic strains (field isolates) of *M. hyopneumoniae* 7448 (MHP_7448) and 7422 (MHP_7422). Neither the attenuated strain J (MHP_J) nor the other species *M. hyorhinis* (MHR) and *M. flocculare* (MFL) produced detectable amounts of this toxic product. The concentration was also standardized based on the average number of cells from each culture. Data are presented as mean and standard deviation of three independent samples and statistical analysis was performed considering *M. flocculare* as a control strain (since it lacks the *glpO* gene). **B. In the presence of different carbon sources:** Pathogenic *M. hyopneumoniae* strains were used to test hydrogen peroxide production in incubation buffer supplemented with either glycerol or glucose after 2 h of incubation. Both strains were able to produce significant amounts of the

toxic product when glycerol was present. Data are represented as mean and standard deviation of four independent samples (* $p < 0.05$; **** $p < 0.0001$).

Fig. 2 Expression levels of *glpO* gene in *M. hyopneumoniae* strains. We did not find any significant difference on the transcript levels of *glpO* from all tested strains. Bars show the average relative quantification normalized against unit mass (500 ng of total RNA) and replicate 2 from strain 7448 was used as the calibrator. Average expression levels were calculated with independent biological triplicates ($p < 0.05$).

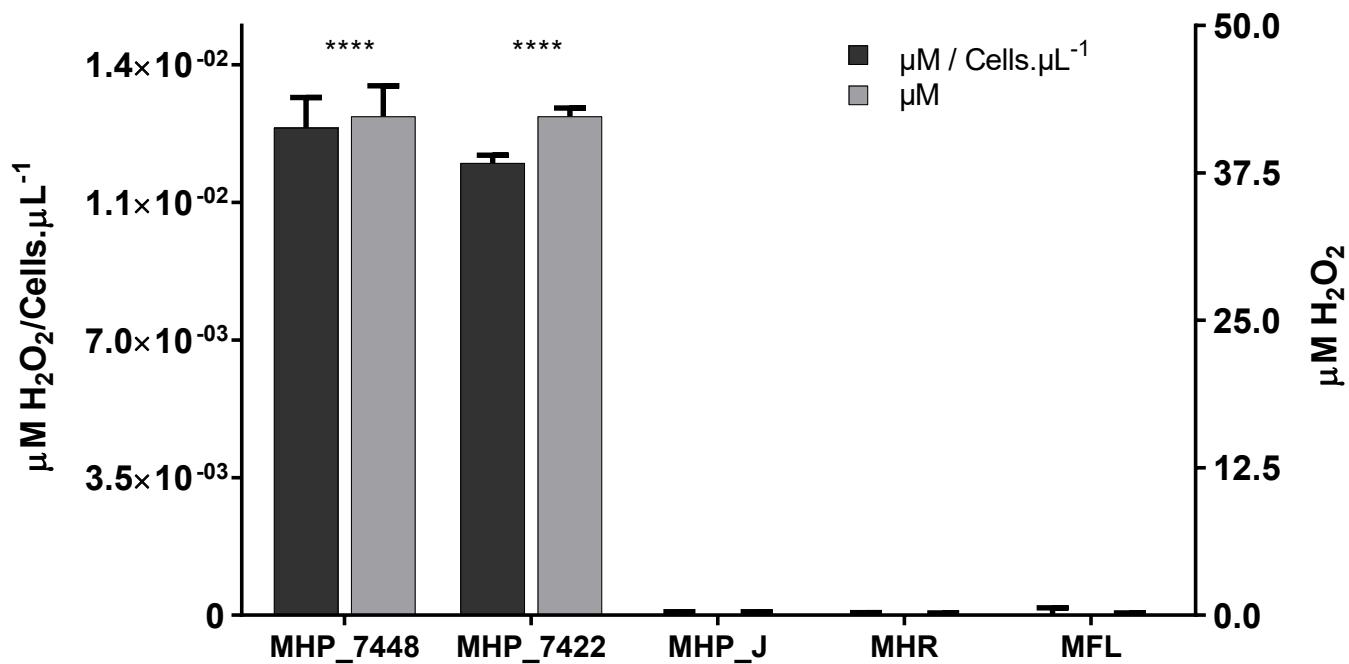
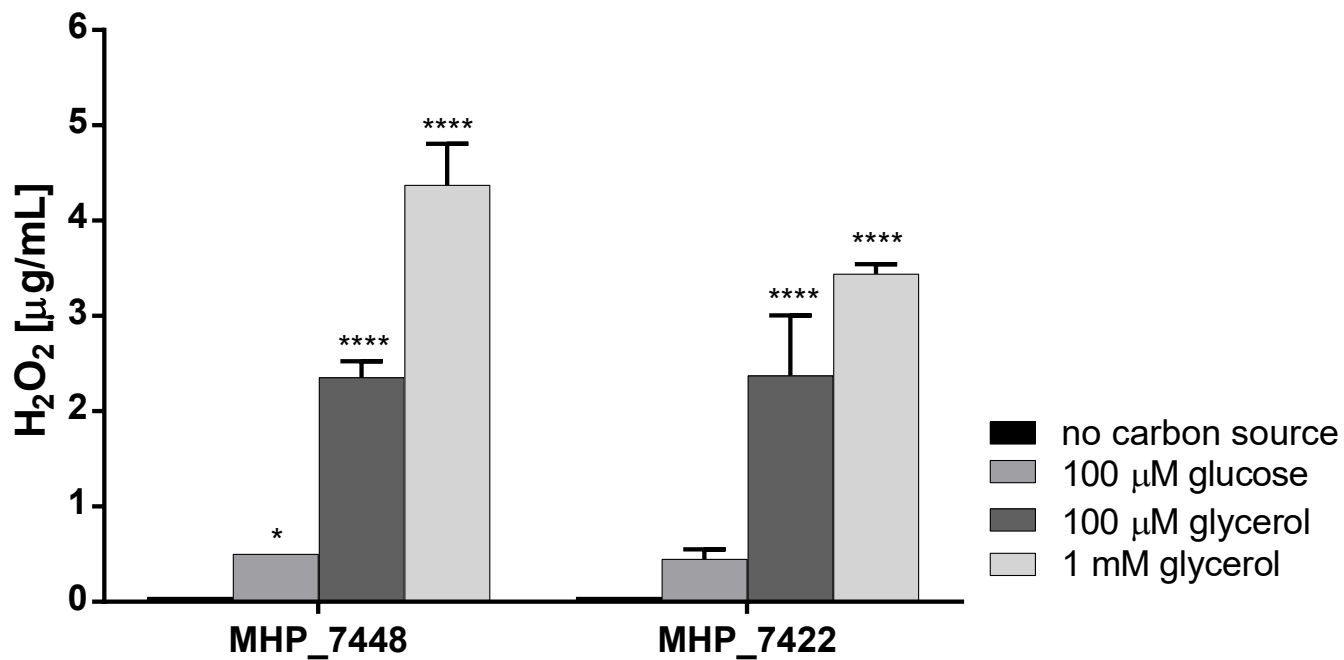
Fig. 3 Myo-inositol catabolism pathway in all *M. hyopneumoniae* strains and its transcriptional unit in *M. hyopneumoniae* strain 7448. Metabolites are depicted in dark green and enzymatic activities present in *M. hyopneumoniae* can be seen in pink. Metabolite abbreviations are as follows: MI (myo-inositol), 2KMI (2-keto-myoinositol), THcHDO (3D-(3,5/4)-trihydroxycyclohexane-1,2-dione), 5DG (5-deoxy-D-glucuronate), DKG (2-deoxy-5-dehydro-D-gluconate), DKGP (6-phospho-5-dehydro-2-deoxy-D-gluconate), MSA (malonate semialdehyde), AcCoA (acetyl coenzyme-A), DHAP (dihydroxyacetone phosphate). EC 1.2.1.27: MSA dehydrogenase; EC 4.1.2.29: DKGP aldolase; EC 2.7.1.92: DKG kinase; EC 5.3.1.30: 5DG isomerase; EC: 3.7.1.22: THcHDO hydrolase; EC 4.2.1.44: Myo-inosose-2 dehydratase; EC 1.1.1.18: MI-2-dehydrogenase; ABC-MI: ABC transporter for myo-inositol.

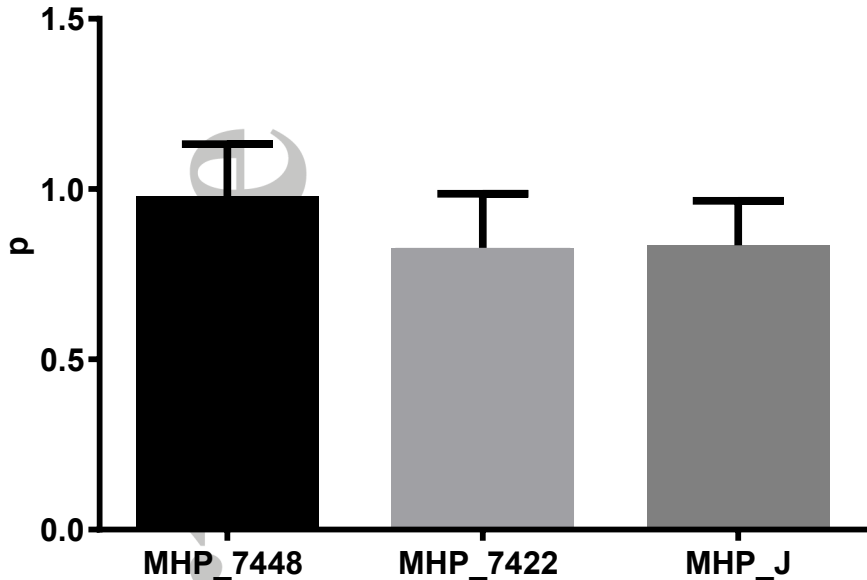
Fig. 4 Substrate cavity prediction for Fba and Fba-1 from *M. hyopneumoniae* strain 7448. Cavities from the comparative models of Fba and Fba-1 from *M. hyopneumoniae* in comparison to the models constructed for Fba and lolJ from *B. subtilis*. The specificity for DKPG in lolJ seems to be strongly associated to the presence of a conserved arginine in position 'a' (R52 in Fba-1 from *M. hyopneumoniae*). In contrast, Fbas generally bear glycines in this position (for complete explanation see Supplementary Figures S3 and S4). While Fba-1 from *M. hyopneumoniae* resembles more the experimentally solved Fba enzymes

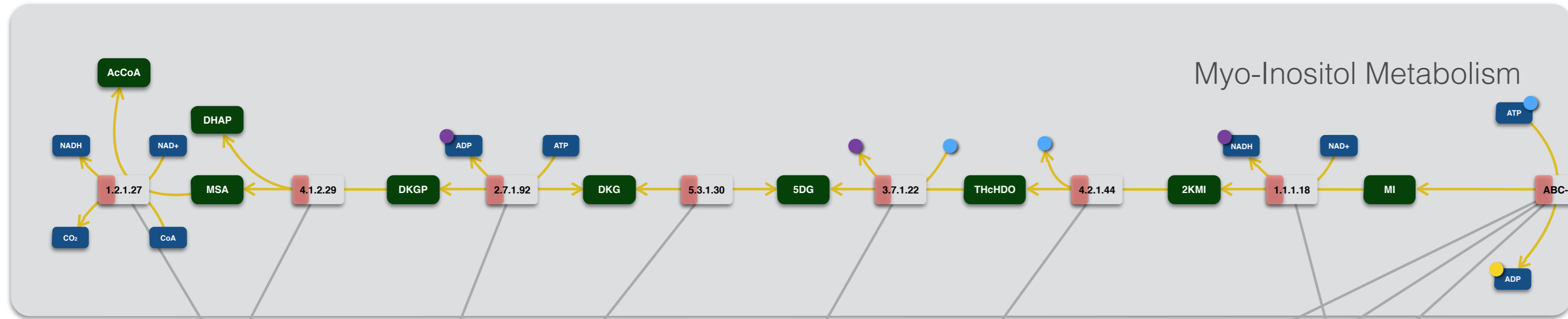
from *B. subtilis*, *E. coli* and, *G. intestinalis*, the predicted structure of Fba from *M. hyopneumoniae* is more similar to the lolJ structure from *B. subtilis*.

Fig. 5 Deuterated myo-inositol-1,2,3,4,5,6- α 6 uptake in complex medium. A. Comparison after 48 h of growth of *M. hyopneumoniae* J ATCC 25934 (MHP_J) and field isolate 7448 (MHP_7448), *M. flocculare* ATCC 27716 (MFL) and *M. hyorhinis* ATCC 17981 (MHR). While there is no significant difference in the concentrations between MFL and MHR and the control medium (CTRL), both *M. hyopneumoniae* strains seem to be able to uptake myo-inositol. **B.** We also collected two extra time points for MHP_7448 and CTRL: 8h and 24h of growth. In all time points there is significant difference between residual marked myo-inositol and the control medium. Data are presented as mean and standard deviation of 4 independent biological replicates. Asterisks indicate statistically significant differences in residual marked myo-inositol (* $p < 0.05$; ** $p < 0.01$).

Fig. 6 Viability of *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* after 8 hours of incubation in myo-inositol defined medium. The viability of cells in myo-inositol defined medium was measured by ATP production in comparison to inoculation in regular defined medium (glucose-containing medium). Data are represented as the ratio between ATP production in each media. There is a significant decrease of ATP production in *M. hyorhinis* and *M. flocculare* whereas at least 75% of the cells from *M. hyopneumoniae* remained viable after cultivation in the myo-inositol defined medium (** $p < 0.001$; **** $P < 0.0001$).

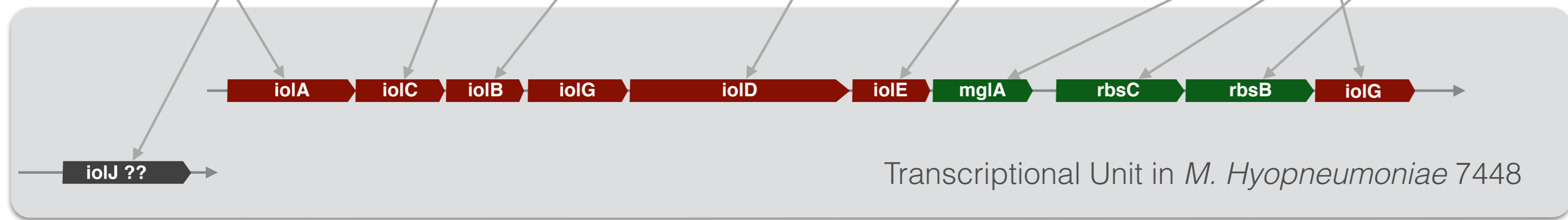
A**B**





Enzyme present in:

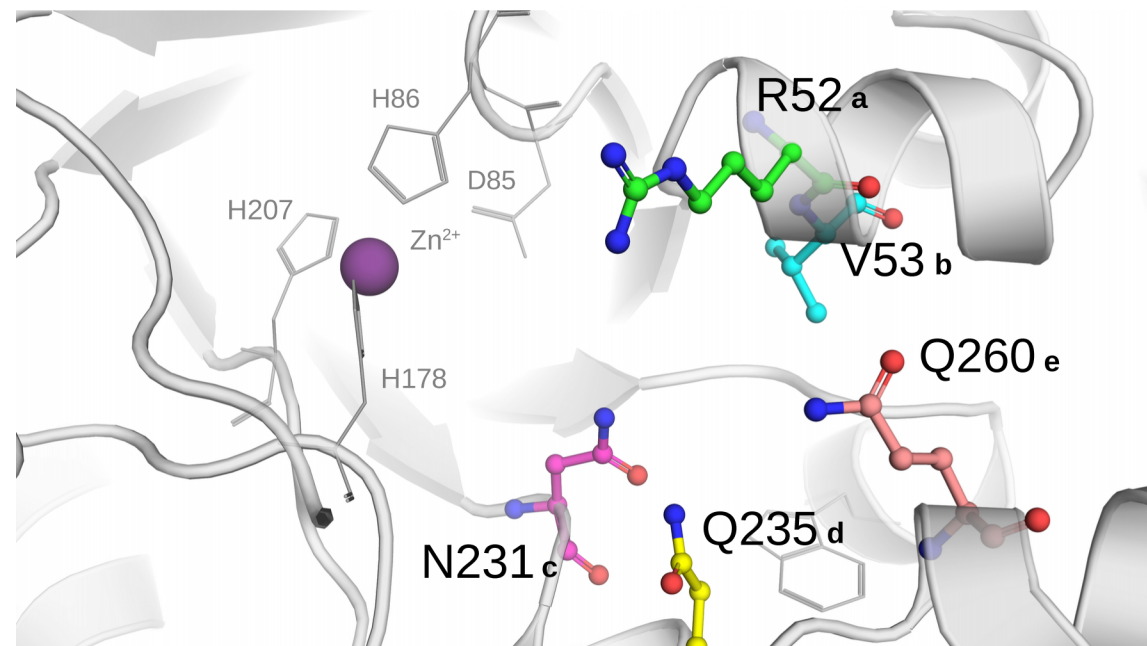
M. hyopneumoniae



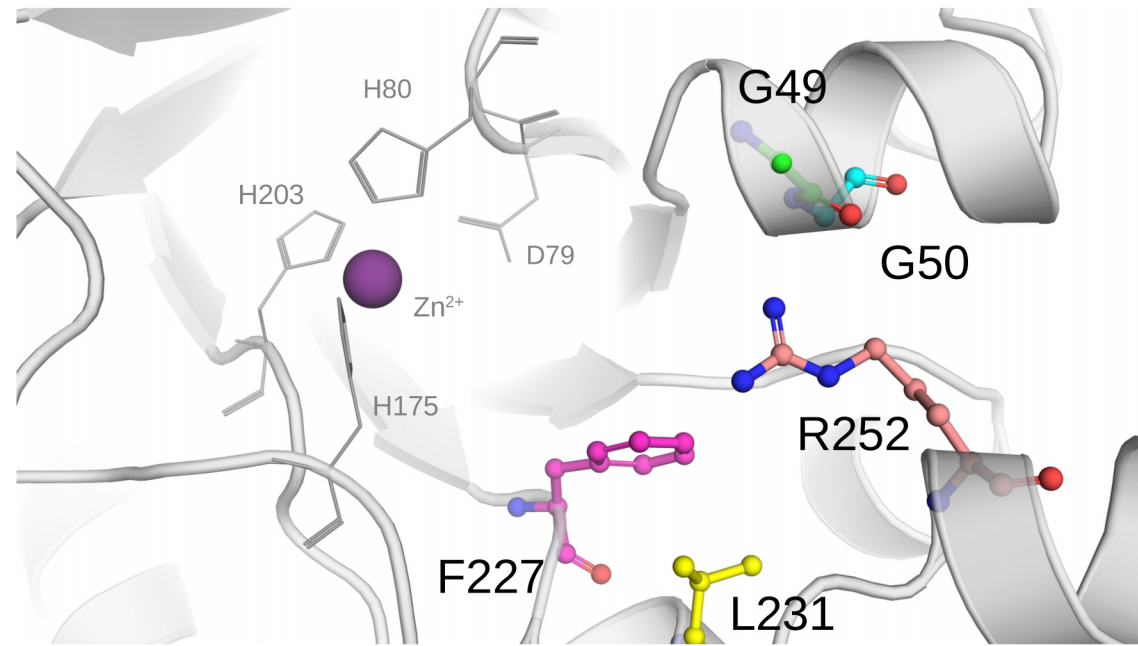
Legend:

- Met Metabolite
- EC Number Enzyme
- Cof Cofactor
- Pi
- Water
- Proton

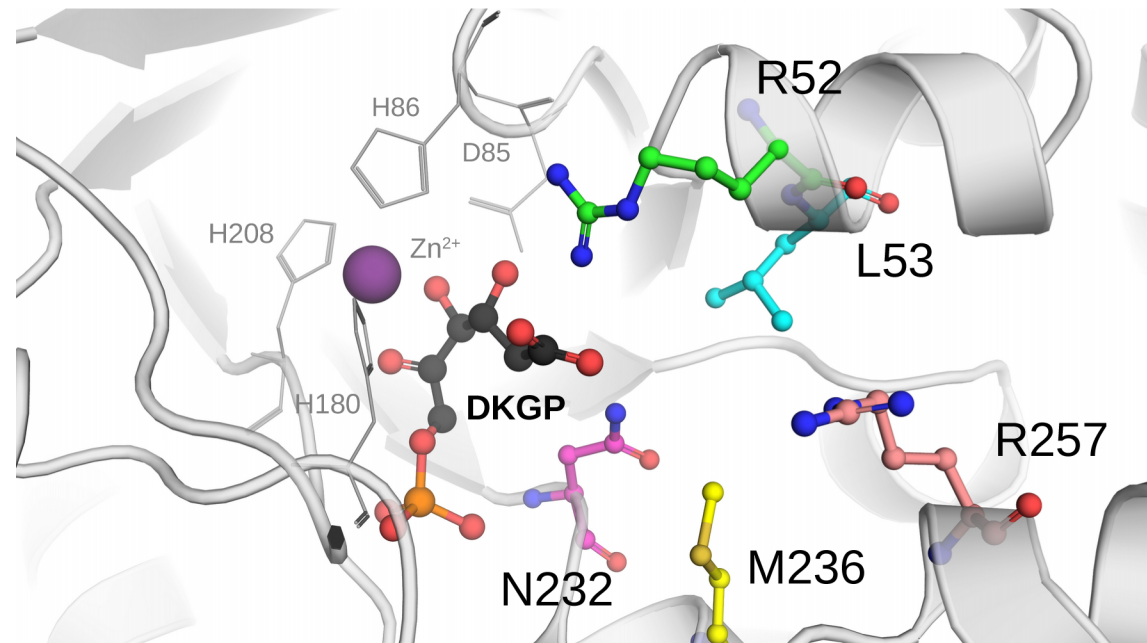
M. hyopneumoniae (Fba)



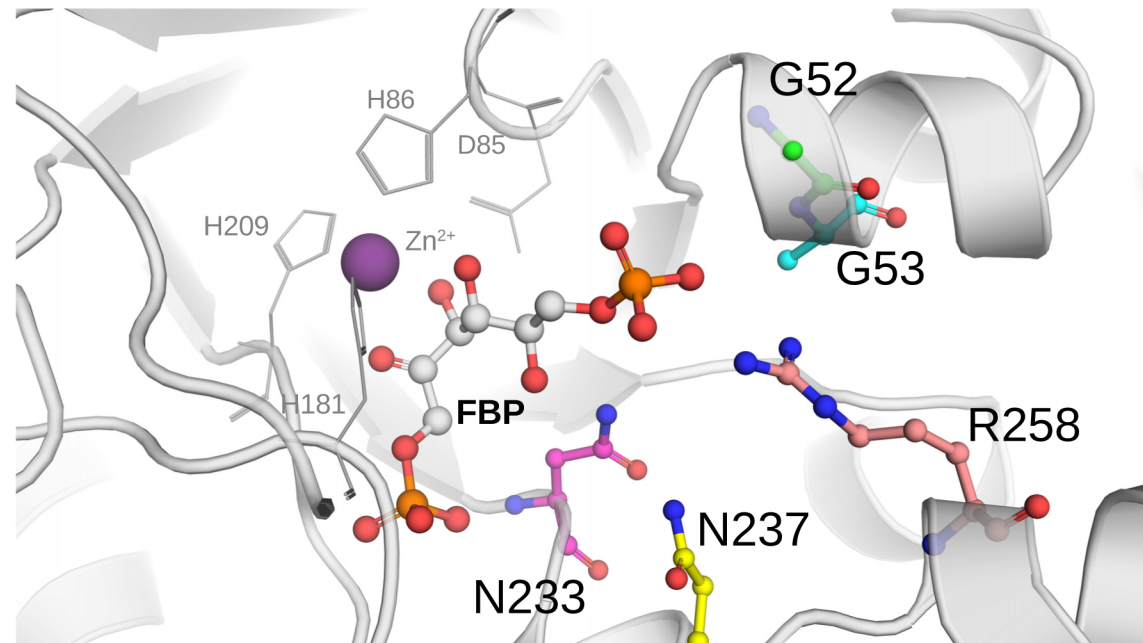
M. hyopneumoniae (Fba-1)

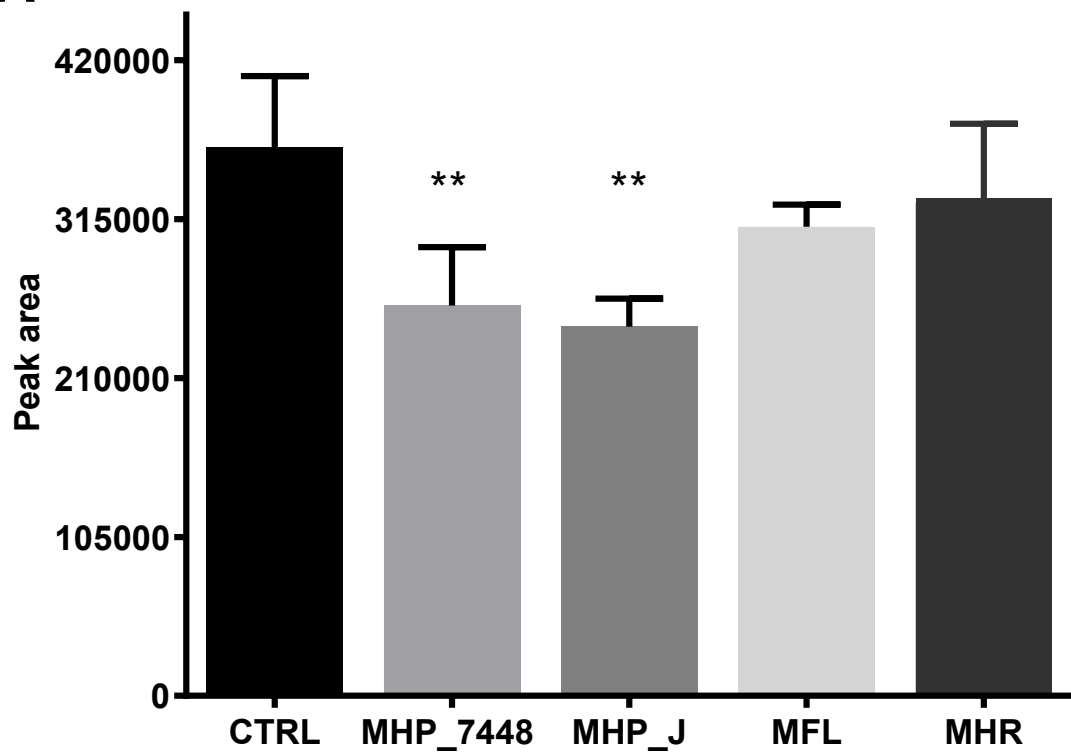


B. subtilis (iolJ)



(Fba)



A**B**