# Metabolic Investigation of the Mycoplasmas from the Swine Respiratory Tract 

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# Metabolic Investigation of the Mycoplasmas from the Swine Respiratory Tract 

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The harder I work, the luckier I get.

$$
\overline{\text { Coleman Cox }}
$$

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## Scope of the PhD Thesis

The swine respiratory tract is mainly colonized by Mycoplasma hyopneumoniae, Mycoplasma flocculare and Mycoplasma hyorhinis [160, 169, 229]. While colonization by M. flocculare is virtually asymptomatic, M. hyopneumoniae is the causative agent of enzootic pneumonia and M. hyorhinis is present in cases of pneumonia, polyserositis and arthritis. Several studies also suggest a synergistic role for M. hyopneumoniae in the initiation of a variety of other bacterial and viral infections [40], which explains why this pathogen is considered as a major cause of economic loss in the pig industry [?]. Nonetheless, the elevated genomic resemblance among these three mycoplasmas [261] combined with their different levels of pathogenicity is an indication that these species, as for most mycoplasmas, have unknown mechanisms of virulence and of differential expression.

In this PhD thesis, we aimed at studying the metabolism of M. hyorhinis, M. hyopneumoniae and $M$. flocculare to try to understand what could influence their different life-styles and pathogenicity. Chapter 1 introduces the biological models in detail and describes the principles of the experimental and mathematical/computational methods used throughout the PhD manuscript.

In so-called systems biology, biological processes are analyzed from a multidisciplinary point of view. Knowledge about the basic biology of the organisms and experimental data are combined with a mathematical modelling of the system. Systemic approaches are usually thought to retrieve information that isolated methods are not able to infer separately [193]. Studying pathogenicity through the metabolism has been well established in systems biology (examples in [37, 186, 164]). Moreover, although this may not be directly linked to the pathology itself, understanding the overall metabolism of a pathogen may lead to the discovery of new drug targets [36].

We therefore reconstructed the metabolic models for several strains of each of the species studied with the intention of comparing the networks and finding possible explanations for the different levels of pathogenicity observed among and within species. Chapter 2 describes the methods used and the results obtained from these reconstructions. We were able to show slight differences in the metabolic networks reconstructed that can partially explain the incapacity of $M$. flocculare to cause disease or the ability of $M$. hyorhinis to grow faster than the other two species. The models for $M$. hyorhinis can uptake a wider range of carbohydrates which, in turn, may reflect the overall better growth rates obtained for this species in culture. This may also explain why this species is considered a common contaminant of cell cultures [180].

As for the lack of pathogenicity of $M$. flocculare, the enzyme responsible for the production of the highly toxic hydrogen peroxide (a well-characterized virulence factor in the lung pathogens Mycoplasma mycoides and Mycoplasma pneumoniae [99, 294]), is absent in this species. On the other hand, M. hyopneumoniae and M. hyorhinis harbor in their genomes this specific enzyme and can use glycerol-sn-3-phosphate as carbon source with the production of hydrogen peroxide as a byproduct of the reaction. Moreover and as mentioned before, $M$.
hyopneumoniae and M. flocculare are closely related genetically, and although both species have been shown to adhere to cilia in a similar way [313], the inability of $M$. flocculare to cause disease corroborates with the hypothesis that it may be due to its incapacity to produce the toxic product, hydrogen peroxide.

However, establishing which are the virulence and pathogenicity factors is generally seen as an open problem in $M$. hyopneumoniae, mostly because non-pathogenic strains are extremely similar to the pathogenic ones. Although surface antigens are a well-characterized virulence mechanism for $M$. hyopneumoniae and $M$. hyorhinis, the lack of experimental information hinders the formulation of hypotheses concerning the specific pathologies of each species.

This is one of the reasons why we chose to perform our own metabolomics experiments for the three species and to investigate possible metabolic differences, even though the metabolic networks were quite similar. Chapter 3 describes these experiments along with growth tests on both complex and defined media. Indeed, through NMR metabolic profiling, we could detect a few metabolites that enabled us to establish a distinction among the species: the production of acetate, for instance, represents one of the major differences among the networks. $M$. hyorhinis seems to lack the turnover of pyruvate to acetate, thus making of pyruvate the final product of glycolysis in the experiments performed in this work; M. hyopneumoniae and $M$. flocculare, on the other hand, exhibit high acetate concentrations at the end of the growth curve. These metabolic differences, together with our previous findings, point to the existence of a machinery for differential gene expression in these species.

After we performed growth tests using a defined media that hypothetically could lead to bacterial growth in silico, we assessed that some cofactors or metabolites were probably missing from this medium. This is why we have to remember that the metabolism of these organisms cannot be completely understood when isolated from its environment. The environmental context may explain the differences between the in silico models and the in vivo behavior.

This was the case for thiamine, for example, which was recently proposed to enter the cells of $M$. hyorhinis in the form of thiamine-pyrophosphate [253]. When we look at the proteome from the airway surface liquid of the swine lungs, we find an enzyme responsible for the specific conversion of thiamine to thiamine-pyrophosphate (a missing activity in the genomes of mycoplasmas). Nonetheless, in the defined media, it is thiamine (instead of thiaminepyrophosphate) the cofactor available for bacterial usage. It is therefore possible that some of the differences among our models if compared to in vivo growth tests are actually the result of metabolites readily available in the swine lung that have not yet been proposed experimentally as cofactors or biomass precursors for these species. This example along with a detailed analysis of the possible exchanges between the host and the mycoplasmas is found in Chapter 4. It is important to point out that, given the complexity of the reconstructed networks, simpler versions (or subnetworks) of the models had to be used to address different problems throughout this chapter.

Metabolic exchanges are not the only possible dialogue between host and pathogens. Other virulence factors such as biofilm formation, surface antigens and polysaccharide capsules are discussed in Chapter 4 along with the interaction between mycoplasmas and other possible pathogens found in the swine lung airways. As we could solve the main problems of metabolite availability (or gaps) of the reconstructed models with the components of the swine lung airways, we proposed the presence of the other species in a simpler interaction model only in diseased states. Supporting this idea that the interaction between mycoplasmas and the other species occurs mainly during disease development, is the fact, reported in the literature, that while the three Mycoplasma species are widespread in pig populations and can easily be found
in healthy hosts [73, 200], other pathogenic species are predominantly prevalent in diseased animals [194]. And this also raises question: what causes the switch from a non-pathogenic Mycoplasma community to a pathogenic one? At the end of Chapter 4, we suggest several hypotheses that are based on carbohydrate availability, host characteristics (such as age, immune system, and vaccination state) and environmental conditions (such as stress, nutrition, and overpopulation). The final conclusion is that the disease is a multifactorial process depending on several factors that include intra-species mechanisms, community composition, host susceptibility and environmental factors.

A key point that is glanced throughout the PhD manuscript and is discussed in the last chapter is the existence of mechanisms for differential expression. It is clear that some kind of control of the gene expression occurs in mycoplasmas. However, they seem to have only one sigma factor and a few regulatory proteins. Control of the gene expression is therefore thought to be mostly quantitative rather than through an on/off switch process [176]. Since it however remains not clear which is actually the case, we decided to investigate the presence of promoter sequences in the genomes of several Mycoplasma species sequenced up to date. With the aid of an unbiased software developed by our team, SMILE, we were able to find the presence of a Pribnow box (approx. 10 bases upstream the beginning of the transcription site), as previously reported [303, 96].

All the results that will be presented in this manuscript aim at better understanding the differential metabolism in each species using different approaches. The reconstructed models from Chapter 2 already show some distinctions among the organisms and serve as a basis for all the hypotheses formulated with the experimental data used in Chapter 3. Metabolic profiling of both complex and defined media also pointed to new differences that we were not able to identify based only on the sequenced genomes. Moreover, in Chapter 4 we identified information missing from the models (gaps) in the actual environment in which these species are found, the airway surface liquid. In this chapter, we also propose a model for the interaction of swine respiratory tract mycoplasmas and other species during disease development. Finally, based on all the indications presented throughout the manuscript and on literature data, which suggests that some kind of regulation of the gene expression indeed exists in these species, we tried to gather information on new promoter sequences in Chapter 5.

In this way, this PhD work will serve as a basis for the study of the differential metabolism and pathologies caused by the swine swine respiratory tract mycoplasmas and may help to propose ways to prevent disease development in the future.

## French Version

Le tractus respiratoire des porcs est principalement colonisé par Mycoplasma hyopneumoniae, Mycoplasma flocculare et Mycoplasma hyorhinis (Mare et Switzer, 1965; Meyling et Friis 1972; Rose, Tully et Wittler 1979). Alors que la colonisation par M. flocculare est pratiquement asymptomatique, M. hyopneumoniae est l'agent causal de la pneumonie enzootique tandis que M. hyorhinis est présent dans les cas de pneumonie, polysérosite et arthrite. Plusieurs études suggèrent également un rôle synergique pour M. hyopneumoniae dans l'initiation d'une variété d'autres infections bactériennes et virales (Ciprian et al. 1994), ce qui explique pourquoi cet agent pathogène est considéré comme une cause majeure de perte économique dans l'industrie du porc. Néanmoins, la ressemblance élevée des génomes de ces trois mycoplasmes (Stemke et al. 1992) combinée avec leurs différents niveaux de pathogénicité est une indication que ces espèces, comme pour la plupart des mycoplasmes, ont des mécanismes inconnus de virulence et d'expression différentielle.

Dans cette thèse, nous avons cherché à étudier le métabolisme de M. hyorhinis, M. hyopneumoniae
et $M$. flocculare pour essayer de comprendre ce qui pourrait influencer leurs différents styles de vie ainsi que la pathogénicité.

Le Chapitre 1 présente les modèles biologiques en détail et décrit les méthodes utilisées dans cette thèse.

Dans ce qu'on appelle la biologie des systèmes, les processus biologiques sont analysés à travers un point de vue multidisciplinaire. Les connaissances sur la biologie fondamentale des micro-organismes et les données expérimentales sont combinées avec une modélisation mathématique du système. Les approches systémiques sont en effet sensées récupérer des informations que les méthodes isolées ne sont pas en mesure de déduire séparément.

L'étude de la pathogénicité à travers le métabolisme est bien établie en biologie des systèmes (voir (Chavali et al. 2008; Oberhardt et al, 2008; Mazumdar et al. 2009)). En outre, bien que cela ne puisse être directement lié à la pathologie elle-même, la compréhension du métabolisme global d'un pathogène peut conduire à la découverte de nouvelles cibles médicamenteuses (Chavali et al. 2012). Nous avons donc reconstitué les modèles métaboliques pour plusieurs souches de chaque espèce avec l'intention de comparer les réseaux et ainsi trouver des explications possibles pour les différents niveaux de pathogénicité entre et au sein des espèces. Le Chapitre 2 décrit les méthodes utilisées et les résultats obtenus à partir de ces reconstructions. Nous avons pu montrer l'existence de légères différences dans les réseaux métaboliques reconstruits ce qui peut en partie expliquer l'incapacité de $M$. flocculare à causer une maladie ou la capacité de M. hyorhinis proliférer par rapport aux deux autres espèces. l'enzyme responsable de la production de peroxyde d'hydrogène qui est hautement toxique (et est un facteur de virulence bien caractérisé dans le poumon et présent dans les bactéries pathogènes Mycoplasma mycoides et Mycoplasma pneumoniae), est absent chez M. flocculare. D'un autre côté, M. hyopneumoniae et $M$. hyorhinis possèdent cette enzyme dans leurs génomes, et peuvent utiliser le glycérol-sn-3-phosphate comme source de carbone avec comme sous-produit de la réaction le peroxyde d'hydrogène. En outre, et comme mentionné précédemment, M. hyopneumoniae et M. flocculare sont étroitement liés génétiquement, et même s'il a été démontré que les deux espèces peuvent adhérer aux cils de façon similaire, l'incapacité de $M$. flocculare à provoquer une maladie corrobore l'hypothèse que cela est dû à son incapacité à produire du peroxyde d'hydrogène.

Les modèles pour $M$. hyorhinis peuvent absorber un plus large éventail de carbohydrates ce qui, à son tour, peut refléter les meilleurs taux de croissance globaux obtenus pour cette espèce dans les cultures. Cela peut aussi expliquer pourquoi cette espèce est considérée comme un contaminant commun de cultures cellulaires.

Cependant, établir quels sont les facteurs de virulence et de pathogénicité est généralement considéré comme un problème ouvert dans $M$. hyopneumoniae, principalement parce que les souches non pathogènes sont extrêmement semblables à celles pathogènes. Bien que les antigènes de surface sont un mécanisme de virulence bien caractérisé chez M. hyopneumoniae et M. hyorhinis, le manque de données expérimentales empêche de formuler des hypothèses concernant les pathologies qui sont spécifiques à chaque espèce.

Ceci est une des raisons pour lesquelles nous avons choisi d'effectuer nos propres expériences de métabolomique pour les trois espèces afin d'explorer de potentielles différences métaboliques, même si les réseaux métaboliques sont assez similaires. Le Chapitre 3 décrit ces expériences ainsi que des tests de croissance sur les deux milieux complexes définis pour les trois espèces. En effet, grâce au profilage métabolique RMN, nous avons pu détecter quelques métabolites qui permettent d'établir une distinction entre les trois espèces: les taux de production de l'cétate, le pyruvate et le formate représentent ainsi des différences majeures entre les réseaux. Alors que M. hyorhinis semble ne pas être capable de produire de l'acétate à partir du pyruvate, ce qui fait du pyruvate le produit final de la glycolyse, $M$. hyopneumoniae et $M$. flocculare possèdent des concentrations élevées d'acétate à la fin de la courbe de croissance. Ces différences métaboliques, ainsi que nos résultats précédents, pointent vers l'existence d'un mécanisme d'expression différentielle des gènes chez ces espèces.

Après avoir effectué des tests de croissance en utilisant un support défini qui par hypothèse pouvait mener à une croissance bactérienne in silico, nous avons pu établir que certains cofacteurs ou métabolites étaient probablement absents de ce milieu. C'est la raison pour laquelle il est important de rappeler que le métabolisme de ces organismes ne peut pas être complètement compris lorsque les bactéries sont isolées de leur environnement. Le contexte environnemental peut expliquer les
différences entre les modèles in silico et le comportement des bactéries in vivo.
Ce fut le cas pour la thiamine, par exemple, qui a été récemment proposée comme pouvant entrer dans les cellules de M. hyorhinis sous la forme de thiamine-pyrophosphate. Lorsque nous analysons le protéome du liquide de surface des voies aériennes du poumon du porc, nous trouvons une enzyme responsable pour la conversion spécifique de thiamine en thiamine-pyrophosphate (une activité absente dans les génomes des mycoplasmes). Néanmoins, dans les médias défini, c'est la thiamine et non la thiamine-pyrophosphate qui est le cofacteur disponible pour une utilisation bactérienne. Il est donc possible que certaines des différences entre nos modèles si on les compare à des essais in vivo de croissance sont en fait le résultat de métabolites facilement disponibles dans le poumon de porc qui n'ont pas encore été proposés expérimentalement comme cofacteurs ou comme des précurseurs de la biomasse chez ces espèces. Cet exemple, ainsi qu'une analyse détaillée des échanges possibles entre l'hôte et les mycoplasmes, sont décrits dans le Chapitre 4. Il est important de souligner que, compte tenu de la complexité des réseaux reconstruits, des versions plus simples des modèles (ie des sousréseaux) ont du être utilisés pour traiter différents problèmes tout au long de ce chapitre.

Les échanges métaboliques ne sont pas le seul dialogue possible entre l'hôte et les pathogènes. D'autres facteurs de virulence tels que la formation de biofilm, les antigènes de surface et les capsules polysaccharidiques sont discutés dans le Chapitre 4 en même temps que l'interaction entre les mycoplasmes et d'autres pathogènes possibles trouvés dans les voies aériennes du poumon du porc. Il est également intéressant de noter que, alors que les trois espèces de mycoplasmes sont très répandues dans les populations porcines et peuvent être facilement trouvées dans les hôtes sains, les autres espèces pathogènes sont majoritairement présentes durant le développement de la maladie.

Comme nous avons pu résoudre les principaux problèmes de disponibilité (ou perte) de métabolite dans les modèles reconstruits à partir des composants des voies aériennes du poumon des cochons, nous n'avons considéré la présence des autres espèces dans un modèle d'interaction plus simple uniquement dans les états malades. Cela pose cependant aussi la question de ce qui provoque le passage d'une communauté non pathogène à une qui est pathogène? à la fin du Chapitre 4, nous proposons plusieurs hypothèses qui sont basées sur la disponibilité des glucides, des facteurs liés à l'hôte (comme l'âge, le système immunitaire, et l'état de vaccination) et des facteurs environnementaux (tels que le stress, la nutrition, et la surpopulation). La conclusion finale est que la maladie est un processus multifactoriel qui dépend de plusieurs facteurs dont des mécanismes intra-espèces, la composition de la communauté, la susceptibilité de l'hôte et des facteurs environnementaux.

Un point clé qui est présent tout le long du manuscrit de thèse et est discuté dans le dernier chapitre est l'existence de mécanismes d'expression différentielle. Il est clair que certains types de contrôle de l'expression des gènes se produit dans les mycoplasmes. Toutefois, ces derniers semblent avoir un seul facteur sigma et seulement quelques protéines régulatrices. L'hypothèse est alors que le contrôle de l'expression des gènes est la plupart du temps quantitatif plutôt qu'à travers un processus discret on/off (Razin et al. 2002). Comme il n'est cependant pas clair lequel de ces cas correspond à la réalité, nous avons décidé de rechercher la présence de séquences promotrices dans les génomes de plusieurs espèces de mycoplasmes séquencés à jour. Avec l'aide d'un logiciel non biasé développé par notre équipe, SMILE, nous avons réussi à trouver la présence d'une boîte de pribnow (env. 10 bases en amont du début du site de transcription), tel que rapporté précédemment. Même si nous ne sommes pas arrivés à une séquence consensus unique pour d'autres séquences promotrices, la présence de plusieurs séquences à une distance conservée de la boîte de Pribnow est aussi une indication de la possible présence de mécanismes distincts de la régulation des gènes dans ces génomes.

Tous les résultats qui seront présentés dans ce manuscrit visent mieux comprendre le métabolisme différentiel dans chacune des espèces à l'aide de différentes approches. Les modèles reconstruits du Chapitre 2 montrent déjà des distinctions entre les espèces et servent de base à toutes les hypothèses formulées avec les données expérimentales utilisées dans le Chapitre 3. Le profilage métabolique des deux milieux, complexe et défini, pointent également sur de nouvelles différences que nous avons pas pu identifier sur la base uniquement des génomes séquencés. En outre, dans le Chapitre 4, nous identifions les informations manquantes à partir de modèles en environnement réel ( BPA ) dans lequel se trouvent ces espèces, le liquide à la surface des voies aériennes. Dans ce chapitre, nous proposons également un modèle pour l'interaction des mycoplasmes respiratoires du porc et d'autres espèces au cours du développement de la maladie. Enfin, sur la base de toutes les indications présentées tout
au long du manuscrit et provenant de données de la littérature qui suggèrent que certains types de régulation de l'expression des gènes existent en effet dans ces espèces, nous avons essayé de recueillir des informations sur de nouvelles séquences promotrices dans le Chapitre 5.

De cette façon, cette thèse pourra servir de base pour l'étude du métabolisme différentiel et des pathologies causées par les mycoplasmes présents dans les voies respiratoires du porc, et pourra aider à proposer des façons de prévenir le développement de certaines maladies à l'avenir.

## Portuguese Version

O trato respiratório de porcos é colonizado prevalentemente por Mycoplasma hyopneumoniae, Mycoplasma flocculare e Mycoplasma hyorhinis [160, 169, 229]. Enquanto a colonização por M. flocculare é considerada assintomática, M. hyopneumoniae é o agente causador da pneumonia enzóotica e $M$. hyorhinis é encontrado frequentemente em casos de pneumonia, poliserosite e artrite. Muitos estudos sugerem um papel sinergístico de M. hyopneumoniae na iniciação de uma variedade de outras infecções bacterianas e virais [40], o que explica por que esse patógeno é considerado uma das maiores causas de perdas econômicas na indústria suína [?]. No entanto, a elevada semelhança genômica entre essas três espécies de micoplasmas [261] combinada aos diferentes níveis de patogenicidade é um indício que essas espécies, assim como a maior parte de micoplasmas, possuem mecanismos de virulência e expressão gênica diferencial distintos.

Nessa tese de doutorado, nós tivemos o intuito de estudar o metabolismo de M. hyorhinis, M. hyopneumoniae e $M$. flocculare para melhor entender o que poderia influenciar seus diferentes estilos de vida e patogenicidade.

O Capítulo 1 introduz os modelos biológicos em detalhe e descreve os princípios dos métodos experimentais e matemático/computacionais que foram usados durante esse estudo.

No que chamamos de biologia dos sistemas, os processos biológicos são analisados por pontos de vista multidisciplinares. Conhecimento sobre a biologia básica dos organismos e dados experimentais são combinados com modelagem matemática do sistema. Abordagens sistêmicas geralmente conseguem obter informações sobre o sistema biológico que os métodos isolados não conseguem inferir separadamente [193]. O estudo da patogenicidade por uma abordagem metabólica já é bem estabelecida em biologia de sistemas (exemplos podem ser vistos em [37, 186, 164]). Além disso, muito embora o metabolismo possa não estar diretamente ligado à patologia em si, o melhor entendimento do mesmo pode levar ao descobrimento de novos alvos para medicamentos e vacinas [36].

Dessa forma, nós reconstruímos os modelos matemáticos referente a diversas cepas de cada espécie escolhida com a intenção de comparar as redes refinadas em busca de possíveis explicações para os diferentes níveis de patogenicidade observados entre espécies e entre cepas de uma mesma espécie. O Capítulo 2 descreve os métodos usados e os resultados obtidos referentes à essas reconstruções. Nós pudemos encontrar pequenas diferenças nas redes metabólicas que podem parcialmente explicar a incapacidade de $M$. flocculare de causar dano às células do hospedeiro e da habilidade de M. hyorhinis de crescer mais rapidamente in vitro se comparado às outras espécies. Os modelos de $M$. hyorhinis conseguem utilizar uma maior gama de carboidratos que, por sua vez, pode refletir nessa capacidade de crescimento acentuada. Isso também pode explicar o porquê essa espécie é considerada um contaminante comum de outras linhagens celulares [180].

No que se refere à falta de patogenicidade de $M$. flocculare, a enzima responsável pela produção do composto tóxico peróxido de hidrogênio (um metabólito caracterizado como fator de virulência nos patógenos de pulmão Mycoplasma mycoides e Mycoplasma pneumoniae [99, 294]) está ausente nessa espécie. Por outro lado, M. hyopneumoniae e M. hyorhinis possuem em seus genomas essa atividade específica e podem usar o glicerol-sn-3-fosfato como uma fonte de carbono e concomitantemente, podem produzir peróxido de hidrogênio. Além disso e como já citado anteriormente, M. hyopneumoniae e M. flocculare possuem genomas altamente similares e aderem de maneira semelhante aos cílios das células epiteliais do hospedeiro [313]. No entanto, o fato de M. flocculare ser não patogênico, pode estar ligado à sua incapacidade de produzir esse metabólito tóxico.

A definição de fatores de virulência e patogenicidade geralmente é vista como um problema aberto em M. hyopneumoniae, principalmente por que as cepas não patogênicas possuem alta sintenia e similaridade às cepas patogênicas. Muito embora antígenos de superfície sejam mecanismos de virulência
bem caracterizados para M. hyopneumoniae e M. hyorhinis, a falta de dados experimentais é um empecilho à formulação de hipóteses relacionadas à patogenicidade específica de cada espécie.

Essa foi uma das razões que nos levou a realizar nossos próprios experimentos de metabolômica para as três espécies, e dessa forma investigar as possíveis diferenças metabólicas mesmo para redes extremamente similares. O Capítulo 3 descreve esses experimentos e também experimentos de crescimento microbiano tanto em meio complexo quanto em meio definido. Por meio da técnica de ressonância magnética nuclear (RMN), nós pudemos detectar algumas distinções entre as espécies: a produção de acetato, por exemplo, representa uma das maiores diferenças entre as espécies. M. hyorhinis aparentemente não consegue produzir grandes quantidades de acetato a partir de piruvato, tornando o piruvato o produto final da glicólise nos experimentos aqui reportados. Por outro lado, em M. hyopneumoniae e M. flocculare nós conseguimos detectar grandes quantidades de acetato no final da curva de crescimento. Essas diferenças metabólicas juntamente aos resultados anteriores apontam para a existência de uma maquinaria de expressão gênica diferencial nessas espécies de micoplasmas.

Após termos feito testes de crescimento em um meio definido que, hipoteticamente deveria permitir a multiplicação celular in silico, nós percebemos que alguns cofatores ou metabólitos estavam provavelmente sendo ofertados às bactérias de forma errônea. É por esse motivo que devemos lembrar que o metabolismo desses organismos não pode ser completamente entendido quando isolados do seu habitat. O contexto ambiental pode explicar algumas diferenças entre os modelos in silico e os comportamentos in vivo.

Esse foi o caso da tiamina, por exemplo, que foi recentemente proposta a ser importada por células de M. hyorhinis na forma de tiamina-pirofosfato [253]. Quando observamos o proteoma do líquido que permeia o pulmão de suínos (ASL), nós encontramos uma enzima responsável pela conversão específica de tiamina em tiamina-pirofosfato (uma atividade ausente nos genomas dessas micoplasmas). No entanto, no meio definido é a tiamina (no lugar de tiamina-pirofosfato) o cofator disponível para a bactéria. Dessa forma, é possível que algumas diferenças in silico de nossos modelos e in vivo dos testes de crescimento são, em realidade, resultado de metabólitos disponíveis na via respiratória de suínos que ainda não foram propostos experimentalmente como cofatores ou precursores de biomassa para essas espécies. Esse exemplo, juntamente a uma análise detalhada das possíveis trocas entre hospedeiro e micoplasmas podem ser encontrados no Capítulo 4. É importante ressaltar que dada à complexidade das redes reconstruídas, versões simplificadas (ou sub-redes) dos modelos tiveram que ser usadas para abordar diferentes problemas ao longo desse capítulo.

Trocas metabólicas não são o único tipo de diálogo entre hospedeiro e patógenos. Outros fatores de virulência como formação de biofilme, antígenos de superfície e cápsulas polissacarídicas são discutidas no Capítulo 4, juntamente com a interação entre micoplasmas e outros possíveis patógenos encontrados nas vias aéreas de suínos. Porém, como nós já havíamos conseguido resolver os maiores problemas relacionados à disponibilidade de metabólitos nas redes reconstruídas (os chamados gaps metabólicos) por meio dos componentes presentes em ASL de suínos, nós propusemos a presença de outras espécies em um modelo simplificado de interação, somente na ocorrência de doença. Corroborando com essa ideia (de que a interação micoplasmas/outras espécies ocorre em hospedeiros doentes) vem o fato reportado na literatura, que enquanto as três espécies de Mycoplasma são prevalentes em populações tanto sadias quanto doentes [73, 200], outros patógenos são prevalentes predominantemente em animais doentes [194]. Isso nos faz questionar: em micoplasmas, o que causa a mudança de uma comunidade não patogênica para uma patogênica? No final do Capítulo 4 nós sugerimos diversas hipóteses baseadas em disponibilidade de carboidratos, características do hospedeiro (idade, sistema imune, estado vacinal) e condições ambientais (estresse, nutrição, temperatura, concentração populacional).

Um ponto chave que permeia todos os capítulos dessa tese de doutorado é discutido no último capítulo: a existência de mecanismos de expressão gênica diferencial. É evidente que algum nível de controle de expressão gênica existe em micoplasmas. No entanto, a maior parte das espécies aparenta possuir apenas um fator sigma e poucas proteínas regulatórias. Pensa-se que o controle da expressão gênica é muito mais relacionado a um controle quantitativo do que um controle on/off [176]. Uma vez que ainda não se tem informações suficientes para suportar nenhuma das hipóteses, nós decidimos investigar a presença de sequências promotoras nos genomas de diversas espécies de micoplasmas. Com o auxílio de um software não tendencioso desenvolvido por nossa equipe, SMILE,
nós conseguimos detectar a presença de um sítio promotor similar ao Pribnow box (aproximadamente 10 bases à montante do sítio de início de transcrição), previamente descrito em [303, 96].

Todos os resultados que s ao apresentados nesse manuscrito visam a um melhor entendimento do metabolismo diferencial de cada espécie de micoplasma, com abordagens distintas. As redes reconstruídas no Capítulo 2 já mostram algumas diferenças entre os microrganismos e servem como uma base para todas as hipóteses formuladas com os dados experimentais do Capítulo 3. Experimentos de metabolômica em meio complexo e definido também apontaram novas distinções, as quais não pudemos identificar baseando-nos apenas nas sequências genômicas. Além disso, no Capítulo 4 nós pudemos complementar as redes reconstruídas com informações da composição do habitat dessas espécies (ASL). Nesse mesmo capítulo, nós propusemos um modelo para a interação entre micoplasmas e outras espécies presentes no trato respiratório de suínos durante o desenvolvimento da doença. Finalmente no Capítulo 5, baseando-nos em todos os indícios presentes ao longo da tese e dados da literatura, que sugerem algum nível de regulação da expressão gênica nessas espécies, nós tentamos obter mais informaçoes sobre sequências promotoras em diversas espécies de micoplasmas.

Dessa forma, essa tese de doutorado poderá servir como base para o estudo do metabolismo diferencial e das patologias causadas por micoplasmas do trato respiratório de suínos e pode ajudar a propor novos meios de tratar e prevenir a incidência dessas doenças no futuro.

## Chapter 1

## Background

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This chapter introduces the biological models, Mycoplasma hyopneumoniae, Mycoplasma hyorhinis and Mycoplasma flocculare, which colonize the respiratory tract of both healthy and diseased swines. I also describe the principles of experimental and mathematical/computational methods used throughout the PhD manuscript. These methods will be further detailed in the methods section of each chapter, whenever needed.

### 1.1 Biological Background

### 1.1.1 The Swine Respiratory Tract Microbiome

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 [62]. The respiratory tract of swines is colonized by several bacteria. Among these, Mycoplasma hyopneumoniae, Mycoplasma flocculare, Mycoplasma hyorhinis are some of the most important species identified so far [160, 169, 229].

In this microbiome we find several pathogenic species. M. hyopneumoniae for instance, is widespread in pig populations and causes enzootic pneumonia, which is considered a major cause of economic loss in the pig industry [?]; M. hyorhinis, although not as pathogenic as the supra-cited species, has already been found as the sole causative agent of pneumonia, polyserositis and arthritis in pigs $[134,53,304,274]$. M. flocculare, on the other hand, has high prevalence in swine herds worldwide, but up to date, no disease has been associated with this species [134].

Several studies also suggest a synergistic role for M. hyopneumoniae in the initiation of a variety of other bacterial and viral infections which occur under field conditions [40]. Other species present in the swine microbiome comprise Actinobacillus pleuropneumoniae, Actinobacillus suis, Streptococcus suis, Bordetella bronchiseptica, Haemophilus Parasuis and Pasteurella multocida. A. pleuropneumoniae is known as the causative agent of hemorrhagic swine pleuropneumonia [83], while A. suis causes actinobacillosis [153] and $S$. suis is generally associated with a wide range of clinical disease syndromes [55, 151]. P. multocida and B. bronchiseptica cause atrophic rhinitis [197, 67] and H. parasuis causes Glässer's disease or polyserositis, as well as pneumonia [16].

Apart from the above, the microbiome of the swine respiratory tract remains largely unknown, and possibly many more unidentified species may decisively influence the infection process of the pathogenic bacteria. To address this issue, our group started recently a metagenomic projet that aims at detecting which species are prevalent in this microbiome in herds from the south of Brazil.

### 1.1.2 Swine Mycoplasmas

Mycoplasmas belong to the Mollicutes class and are the smallest self-replicating organisms that are known [173], with genome sizes ranging from 580 kb (Mycoplasma genitalium) [263] to more than $1,358 \mathrm{~kb}$ (Mycoplasma penetrans) [237]. Mollicutes in general are characterized by a high AT content of their genomes ( $60-77 \mathrm{~mol} \%$ ) as well as a lack of cell wall [117]. Mycoplasmas have evolved by genome reduction from Gram-positive bacterial ancestors common to some Clostridia [17]. This significant genome reduction was only possible by adopting a parasitic mode of life [222]. It is possible that after an initial symbiotic phase, the availability of a broad range of metabolites derived from the host enzymatic activities, associated to the ability of the bacteria to uptake such compounds, made several activities dispensable for the bacterial life. These bacteria would therefore lose some of their genes unnecessary for living in an environment conditioned by another genome, becoming obligate parasites [7].

Although the three species of respiratory tract swine mycoplasmas are closely related genetically [261], it is not yet clear what causes the specific pathogenicity - or lack thereof in each of them. 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 $[155,74,249]$ and many can become carriers of the pathogen throughout their entire life without developing the acute disease. M. hyopneumoniae can also persist in healthy animals even after successful treatment of the disease [232, 73].

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 that are known to be attenuated by culture passages. These strains cannot cause disease in vivo and up until now it is not clear why. But, while the diseases caused by these mycoplasmas have been extensively studied, neither the species nor the diseases have been explored from a mathematical and computational point of views, mostly because the genome sequences of M. hyopneumoniae and M. flocculare were not available until recently [171, 289, 257].

Besides being an interesting disease model and because they are composed of a minimum set of organelles (plasma membrane, ribosomes and a circular DNA molecule [222]), these swine mycoplasmas are also suitable candidates for the study of the basic functions of prokaryotes from a systems biology point of view.

### 1.1.3 Metabolism of Swine Mycoplasmas

Respiratory tract swine mycoplasmas are facultatively anaerobic bacteria, usually grown in microaerophilic conditions. They are fermentative bacteria and may use glucose as their main energy source [222]. In general, they can also use a wide range of carbohydrates instead of glucose (fructose, maltose, dextrin, glycogen, starch, sucrose, mannose and galactose) to produce various types of acids (such as acetic, pyruvic, lactic). All Mollicutes, except for Ureaplasma species, are also considered to have an oxygen-dependent flavin terminated respiratory system, but it is not entirely understood up to date [203]. Like other Mycoplasma species, these bacteria salvage and interconvert but do not synthesize de novo nucleosides [203]. They lack cytochromes, functional tricarboxylic acid and glyoxylate cycles, and they cannot synthesize fatty acids [202]. Mollicutes in general seem to compensate for this apparently limited coding capacity with the presence of promiscuous, or non-specific enzymes that fulfill some of the missing functions [203]. An extensive literature research on the metabolism of $M$. hyopneumoniae, M. flocculare and M. hyorhinis and other related species took place and, if relevant, will be mentioned in Chapter 2.

### 1.1.4 Cultivation of Mycoplasmas

Mycoplasmas contain sterols in their membranes, a unique property among prokaryotes. Since they cannot synthesize them, cholesterol is required for growth [221]. Serum is often used as an exogenous source for cholesterol and fatty acids, increasing even more the complexity of an already rich and undefined growth medium. This is one of the reasons for the scarcity of metabolic studies on these organisms [203]. Many efforts were made in order to remedy this lack of knowledge, and defined media have been developed for many species, such as Acholeplasma laidlawii, Mycoplasma gallisepticum, Mycoplasma sp. strain Y and Mycoplasma pneumoniae [282, 279, 226, 314]. Mycoplasmas have also been cultivated in semi-defined rich mammalian media. These media are nucleoside and vitamin-rich; two examples are Eagle's basal media [210] and CMRL-1066 usually supplemented with serum [87]. Concerning swine mycoplasmas, to our knowledge, no defined media has been used for their growth up to now. Instead, the complex Friis medium [86] is still the most widely used for M. hyopneumoniae, M. flocculare and M. hyorhinis. Another setback for these species is that quantification of cells is not possible by using turbidity measurements or colorimetric assays based on acid color shift. Instead, we have to resort to the color changing unit method (CCU) [208, 262], in which 1 CCU represents the highest dilution of a cell suspension that can produce a change in color from red to yellow in broth medium. In search for more metabolic information, we also performed growth experiments on complex and defined growth media for the selected species. The results obtained can be seen in Chapter 3.

### 1.1.5 Virulence Factors in Swine Mycoplasmas

Virulence is one of the possible outcomes of a host-pathogen interaction and may involve a diverse set of mechanisms from both pathogen and host. Virulence genes, on the other hand, are classically seen as genes that encode for products - virulence factors - that have a direct pathological effect in the host tissue [27]. However, in contrast to other pathogenic bacteria, and as revealed by the analysis of sequenced genomes from several mycoplasmas [105, 34, 171, 289, 257], pathogenic Mycoplasma species seem to lack typical primary virulence factors such as toxins, invasins, and cytolysins [201]. 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, that 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 [220], invasion [30], cytotoxicity [294, 99], host-evasion [250] and host-immunomodulation (immunostimulation or immunosuppresion) [124, 297].

As for M. hyopneumoniae and M. hyorhinis, adhesion factors such as antigen surface proteins or the ability of these organisms to produce a lipopolysaccharide capsule have already been described in the literature $[268,43,64,247,304]$. What has yet to be better understood from our point of view is the direct participation of metabolism in the development of the diseases caused by these two species. 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 [255] and have been shown to adhere to cilia in a similar way [313]. Thus, it remains unclear what prevents M. flocculare to cause disease in this context.

### 1.2 Methodological Background

### 1.2.1 Systems Biology

Systems biology combines experimental data and computational modeling to understand complex biological systems [193]. This branch of science has gained more attention recently along with the increase in the number of large-scale datasets from high-throughput technologies [128]. An important factor for this is that while the achievement of sequencing complete genomes has been of great benefit to fundamental science (for example comparative genomics and evolutionary biology), reductionist approaches did not lead to the expected rapid and simple solutions to multifactorial diseases [259].

This is mainly possible because systems biology assumes that in order to understand the biological complexity of an organism (or a collection of organisms), one has to look at its whole, bringing together the knowledge available (theoretical and experimental) from all fields of biology, chemistry and physics to help explain the biological system [181]. In order to integrate all available data, and retrieve information from large-scale experimental datasets, it is essential develop mathematical models that help represent the entire system. Biological systems include but are not limited to metabolism, protein interaction networks (interactome) and regulation networks (regulome).

An appropriate mathematical approach has to be suitable for the size of the biological system, the type of experimental data to be integrated and the question to be addressed. All models are initially based on hypotheses and literature data, and are further refined by iterative cycles of model adjustments and experimental validation[129]. Such steps should be carried out until the model is able to accurately reproduce the experimental data for the biological system. The integration of other available experimental data, along with the design of new experiments to validate the in silico predictions, are usually used to achieve a much more accurate final model [284].

### 1.2.2 Modeling Metabolism

Metabolism is known as the set of chemical transformations - known as reactions - occurring within the cells of living organisms. These reactions comprise catabolic pathways - the breakdown of substrates into common metabolites - and anabolic pathways - the synthesis of macromolecules such as amino acids, nucleic acids and fatty acids. Usually, catabolism generates energy from carbon sources (in the form of ATP) and provides reducing power (in the form of $\mathrm{NAD}(\mathrm{P}) \mathrm{H})$ to transform carbon intermediates into reduced end products and building blocks for biomass growth [213]. Most of these chemical reactions are not spontaneous and need biocatalysts - enzymes - in order to happen. Although in general each enzyme is specific to one reaction, one reaction can be catalyzed by different enzymes and some enzymes can also act promiscuously, using a wide range of substrates to generate just as many products.

Overall, a cell needs approximately as many enzymes as there are reactions in order to grow. In essence, every chemical reaction in a cell occurs at a useful rate because enzymes lower the activation energy only for the reaction(s) they catalyze, enhancing these rates specifically [179]. Enzymes are
neither consumed nor produced during these steps and generally do not need to be recycled after a reaction is over. Moreover, although in many enzyme-catalyzed reactions, the equilibrium is favored towards one side, depending on the thermodynamic constraints, most reactions can be reversible [168].

The biochemical principles and many times, even the sequence of reactions, remain the same for organisms as distinct as human and bacteria. This is known as the unity of biochemistry, and is a key concept when we try to infer knowledge from well-studied model organisms to species lacking experimental data [243]. It is also important to notice that recent studies by Kelkar and Ochman [125] have shown that reduced genomes are able to increase their functional complexity through protein multitasking. When the authors compared the interactomes (protein-protein interaction networks) from reduced and large genomes, they found out that proteins from organisms with small genomes interact with proteins that have a broader range of functions than their orthologs from larger genomes. In this way, many genes end up adopting new roles to counteract gene loss; this ability is also called protein moonlighting.

### 1.2.3 Metabolic Network Reconstruction

Network reconstruction is the process of identifying all the reactions present in a biological system [193]. A detailed protocol was described by Thiele and Palsson [277] and consists of a series of 96 steps showed in Figure 1.1 and succinctly addressed below.


Figure 1.1: Overview of the procedure to iteratively reconstruct metabolic networks. Extracted from [277].

A genome-scale metabolic network reconstruction usually relies on the functional annotation of genes based on sequence homology. The presence of an enzyme with high similarity to a characterized enzyme (from other organisms) may be used as a strong evidence for the presence of a reaction in the organism of study. The first step is the creation of a draft reconstruction, which may be achieved by a variety of automatic reconstruction tools, listed and detailed in various reviews [142, 68, 76]. None of the methods succeed in obtaining a high-quality model without manual curation [277, 235]. In this way, additional experimental evidence (physiological or biochemical characterization) is used for the refinement of draft reconstructions. Diverse omics data such as transcriptomics, proteomics and metabolomics can also be included to gain information on the enzymatic capabilities of an organism and thus to enrich the model.

The curation process is iterative and laborious, and can be briefly summarized in three steps: refinement, modeling and evaluation. Each new modification (refinement) should be tested (modeling) and evaluated based on topological and functional properties of the network; gaps and inconsistencies
should be addressed in a case-by-case manner. It is essential that the refinement process should be repeated until the model reproduces well the phenotypic characteristics of the target organism [277].

### 1.2.4 Metabolomics in Systems Biology

A great number of different technologies have emerged after the genome sequencing era to facilitate the understanding of these large sets of data. Analysis of the gene expression (transcriptomics) or protein translation (proteomics) levels helps us make sense of the genomes from sequenced organisms. However, even the development of these fast evolving technologies is not sufficient for us to assign functions to hypothetical genes, for instance [299]. In this way, metabolomic data serve as a complement to genomics, transcriptomics and proteomics by trying to identify the metabolic capabilities of an organism.

Metabolomics is the global analysis of all (or a large number of) cellular metabolites from a biological sample [248]. While proteins and RNAs are composed by homogeneous building blocks and are thus detectable by a single technical approach (proteomics and transcriptomics methods, respectively), metabolites are difficult to assess when applying a single analytical method, due to chemical diversity and variable cellular abundance [92]. In addition, metabolites can be unstable or have short lives, which can pose challenges for sample preparation and processing. There are three major approaches in metabolomics: target analysis, metabolite profiling and metabolic fingerprinting. Target analysis is usually limited to a small number of identifiable compounds and metabolic fingerprinting does not attempt to identify or precisely quantify all metabolites in a sample [248]. In this way, metabolite profiling is the preferred approach for systems biology. The different techniques used for identification and quantification of cellular components are usually nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS).

MS is known for its greater sensitivity; however, its cost, especially when heavy labelled internal standards are needed (for absolute quantification) is a setback for the use of MS with high-throughput approaches. On the other hand, sample preparation in NMR is generally faster and the analysis time is shorter, resulting in lower analysis costs. NMR is a robust, reproducible technique that can provide absolute quantification of many metabolites. [212]. NMR has two other great advantages: it is a non-destructive technique and it can identify all types of molecules (lipids, carbohydrates, and so on) with similar efficiency [209]. Its biggest disadvantage is that it is usually not very sensitive, therefore lowly abundant compounds are difficult to be detected.

In this work, we have chosen to use a metabolic profiling approach through NMR spectroscopy to further refine the models reconstructed in silico.

### 1.2.5 Modeling of Microbial Communities

Microbial communities are ubiquitous in nature and have a major impact on every aspect of life in our planet. In spite of this importance, little is known about the principles that determine the functioning, robustness, evolution and control of microbial communities. Each community has strikingly different characteristics, which in turn, makes each model unique and not easily inferable from others.

The most widely studied microbiome is the human gut. And while gut and lung are both mucosalined luminal organs, they present remarkable differences in morphology and composition, yielding completely different populations of bacteria. For instance, the migration of microbes in the digestive tract is unidirectional (from the mouth to the anus) with many physico-chemical barriers (differences in pH ). On the other hand, the movement of air, mucus and microbes in the lung is bidirectional, with no apparent physical barrier. Hence the microbiome of the respiratory tract is more dynamic and transient than the one from the lower gastrointestinal tract [62]. The gut temperature is uniform ( $37^{\circ} \mathrm{C}$ ) and bacteria live in anaerobiosis, while the airway temperatures vary according to the ambient temperature [112] and the lung environment is oxygen-rich. While the gut is heavily packed with bacteria, the bacterial density in the airways is quite modest [104]. The inter-bacterial metabolic interactions are therefore clearly different.

Because of this complexity and singularity of these communities, it is clear that experimental data alone do not allow us to fully understand the global capacities and functions of these organisms.

Hence, mathematical models are of high importance on this matter [100]. Currently, we are unable to decipher what role individual microorganisms have and how their individual actions influence others in the community [315].

Hanemaaijer and collaborators [100] postulate that in many relevant cases, the metabolic component of the community is dominant and many species-species interactions are metabolically driven. However, communities are not entirely structured by metabolism. Social traits such as quorum sensing, antibiotic production, signaling mechanisms among others are usually left out of the models because they are thought to have minor effects on the phenotype of the organisms [189, 186].

In communities as complex as the human gut, oral cavity, lungs and skin (ranging from 500 to 1000 species) $[88,94,104,61]$, the modeling itself may be a laborious and almost infinite work, more suitable for the computational/mathematical domain than for the actual biologist. Of course, it is essential to integrate existing genomic, physiological, and physicochemical information with metagenomics data so as to maximize the information content and predictive power. However, the resulting model should be simplified to a "coarse-grained" version, tailored to solve the inference problem from experimental data. In this way, the modeling of communities would be less of a theoretical/computational exercise and a more direct method for the experimentalist [100].

### 1.3 Mathematical Background

Mathematical models link model variables by mathematical relations [111]. The mathematical representation of a set of reactions, for instance, is usually made in a tabular representation, called the stoichiometric matrix $S$ (see Figure 1.2).


Figure 1.2: Representation of a metabolic model in the form of a stoichiometric matrix $S$. After the metabolic network has been assembled (on the left), it must be converted into a mathematical representation (table on the right). The result of this conversion is performed using a stoichiometric matrix $([S])$ in which the stoichiometry of each metabolite involved in a reaction is enumerated. Reactions form the columns of this matrix and metabolites, the rows. Each entry of a metabolite corresponds to its stoichiometric coefficient in the corresponding reaction. Negative coefficient substrates are consumed (reactants), and positive coefficients are produced (products). Exchange reactions are inserted to the networks to allow source (EX1) and sink (EX2) reactions, and represent the real substrates and products of the model.

When information about time-dependent quantitative changes of the system is not available, one has to resort to static modeling. These models assume that the system is in a steady state condition,
which means that the concentration of metabolites is constant throughout the predictions [187]. The constraint-based approach is a static modeling that has been developed over the last 30 years and will be discussed in detail below [77, 239].

## Constraint-based models

Metabolism is a complex network of biochemical reactions. Each reaction is also limited by many natural constraints, such as metabolite availability, mass and charge conservation and thermodynamics [146]. A constraint-based model is a union of stoichiometrically balanced reactions, a set of constrained metabolic fluxes and information on the possible genes responsible for each reaction. Detailed information on the metabolite concentrations, reaction mechanisms and kinetic parameters is not required for this type of modeling [187]. Each reaction may receive upper and lower bounds, which define the interval acceptable for the reaction fluxes to take place. The lower and upper bounds account for two sets of information: enzyme reversibility and enzyme turnover/effectiveness (Figure 1.3).


Figure 1.3: Representation of reaction fluxes. $V R$ and $V R$ ' represent the reaction rates of the forward and reverse reactions respectively and $x$ represents a hypothetical constant. If the absolute upper and lower bounds are equal (reaction R1), the enzyme has no preference for either direction; If the reaction is irreversible, the lower bound is equal to zero (reaction R2, on the table). If a reaction has different absolute upper and lower bounds, it means that the enzyme has a higher affinity for either the substrate of forward or substrate of the reverse reaction. As an example, in reaction R3 the rate of conversion of C into $\mathrm{D}(v R 3)$ is two times higher than the reverse reaction (vR3').

Under steady-state conditions, the stoichiometric matrix $S$ can be related to the change in concentrations of the substrates through the following equation:

$$
S * v=0
$$

where $v$ is the flux distribution (the possible rates of every reaction in the network). Each of these fluxes can vary between the upper and lower bounds determined previously. The flux vector, a mathematical object, is a list of all flux values for one single state (out of infinite states) that the network can assume. Substrate uptake and secretion rates can also be added as constraints. All constraints and fluxes define an allowable solution space (Figure 1.4). The flux vectors consistent with all imposed constraints are thus candidate physiological states for the conditions considered.
There are several analytical methods to study the properties of this solution space, such as maximal growth and product yields, gene essentiality and behavior prediction under stress conditions [187]. Gene essentiality studies can be performed by the elimination of all reactions related to a single gene (knockout mutant simulation) [266] while maximal growth and product yields can be tested through flux balance analysis [190] and will be described below.


Figure 1.4: Graphic representation of a solution space for three reactions. On the left, in unconstrained models, every point in the graph may be a solution for steady-state conditions. On the right, when applying constraints to the model, the solution space is defined and every point inside this allowable space may be a feasible solution.

## Flux Balance Analysis

Since the solution space is very large even under constrained conditions, flux balance analysis (FBA) uses linear programming to optimize the flux distribution towards one or several objective functions. The assumption behind FBA is that metabolism maximizes or minimizes a set of reaction fluxes. Common objective functions include maximal biomass yield, maximal and minimal energy and metabolite production, detoxification, among others (Figure 1.5).


Figure 1.5: Examples of objective functions. FBA can be used to calculate the optimal flow of metabolites from a network input to a network output. The desired output is described by an objective function. (A) If the objective is to optimize flux through reaction Biomass, the optimal flux distribution would correspond to the levels of biomass reaction and oxygen uptake reaction at the red point circled in the figure. (B) If, on the other hand, the desired output is to maximize oxygen uptake, optimal fluxes correspond to the yellow circle.

In nature, it is known that many suboptimal flux distributions may also exist to help the organism during adaptation to specific environmental conditions [143]. Moreover, analysis of the flux distributions showed that for complex networks, it is highly probable that multiple optimal solutions may exist [156].

### 1.3.1 Biomass Composition

In order to perform growth simulations, it is necessary to delineate the composition of biomass for the selected organism. In general, the biomass contains all macromolecules (in their stoichiometric quantities) necessary to build up an entire cell. The biomass equation (or reaction) is the mathematical representation of the biomass composition along with the amount of energy necessary to sustain growth (growth associated maintenance or GAM) [298]. GAM is incorporated to the biomass reaction with the following general form:

$$
\mathrm{xATP}+\mathrm{xH}_{2} \mathrm{O} \longrightarrow \mathrm{xADP}+\mathrm{xPi}+\mathrm{xH}^{+}
$$

where $x$ represents the ATP requirements for macromolecule synthesis and other growth-related processes. In order to accurately predict the biomass growth, one therefore has to estimate from the literature (or measure experimentally) the overall composition of the cell and incorporate it into the biomass reaction. The amino acid and nucleic acid composition may be estimated from the genome, while the lipid content is usually inferred from experimental data. It is also necessary to estimate and incorporate the composition of the soluble pool (co-factors, vitamins, etc) as well as ion content [298]. This results in a generic biomass reaction as follows:

$$
\text { dNTPs }+ \text { AAs }+ \text { LipidPrecursors }+ \text { Ions }+ \text { SolublePool }+\mathrm{ATP}+\mathrm{H}_{2} \mathrm{O} \longrightarrow \mathrm{ADP}+\mathrm{Pi}+\mathrm{H}^{+} .
$$

The non-growth associated maintenance (NGAM) must be experimentally determined and represents the energy needed for maintaining homeostasis in the cell. It is generally included as a separate reaction with the following formula:

$$
1 \mathrm{ATP}+1 \mathrm{H}_{2} \mathrm{O} \longrightarrow 1 \mathrm{ADP}+1 \mathrm{Pi}+1 \mathrm{H}^{+}
$$

### 1.3.2 Gene Essentiality

Essential genes consist of genes whose punctual deletion renders the organism unable to grow in specific growth conditions. Complex biological systems are inherently robust to genetic and environmental perturbations [127]. In order to study the robustness of a living system, one could resort to gene essentiality experiments followed by a series of growth tests and complementation studies. Since this is generally a time-consuming method, the availability of genome-scale metabolic models has provided the tools to rapidly predict the effect of multiple genetic manipulations on the strain growth phenotype in different environmental conditions [266]. In practice, simulation of gene knockouts in silico consists in the exclusion of all reactions performed by the same gene throughout the model with subsequent testing for feasible growth. Comparison of the mutant and wild-type fitness gives information on which genes influence or are essential for biomass production. Several approaches use this technique for strain design to collapse certain unwanted pathways from the network and guarantee enhanced metabolite production [29, 131, 152].

## Chapter 2

## Metabolic Network Reconstruction

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#### Abstract

In order to search for possible explanations for the different levels of pathogenicity observed among and within M. hyopneumoniae, M. hyorhinis and M. flocculare, we decided to study their metabolism from a systems biology point of view. This chapter introduces the reconstruction and analyses of the whole metabolic networks for several sequenced strains of the three selected species. The aim here was to have a global view of the major differences between the metabolism of these species. At first, I describe in detail the reconstruction and refinement processes; Then, I discuss how the metabolic differences of each species may impact on their specific pathogenicity. Although the metabolism may not be directly linked to the pathology itself, understanding the basic biology of a pathogen may lead to the discovery of new drug targets. Moreover, we were able to find slight differences in the reconstructed models that can partially explain the incapacity of $M$. flocculare to cause disease, the ability of $M$. hyorhinis to grow faster than the other two species, and the enhanced level of pathogenicity of $M$. hyopneumoniae.


### 2.1 Overview

In general, and similarly to all mycoplasmas, the selected species have limited metabolic activity. Little experimental information that is species-specific was available in the literature. We found partial data only for $M$. hyopneumoniae and $M$. hyorhinis (Figure 2.1). Thus, for the whole-genome metabolic reconstructions, we also based the research on closely related species, specially M. pneumoniae and M. genitalium, which had their whole-genome metabolic reconstructions made recently available [314, 307, 265].

Tourtellotte and Jacobs [281] were able to demonstrate that M. hyorhinis could metabolize a wide range of carbohydrates into three end products: lactic acid (in high concentrations), pyruvic acid and acetic acid (in small concentrations). They also showed that this species was unable to produce acetaldehyde or ethanol. No succinate, malate or formate production was reported experimentally for any of the species up to date.

Constantopoulos and collaborators [46] have measured the activities of oxidative enzymes (enzymes that catalyze oxidation reactions) in M. hyorhinis strain GDL-1. They showed the presence of pyruvate dehydrogenase (EC 1.2.1.-), $\alpha$-ketoglutarate dehydrogenase (EC 1.2.4.2) and lactate dehydrogenase (EC 1.1.1.27) activities. Even though this was not tested in swine mycoplasmas, acetate kinase activity has been measured in several closely related extracts of mycoplasmas [175].


Figure 2.1: Schematic diagram of enzyme activities and pathways detected in extracts of M. hyopneumoniae and M. hyorhinis. Data extracted from [281, 159, 46].

Manolukas and collaborators [159] have shown the absence of the tricarboxylic acid cycle in $M$. hyopneumoniae extracts, with the exception of malate dehydrogenase activity (EC. 4.2.1.2). They also showed the presence of several enzymatic activities for pyruvate degradation (Figure 2.1). All enzymatic activities previously mentioned are summarized in Table 2.1.

Table 2.1: Enzyme activities in extracts of M. hyopneumoniae and M. hyorhinis. Data extracted from [281, 159, 46].

| Enzyme | EC Number | M. hyopneumoniae | M. hyorhinis |
| :---: | :---: | :---: | :---: |
| Malate dehydrogenase | 1.1.1.37 | + ${ }^{1}$ | NA |
| Fumarase | 4.2.1.2 | $+{ }^{1}$ | NA |
| Aspartate aminotransferase | 2.6.6.1 | + ${ }^{1}$ | NA |
| Malate synthase | 2.3.3.9 | $+{ }^{1}$ | NA |
| PEP carboxylase | 4.1.1.31 | - 1 | NA |
| Pyruvate carboxylase | 6.4.1.1 | $+{ }^{1}$ | NA |
| Pyruvate dehydrogenase | 1.2.1.- | NA | $+^{2}$ |
| Pyruvate kinase | 2.7.1.40 | $+1$ | $+{ }^{2}$ |
| Lactate dehydrogenase | 1.1.1.27 | $+{ }^{1}$ | $+{ }^{2}$ |
| a-ketoglutarate dehydrogenase | 1.2.4.2 | NA | $+^{2}$ |
| Acetaldehyde dehydrogenase | 1.2.1.10 | NA | - 3 |
| Alcohol dehydrogenase | 1.1.1.1 | NA | 3 |
| Phosphate acetyltransferase | 2.3.1.8 | NA | $+{ }^{3}$ |
| Acetate kinase | 2.7.2.1 | NA | $+{ }^{3}$ |

Notes: +: Detected activity; -: No detected activity; NA: Activity not tested.
References: [1] Manolukas et al., 1988; [2] Constantopoulos et al., 1987; [3] Tourtellotte et al., 1960.

One could also infer from the pathways present in most organisms that AcCoA would rather be used for lipid metabolism instead of acetate production given the small detected concentration of this end product in M. hyorhinis. Usage of Acetyl coenzyme-A in lipid biosynthesis is dependent on the presence of the acyl carrier protein (ACP) [231]. ACP acts as an acyl chain carrier/donor in lipid biosynthesis [305]. Since M. hyopneumoniae and M. flocculare lack the gene coding for ACP, it is likely that this activity is absent in these two organisms. Although M. hyorhinis possesses a gene coding for ACP, it is yet undefined experimentally if AcCoA could be directed towards lipid metabolism in this organism. In Mycoplasma gallisepticum, and many other mycoplasmas, the synthesis of long-chain fatty acids from AcCoA is impaired due to low levels of ACP activity [231]. Thus, the sole presence of the ACP gene (or any other enzyme-coding gene for that matter) is not directly related to enzyme activity.

As for cofactor metabolism, the only experimental data available is related to thiamine metabolism in M. hyorhinis [253]. The protein p37 was described as an extracytoplasmic thiamine-pyrophosphate binding lipoprotein, responsible for the uptake of this cofactor into the cells.

After the reconstruction of the networks, we also decided to retrieve more experimental data to enrich our analyses and validate our reconstructed models. These results will be presented in the next chapter, but, keep in mind that even though the networks were reconstructed prior to experiments, we had to slightly modify some reactions at a final step, based on the experimental data.

### 2.2 Methods

### 2.2.1 Selected Species

We chose a total of 12 strains that had their genomes available from M. hyopneumoniae ( 6 strains), M. hyorhinis (4 strains) and M. flocculare (2 strains). Detailed information on each of them may be
seen in Table 2.2).
Table 2.2: Selected species and strains, abbreviation, NCBI accession numbers and experimental availability of strains.

| Species | Strain | Accession Number | Abbreviation | Pathogenicity Level | Experimental <br> Availability ${ }^{* 1}$ | Reference |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Mycoplasma hyopneumoniae | 168 | NC_017509 | MHP168 | Pathogenic | Not available | Liu et al., 2011 |
|  | 168L | CP003131 | MHP168L | Attenuated | Not available | Liu et al., 2013 |
|  | 232 | AE017332 | MHP232 | Pathogenic | Not available | Minion et al., 2004 |
|  | 7422 | NC_021831 | MHP7422 | Pathogenic | Available in Brazil | Siqueira et al., 2013 |
|  | 7448 | AE017244 | MHP7448 | Pathogenic | Available in Brazil | Vasconcelos et al., 2005 |
|  | J, ATCC 25934 | AE017243 | MHPJ | Attenuated | Available in Brazil and France | Vasconcelos et al., 2005 |
|  | All strains | NA | MHP | NA | NA | NA |
| Mycoplasma hyorhinis | HUB-1 | NC_014448 | MHRHUB1 | Pathogenic | Not available | Liu et al., 2010 |
|  | GDL-1 | NC_016829 | MHRGDL1 | $N A^{*}{ }^{2}$ | Not available | Calcutt et al., 2012 |
|  | SK76 | NC_019552 | MHRSK76 | Pathogenic | Not available | Goodison et al., 2013 |
|  | ATCC 17981 | ARTL00000000 | MHR17981 | Attenuated | Available in Brazil and France | Kyrpides et al., 2013 ${ }^{* 3}$ |
|  | All strains | NA | MHR | NA | NA | NA |
| Mycoplasma flocculare | ATCC 27399 | CP007585 | MFL27399 | Non pathogenic | Available in France | Calcutt et al., 2015 |
|  | ATCC 27716 | AFCG01000000 | MFL27716 | Non pathogenic | Available in Brazil | Siqueira et al., 2013 |
|  | All strains | NA | MFL | NA | NA | NA |
| All species | All strains | NA | Pan-Network | NA | NA | NA |

Notes:
NA : Not available
${ }^{*}$ 1: Strains available only in Brazil are protected strains which are property of the government and cannot be used outside authorised laboratories.
Strains available in France were purchased from the ATCC repository; these strains were also available for testing in the laboratory in Brazil.
*2: M. hyorhinis strain GDL-1 was retrieved from a contaminated cell line and, to our knowledge it has never been reported either as pathogenic or as an attenuated strain.
*3: The genome of M. hyorhinis strain ATCC 17981 is available online but not published in any paper up to now. (source: http://genomeportal.jgi.doe.gov/Mychy1/Mychy1.info.html)

It is interesting to note here that while M. hyopneumoniae strains 232, 7422, 7448 and 168 are considered pathogenic, strains J and 168L became attenuated after serial passages of in vitro culture. M. hyorhinis strains HUB-1, SK76 and ATCC 17981 were isolated from swines, while GDL-1 was isolated from a tissue cell line. M. flocculare strain ATCC 27399 was isolated directly from swine pneumonia and ATCC 27716 is derived from the 27399 strain. A diagram showing the information on pathogenicity can be seen in Figure 2.2. Throughout the thesis from now on we will refer to each strains as abbreviated in Table 2.2).


Figure 2.2: Schematic diagram to show the pathologicity of each strain from M. hyopneumoniae, M. hyorhinis and M. flocculare.

### 2.2.2 Biomass Composition and Biomass Reaction Assembly

In order to simulate growth, we had to estimate the average cell composition of the three mycoplasmas (biomass composition). To this end, we used a general macromolecule Mycoplasma cell composition from Razin and collaborators [218]. Depending on the species, the authors showed that the solid residue contained $54-62 \%$ of proteins, $12-20 \%$ of lipids, $3-8 \%$ of carbohydrates, $8-17 \%$ of RNA, and $4-7 \%$ of DNA. The membranes comprised around $35 \%$ of dry weight of the organisms and contained $47-60 \%$ of proteins, $35-37 \%$ lipids, $4-7 \%$ carbohydrates and small amounts of DNA and RNA [218]. Since no information on metal ions and cofactors was available, we included them quantitatively based on the metabolic networks reconstructed for related mycoplasmas [307, 266]. Membrane and lipid components were added based on the literature composition for the selected species [38, 158, ?].

Based on the previous information, we assumed the following fractions of macromolecules: $55 \%$ proteins, $15 \%$ lipids, $6.88 \%$ carbohydrates, $12 \%$ RNA, $6 \%$ DNA, and $5.12 \%$ of ions and cofactors. To create a biomass elemental formula, we took into account the percentage contribution of each of the components to the overall cell. For example, if we had a biomass that consisted of $50 \%$ of glucose and $50 \%$ water, the elemental contribution of each one to the overall elemental formula would be:

$$
\begin{aligned}
50 \% \text { of Glucose } & =0.00278 \mathrm{~mol} \text { of glucose } / \mathrm{g} \text { of biomass; } \\
50 \% \text { of Water } & =0.02778 \mathrm{~mol} \text { of water } / \mathrm{g} \text { of biomass. }
\end{aligned}
$$

The biomass elemental formula would be:

$$
0.00278 *\left(\mathrm{C}_{6} \mathrm{H}_{12} \mathrm{O}_{6}\right)+0.02778 *\left(\mathrm{H}_{2} \mathrm{O}\right)=C_{0.00167} H_{0.0889} O_{0.445}
$$

The biomass reaction, on the other hand, represented the drain of these components into biomass production. It assembled implicitly DNA replication, RNA transcription, and protein synthesis into one single reaction:
Amino acids + Nucleic Acids + Lipids +
Carbohydrates + lons + Cofactors + Water + Energy
Biomass +
Carbohydrates + lons + Cofactors + Water + Energy $\longrightarrow$ Subproducts

Amino acids were indirectly included in the biomass reaction: charged tRNAs were accounted as substrates and uncharged tRNAs as products. Growth and non-growth associated maintenance (GAM and NGAM) were estimated based on the literature and were manually added to the biomass equation [75, 178].

### 2.2.3 Network Reconstruction and Refinement

We reconstructed the metabolic networks of the 6 strains of M. hyopneumoniae ( $\mathrm{J}, 232,7422,7448$, 168 and 168L), 4 strains of M. hyorhinis (HUB-1, GDL-1, SK-76 and ATCC 17981 strain BTS7) and 2 strains of M. flocculare (ATCC 27716 and ATCC 27399). The semi-automated reconstructions were generated by the Pathologic tool from the Pathway Tools software [121] using the complete genomes available online (Table 2.2).

Pathway Tools automatically associated genes with reactions, based on the annotation names, Gene Ontology (GO) terms and enzyme code (EC) numbers contained in the GenBank files. The software assembled the reactions into pathways by comparing them with the reference database, MetaCyc [121]. The software automatically added missing reactions from the reference database to the model, creating many orphan reactions (reactions that did not have an enzyme associated to them). The Pathway Hole Filler tool, included in the Pathway Tool software, attempted to identify genes associated with these orphan reactions.

After this initial draft reconstruction, we deleted the remaining orphan reactions and compared the resulting set of reactions from all organisms by using the subsystems approach [191] modified to reaction annotation. This approach improves accuracy in high-throughput annotation by having one single expert annotating one subsystem at a time over multiple genomes simultaneously, instead of
having one expert per genome. In our work, since the genomes were already annotated, refinement of the networks was made in a subsystems approach simultaneously on all organisms. As a result of different genomic annotations, slightly different reactions arose throughout the models. These reactions were checked for consistency, and after validation of one of them, the duplicates were deleted. Whenever only one organism was missing an essential enzyme for in silico growth, that all other organisms had, we assumed as an indication that such an activity was likely to occur in all organisms. This was assumed only when the alternative way for the biomass to grow was to add a large set of orphan reactions to the model.

Gene-Protein-Reaction (GPR) associations were systematically validated or included based on experimental data, then on the information provided by the Pfam functional domain database [12] and by reciprocal sequence homology (BLASTp [5]) searchesintegrated with a synteny analysis (gene context analysis). Homologous proteins with equivalent functional domains were assigned as isozymes to a particular reaction, while proteins with distinct functional domains were assigned as subunits of a multi-protein complex. Non-metabolic reactions such as DNA polymerization, replication and repair, protein synthesis and modification, RNA synthesis and turnover, among others, were explicitly deleted from the network but implicitly included in the biomass assembly. Generic reactions such as Carbohydrate + ATP $=>$ ADP + Carbohydrate -P were either specified or excluded from the models.

The resulting models were further refined in accordance with a detailed protocol from Thiele and Palsson (2010). Reactions were computationally balanced for mass and charge, while cofactor usage was determined based on literature data for the studied species or for closely related ones.

Reaction directionality was thermodynamically checked and validated. The standard transformed Gibbs energy of a reaction $\left(\Delta r G^{\prime}\right)$ determines its direction of net flux. Although all biochemical reactions in nature are, in principle, reversible [179], at physiological concentrations of the metabolites, the $\Delta r G^{\prime}$ of some reactions has always the same sign. A negative $\Delta r G^{\prime}$ corresponds to a forward net flux, and vice-versa ( $\Delta r G^{\prime}>0$ imposes a reverse flux); these are called irreversible reactions. Quantitatively reversible reactions have a physiological range of transformed Gibbs energy that spans the zero line [82]. In this way, reaction directionality was determined based on the component contribution method [183, 184], which extends the group contribution method [114] and achieves a significant improvement in accuracy in estimations of standard Gibbs energies. An online search and calculation interface called eQuilibrator along with metabolite and reaction thermodynamic databases are available at www.equilibrator.weizmann.ac.il [81]. Heuristic rules were used to improve the directionality assignment. Generally, a reaction is irreversible in the direction of consumption of a high-energy co-substrate [139], which includes phosphorylation, coenzyme-A/ACP ligase, carboxylation, and ATP-dependent reactions.

Gaps in the metabolic network were filled only to allow known functionalities of the organisms or biomass production in the reconstructed models (see next section for biomass assembly and composition). A wide range of automated gap filling approaches are available to enable filling-in network gaps based on a comparison with reference databases. Based on a methodology established by Kumar and collaborators [238], we incorporated the minimal number of gap filling reactions into the models to limit unusual behaviors. We analyzed the network topology to identify compounds that were only produced or consumed in the network, the so-called dead-end metabolites (DEM). Whenever a DEM was found, we either (i) added a gap filling reaction to reconnect it to the rest of the network, or (ii) removed the reaction from the model when both substrate and product were disconnected from the network and did not affect the overall metabolism.

Transporters were predicted at first from genome annotation using the Transport Identification Parser, from Pathway Tools [144]. Specific transport reactions required for production of biomass components were also manually added to the final version of the networks.

### 2.3 Results

### 2.3.1 Biomass Composition and Biomass Reaction Assembly

## Biomass Composition

The biomass reaction drained all precursors (in their molar biological ratios) into biomass. Based on the previously mentioned macromolecule composition (Table 2.3), we were able to separately calculate the biomass components per gram dry weight of biomass (g DW). The nucleotide and protein compositions were derived from the genome sequence; the lipid, carbohydrate, ion and cofactor compositions were based on literature data.

Table 2.3: Average cellular composition of M. flocculare, M. hyorhinis and M. hyopneumoniae.

| Macromolecule | $\%(w / w)$ | $\%(g / g$ DW $)$ |
| :---: | :---: | :---: |
| DNA | 6.00 | 0.0600 |
| RNA | 12.00 | 0.1200 |
| Proteins | 55.00 | 0.5500 |
| Lipids | 15.00 | 0.1500 |
| Cofactors | 1.68 | 0.0168 |
| lons | 3.44 | 0.0344 |
| Carbohydrates | 6.88 | 0.0688 |

## DNA composition

An average DNA composition based on the genome sequence for all strains was defined (Table 2.4).
Table 2.4: Guanine-Cytosine (GC) and Adenine-Thymine (AT) content from all strains, except for MHR17981. Since there was no genome announcement for MHR17981 and no complete chromosome, this strain was not taken into account.

| Strain | \%AT | \%CG |
| :---: | :---: | :---: |
| MHP168 | 71.54 | 28.46 |
| MHP168L | 71.54 | 28.46 |
| MHP232 | 71.51 | 28.49 |
| MHP7422 | 71.50 | 28.5 |
| MHP7448 | 71.50 | 28.5 |
| MHPJ | 72.11 | 27.89 |
| MHRHUB1 | 74.12 | 25.88 |
| MHRGDL1 | 71.91 | 28.09 |
| MHRSK76 | 72.21 | 27.79 |
| MRH17981 | NA | NA |
| MFL27716 | 71.10 | 28.90 |
| MFL27399 | 71.04 | 28.96 |
| Average | $\mathbf{7 1 . 8 3}$ | $\mathbf{2 8 . 1 7}$ |

The average molecular weight of a nucleotide residue (inside DNA) was calculated from the molecular weight of nucleotides (Table 2.5).

Based on an average molecular weight of $306.82 \mathrm{~g} / \mathrm{mol}$ of DNA residue, we concluded that 0.06 g of DNA (Table 2.3) corresponded to 0.1955 mmol of DNA per g dW . The concentration of each base in $\mathrm{mmol} / \mathrm{gdW}$ was estimated and may be found in the biomass total composition (available in the end of this section, in Table 2.13).

Table 2.5: Average DNA content from all strains and average molecular weight of nucleotide residue in DNA.

| Nucleotide <br> in DNA | \% ( $\mathrm{mol} / \mathrm{mol}$ ) | MW of residue ( $\mathbf{g} / \mathrm{mol}$ ) |
| :---: | :---: | :---: |
| dAMP | 35.915 | 311.19 |
| dTMP | 35.915 | 302.18 |
| dGMP | 14.085 | 327.19 |
| dCMP | 14.085 | 287.17 |
| Average MW of DNA residue | 306.82 |  |

## RNA composition

The RNA composition was calculated similarly to the DNA composition. The GC and AT contents were based only on open reading frame (ORF) sequences annotated in GenBank files (Table 2.6). An average nucleotide molecular weight inside the RNA molecules was calculated and can be found in Table 2.7.

Table 2.6: Theoretical RNA nucleotide content from all strains.

| Strain | AMP | UMP | CMP | GMP |
| :---: | :---: | :---: | :---: | :---: |
| MHP168 | 38.47 | 32.98 | 13.73 | 14.82 |
| MHP168L | 38.43 | 32.97 | 13.77 | 14.83 |
| MHP232 | 38.37 | 32.94 | 13.87 | 14.82 |
| MHP7422 | 38.37 | 32.99 | 13.80 | 14.84 |
| MHP7448 | 38.30 | 33.01 | 13.85 | 14.84 |
| MHPJ | 38.40 | 32.88 | 13.88 | 14.84 |
| MHRHUB1 | 40.14 | 33.16 | 12.85 | 13.85 |
| MHRGDL1 | 39.86 | 33.42 | 12.86 | 13.86 |
| MHRSK76 | 40.25 | 33.12 | 12.84 | 13.79 |
| MRH17981 | 40.65 | 33.07 | 12.56 | 13.72 |
| MFL27716 | 38.06 | 32.09 | 14.45 | 15.40 |
| MFL27399 | 37.96 | 32.18 | 14.46 | 15.40 |
| Average | 38.94 | 32.90 | $\mathbf{1 3 . 5 8}$ | 14.58 |

Table 2.7: Average RNA content from all strains and average molecular weight of the nucleotide residue in RNA.

| Nucleotide <br> in RNA | \% (mol/mol) | MW of residue (g/mol) |
| :---: | :---: | :---: |
| AMP | 38.94 | 327.19 |
| UMP | 32.9 | 304.15 |
| GMP | 14.58 | 343.19 |
| CMP | 13.58 | 303.17 |
| Average MW of RNA residue |  | $\mathbf{3 1 8 . 6 8}$ |

Based on an average molecular weight of $318.68 \mathrm{~g} / \mathrm{mol}$ of RNA residue, we concluded that 0.12 g of RNA (Table 2.3) corresponded to 0.3765 mmol of RNA per gdW; the concentration of each base in $\mathrm{mmol} / \mathrm{g}$ dW was estimated and is found in Table 2.13.

## Amino acid composition

Amino acid composition accounted for all cellular proteins and was estimated by sequence analysis of translated mRNAs (Table 2.8). The average molecular weight of an amino acid residue was calculated and can be found in Table 2.9.

Table 2.8: Theoretical amino acid content from all analyzed strains (in \%).

| Amino acid | MHP168 | MHP168L | MHP232 | MHP7422 | MHP7448 | MHPJ | MHRHUB1 | MHRGDLI | MHRSK76 | MHR17981 | MFL27716 | MFLL27399 | AVg |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Alanine | 5.12 | 5.07 | 5.05 | 5.05 | 4.97 | 5.04 | 5.06 | 5.05 | 5.05 | 5.06 | 5.34 | 5.35 | $\mathbf{5 . 1 0}$ |
| Arginine | 2.96 | 2.97 | 2.97 | 2.97 | 3.00 | 2.97 | 2.92 | 2.94 | 2.89 | 2.87 | 2.89 | 2.91 | $\mathbf{2 . 9 4}$ |
| Asparagine | 7.66 | 7.64 | 7.62 | 7.62 | 7.62 | 7.61 | 7.61 | 7.60 | 7.62 | 7.61 | 7.71 | 7.68 | $\mathbf{7 . 6 3}$ |
| Aspartic Acid | 4.74 | 4.77 | 4.78 | 4.78 | 4.85 | 4.80 | 4.87 | 4.83 | 4.89 | 4.90 | 4.62 | 4.67 | $\mathbf{4 . 7 9}$ |
| Cysteine | 0.43 | 0.42 | 0.41 | 0.41 | 0.41 | 0.41 | 0.41 | 0.42 | 0.41 | 0.41 | 0.45 | 0.42 | $\mathbf{0 . 4 2}$ |
| Glutamic Acid | 6.44 | 6.47 | 6.47 | 6.47 | 6.52 | 6.48 | 6.49 | 6.48 | 6.50 | 6.50 | 6.36 | 6.44 | $\mathbf{6 . 4 7}$ |
| Glutamine | 3.99 | 4.01 | 4.04 | 4.05 | 4.06 | 4.06 | 4.12 | 4.08 | 4.14 | 4.14 | 3.92 | 3.94 | $\mathbf{4 . 0 5}$ |
| Glycine | 4.57 | 4.57 | 4.58 | 4.58 | 4.62 | 4.59 | 4.50 | 4.54 | 4.47 | 4.43 | 4.58 | 4.57 | $\mathbf{4 . 5 5}$ |
| Histidine | 1.24 | 1.23 | 1.22 | 1.22 | 1.22 | 1.22 | 1.27 | 1.25 | 1.29 | 1.31 | 1.27 | 1.28 | $\mathbf{1 . 2 5}$ |
| Isoleucine | 9.91 | 9.91 | 9.90 | 9.88 | 9.86 | 9.87 | 9.71 | 9.80 | 9.66 | 9.63 | 9.88 | 9.89 | $\mathbf{9 . 8 3}$ |
| Leucine | 9.93 | 9.96 | 9.97 | 9.97 | 9.95 | 9.95 | 9.92 | 9.94 | 9.90 | 9.90 | 9.84 | 9.81 | $\mathbf{9 . 9 2}$ |
| Lysine | 10.75 | 10.77 | 10.77 | 10.76 | 10.70 | 10.76 | 10.74 | 10.73 | 10.74 | 10.73 | 10.68 | 10.74 | $\mathbf{1 0 . 7 4}$ |
| Methionine | 1.25 | 1.25 | 1.25 | 1.25 | 1.29 | 1.25 | 1.28 | 1.27 | 1.29 | 1.30 | 1.24 | 1.23 | $\mathbf{1 . 2 6}$ |
| Phenylalanine | 6.93 | 6.93 | 6.93 | 6.92 | 6.89 | 6.91 | 6.76 | 6.83 | 6.71 | 6.68 | 6.93 | 6.88 | $\mathbf{6 . 8 6}$ |
| Proline | 3.00 | 3.01 | 3.03 | 3.03 | 3.07 | 3.04 | 2.94 | 2.99 | 2.90 | 2.88 | 2.98 | 2.96 | $\mathbf{2 . 9 9}$ |
| Serine | 7.11 | 7.09 | 7.09 | 7.09 | 7.04 | 7.09 | 7.11 | 7.09 | 7.12 | 7.12 | 7.18 | 7.10 | $\mathbf{7 . 1 0}$ |
| Threonine | 4.54 | 4.52 | 4.51 | 4.52 | 4.44 | 4.51 | 4.67 | 4.60 | 4.74 | 4.78 | 4.66 | 4.66 | $\mathbf{4 . 6 0}$ |
| Tryptophan | 0.99 | 0.99 | 1.00 | 1.00 | 1.03 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.99 | 0.95 | $\mathbf{0 . 9 9}$ |
| Tyrosine | 3.67 | 3.68 | 3.69 | 3.69 | 3.75 | 3.70 | 3.72 | 3.71 | 3.74 | 3.75 | 3.61 | 3.64 | $\mathbf{3 . 7 0}$ |
| Valine | 4.76 | 4.74 | 4.73 | 4.74 | 4.71 | 4.73 | 4.90 | 4.83 | 4.95 | 5.01 | 4.87 | 4.87 | $\mathbf{4 . 8 2}$ |

Table 2.9: Average amino acid content from all strains and average molecular weight of the amino acid residues inside proteins.

| Amino acid | \% (mol/mol) | MW of residue (g/mol) |
| :---: | :---: | :---: |
| Alanine | 5.10 | 71.08 |
| Arginine | 2.94 | 157.19 |
| Asparagine | 7.63 | 114.10 |
| Aspartic Acid | 4.79 | 114.08 |
| Cysteine | 0.42 | 103.14 |
| Glutamic Acid | 6.47 | 128.11 |
| Glutamine | 4.05 | 128.13 |
| Glycine | 4.55 | 57.05 |
| Histidine | 1.25 | 137.14 |
| Isoleucine | 9.83 | 113.16 |
| Leucine | 9.92 | 113.16 |
| Lysine | 10.74 | 129.18 |
| Methionine | 1.26 | 131.20 |
| Phenylalanine | 6.86 | 147.17 |
| Proline | 2.99 | 97.12 |
| Serine | 7.10 | 87.08 |
| Threonine | 4.60 | 101.10 |
| Tryptophan |  | 0.99 |
| Tyrosine | 3.70 | 186.21 |
| Valine |  | 4.82 |
| Average MW of Amino Acid Residue |  | 163.17 |
|  |  | 99.13 |
|  |  | 114.99 |

Based on an average molecular weight of $114.99 \mathrm{~g} / \mathrm{mol}$ of amino acid residue, we concluded that 0.55 g of proteins (Table 2.3) corresponded to 4.78 mmol of proteins per gdW ; the concentration of each amino acid in $\mathrm{mmol} / \mathrm{gdW}$ was estimated and may be found in Table 2.13.

## Lipid composition

Lipids were divided in three categories: sterols, phospholipids and glycolipids [158, 137]. Phospholipids and glycolipids were separated into two fractions: elementary portion and fatty acid radicals (Figure 2.3). Based on the fatty acid composition (Table 2.10 from literature data [38, 158, 137], we were able to estimate an average fatty acid molecular weight to incorporate into the elementary lipids portion. The fatty acids associated with elementary portions of lipids seem to mimic the total fatty acid composition of the organism and are dependent on the composition of the culture medium [52, 158].


Figure 2.3: Lipids were split into an elementary portion fatty acid radicals. Phospholipids and glycolipids were taken into account as an elementary part and two or more fatty acids: Sphingomyelin (SPM), phosphatidylcholine (PC), phosphatidylglycerol (PG), monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are composed by the elementary fractions and two fatty acid radicals, cardiolipin (CL) consists of its elementary portion and 4 fatty acid radicals. Cholesterol has no fatty acid radicals. Fatty acid radicals can occur in saturated (XX:1 or XX:2) or unsaturated form (XX:0). For nomenclature and details see Table 2.10. Multiple combinations of fatty acids and elementary portions can occur, this is why we treated each of them separately.

The main difference between M. hyorhinis and M. hyopneumoniae is that M. hyorhinis does not possess glycolipids [158]; Conversely, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) have been previously detected in M. hyopneumoniae [38]. Since no information was available for M. flocculare, we extrapolated the data from M. hyopneumoniae, because of the proximity between species, and included both glycolipids in its biomass composition.

Table 2.10: Fatty acid saturation and composition in M. hyopneumoniae (data extracted from [38] and [158]).

| Fatty Acids | Size:Saturation | $\%(w / w)$ |
| :---: | :---: | :---: |
| Lauric | $12: 0$ | 8.8 |
| Myristic | $14: 0$ | 7.6 |
| Palmitic | $16: 0$ | 32.9 |
| Palmitoleic | $16: 1$ | 1.7 |
| Stearic | $18: 0$ | 7.0 |
| Oleic | $18: 1$ | 34.5 |
| Linoleic | $18: 2$ | 7.5 |

Table 2.11: Overall lipid composition and average molecular weight of a complete lipid of M. hyopneumoniae (MHP), M. hyorhinis (MHR) and M. flocculare (MFL).

| Lipid | MW (g/mol) | MHR <br> $\%(\mathbf{w} / \mathbf{w})$ | MHP/MFL <br> $\%(w / w)$ |
| :---: | :---: | :---: | :---: |
| Cholesterol | 370.65 | 50.0 | 25.0 |
| CL | 1378.00 | 20.7 | 20.7 |
| PC | 746.11 | 6.3 | 6.3 |
| PG | 735.04 | 6.2 | 6.2 |
| SPM | 927.52 | 16.8 | 16.8 |
| MGDG | 744.13 | 0 | 16.7 |
| DGDG | 906.28 | 0 | 8.3 |
| Average Complete Lipid MW (g/mol) | $\mathbf{7 1 9 . 0}$ | $\mathbf{8 2 5 . 8}$ |  |

Notes: MW includes the elementary molecular weight of each lipid and the average fatty acid molecular weight in the correct quantities (as described in Figure 2.3). When present, the percentage of phospholipids equals the combined percentages of neutral and glycolipids. When absent, neutral lipid content equals the phospholipid content [158]. Lipid composition was based on [?], [158] and [38]. We corrected the composition of MHP and MFL to accommodate the presence of glycolipids (MGDG and DGDG), but kept the relative ratio between phospholipids. CL: Cardiolipin; PC: Phosphatidylcholine; PG: Phosphatidylglycerol; SPM: Sphyngomyelin; MGDG: Monogalactosyldiacylglycerol; DGDG: Digalactosyldiacylglycerol.

Based on an average molecular weight of $719.0 \mathrm{~g} / \mathrm{mol}$ for M. hyorhinis (Table 2.11, we estimated that 0.15 g of lipids (Table 2.3) corresponded to 0.2086 mmol of lipids per g DW; the concentration of each elementary lipid and each fatty acid in $\mathrm{mmol} / \mathrm{g}$ DW was estimated and may be found in Table 2.13. Based on an average molecular weight of $789.3 \mathrm{~g} / \mathrm{mol}$ for M. hyopneumoniae and M. flocculare, we estimated that 0.15 g of lipids (Table 2.3) corresponded to 0.1816 mmol of lipids per g DW; the concentration of each elementary lipid and each fatty acid in mmol/g DW was estimated and may be found in Table 2.13.

## Ions and Cofactors

We adapted the ions and cofactors composition from the metabolic network available for M. pneumoniae [307]. The concentration of each compound can be found directly in Table 2.13.

## Carbohydrate composition

The structural unit for carbohydrate/polysaccharide fraction was fixed only as glucose for simplicity reasons. However, the polysaccharide composition in these species was not yet determined. The concentration of glucose can be found directly in Table 2.13.

## Biomass Elementary Composition

Considering the percentage contribution of each of the components to the overall cell, an approximate biomass elementary composition for $M$. hyorhinis was computed as follows:

$$
\mathrm{CH}_{1.57} \mathrm{O}_{0.36} N_{0.21} P_{0.02} S_{0.02} ;
$$

We had slight differences for M. hyopneumoniae and M. flocculare:

$$
C H_{1.59} O_{0.34} N_{0.21} P_{0.16} S_{0.02},
$$

with traces of calcium, chlorum, cobalt, copper, iron, potassium, magnesium, manganese, molibdenium and zync. The molecular weight of the biomass for the three reconstructed species on a C-mole basis was approximated to $23.5 \mathrm{~g} / \mathrm{C}-\mathrm{mol}$.

## Biomass Reaction Assembly

In order to create the biomass, the cell must unwind and replicate DNA, transcribe and degrade RNA, translate and modify proteins, among others. The approximate costs for several maintenance functions associated with growth (GAM) were calculated and are presented in Table 2.12.

Table 2.12: Energy requirements for biomass assembly (adapted from [178]).

| Synthesis and Processing |  | umol ATP / $\mu \mathrm{mol}$ macromolecule | umol ATP/ gDW |
| :---: | :---: | :---: | :---: |
| DNA | Unwinding helix | 1.000 | 195.60 |
|  | Proofreading | 0.360 | 70.40 |
|  | Negative supercoiling | 0.100 | 19.60 |
|  | Discontinuous synthesis | 0.006 | 1.20 |
|  | Methylation | 0.001 | 0.20 |
| RNA | Discarding segments | 0.380 | 143.10 |
|  | Modification | 0.020 | 7.50 |
| Protein | mRNA Synthesis | 0.200 | 956.60 |
|  | Activation and incorporation | 2.000* | 9565.70 |
|  | Proofreading | 0.100 | 478.30 |
|  | Assembly and modification | 0.006 | 28.70 |
| TOTAL umol of ATP |  |  | 11466.90 |

Notes: To adjust the ATP requirements and accommodate tRNA charging directly into the models, activation and incorporation were discounted 2 ATPs per $\mu \mathrm{mol}$ of protein.

We also had to take into consideration that for every polymerization step (and every ATP used), we have extra substrates and products. For instance, even if we consider that in DNA, cytosine is in the form of dCMP, the reaction in vivo uses a dCTP as substrate and produces a pyrophosphate $(\mathrm{PPi})$, and finally incorporates a molecule of dCMP into the biomass:

$$
\begin{gathered}
\mathrm{n} \mathrm{dNTPs}+\mathrm{n} \mathrm{ATP} \rightarrow \mathrm{DNA}+\mathrm{n} \mathrm{PPi}, \\
\mathrm{n} \mathrm{NTP}+\mathrm{n} \text { ATP } \rightarrow \mathrm{RNA}+\mathrm{n} \mathrm{PPi}, \\
\mathrm{n} \text { Amino Acids }+\mathrm{n} \text { ATP } \rightarrow \text { Protein }+\mathrm{n} \text { ADP }+\mathrm{n} \mathrm{Pi}+\mathrm{n} \mathrm{H}_{2} O,
\end{gathered}
$$

From all these estimated quantities, we were able to assemble the biomass reaction into the following form:

Biomass Precursors +11.53 ATP $+6.11 \mathrm{H}_{2} \mathrm{O}+$ Charged tRNAs $\rightarrow$ (cont) (cont) $\rightarrow$ Biomass +11.46 $\mathrm{ADP}+11.45 \mathrm{Pi}+11.46 \mathrm{H}^{+}+0.57 \mathrm{PPi}+$ Uncharged tRNAs,
where the stoichiometric coefficients for all biomass precursors can be found in Table 2.13 (with the exception of ATP, Pi and Ppi, which were adapted for ATP maintenance functions).

### 2.3.2 Model Reconstruction and Refinement

Based on the published genomes of 6 strains of M. hyopneumoniae, 4 strains of M. hyorhinis and 2 strains of $M$. flocculare, we built 16 genome-scale metabolic reconstructions: one model for each strain separately, one for each species and a pan-reconstruction for all three species (Table 2.2). The semi-automated reconstructions were manually refined according to the description given in Methods section. The Pan-Swine mycoplasma network (representing the merge of all strains, and called pannetwork) was initially composed by 829 reactions, of which 269 consisted of orphan enzymes even after the Pathway Hole Filler step. These were excluded from the models. Duplicate reactions arose from the fact that Pathway Tools is based only on the annotations contained in the GenBank files. Since the software does not include sequence alignments against known databases, slightly different annotations in the GenBank files resulted in similar reactions with different IDs. In a preliminary comparison between one member of each species, we show here that out of 560 gene-associated reactions (from all strains), 48 were exclusive of MFL27716, 78 were exclusive of MHRHUB1 and 30 were exclusive of MHP7448 (Figure 2.4, A). The comparison between strains also revealed strain-specific reactions (Figure 2.4, B). Reactions present in each strain in this draft-automated reconstruction can be found in Table 2.14.


Figure 2.4: Venn diagrams representing the comparison of the draft reconstructions (A) between species and (B) between 4 selected strains of M. hyopneumoniae. This analysis revealed distinct metabolic capabilities (which further on we will explain as an error). MHR: M. hyorhinis; MHP: M. hyopneumoniae; MFL: M. flocculare.

Table 2.13: Biomass composition and element composition of each biomass precursor. Differences between species are marked with a yellow background.


Table 2.14: Preliminary comparison between reactions in all strains of all species.

| Strain | Rxns | Rxns Absent | Exclusive |
| :---: | :---: | :---: | :---: |
| MHP168 | 203 | 357 | 0 |
| MHP168L | 240 | 320 | 10 |
| MHP232 | 211 | 349 | 15 |
| MHP7422 | 227 | 333 | 7 |
| MHP7448 | 207 | 353 | 11 |
| MHPJ | 213 | 347 | 12 |
| MHRHUB1 | 248 | 312 | 32 |
| MHRGDL1 | 234 | 326 | 23 |
| MHRSK76 | 255 | 305 | 25 |
| MRH17981 | 309 | 251 | 67 |
| MFL27716 | 200 | 360 | 1 |
| MFL27399 | 199 | 361 | 0 |

After we ran reciprocal homolog searches between all organisms, we realized that most exclusive reactions had indeed duplicate reactions with different IDs in the other models (an example can be found in Figure 2.5).


Figure 2.5: An example of duplicate and generic reaction for cation import. Most organisms had predicted cation transport through the same homolog, but different IDs were designated in each organism. We curated the models by excluding generic reactions and including specific reactions for calcium, iron, copper and sodium transport since the genes were homologs.

In this way, we excluded generic and duplicate reactions and replaced them with specific and validated ones. Non-metabolic reactions were also excluded at this point, along with reactions that are known to correspond to absent capabilities of these mycoplasmas, such as heme, quinone or cytochrome dependent reactions [222]. The networks were also tested for the presence of DEM and the ability to produce all biomass precursors. DEMs were analyzed on a case-by-case manner. From initially 157 DEMs, 124 disconnected metabolites (along with 58 reactions) were excluded from the models. From these 124 excluded DEMs, 7 came from spontaneous reactions; 59 were carbohydrate
substrates derived from wide range transport reactions and were not used by any other reaction in the network; 58 were excluded as they did not interfere with the overall metabolism and the enzyme had already been assigned to one or many other reactions. It is interesting to point out that other unknown enzymes (or even moonlight enzymes) from these organisms may indeed use some of the excluded DEMs; however, since we have no experimental evidence at present, we could not assess their interference in the metabolic models. The remaining DEMs consisted mainly of cofactors and biomass precursors (such as nucleotides, amino acids, fatty acids) disconnected from the rest of the network. They were solved along with the biomass precursor check: 141 reactions were added to allow growth: the biomass reaction, 32 enzymatic reactions, 1 drain-synthetic reaction, 105 transport reactions and 5 spontaneous reactions. Transport reactions were considered as such even if an enzymatic activity was also present (i.e. the import of sugars with a concomitant phosphorylation of substrate). In order to correctly assign possible transporters to reactions, we performed an extensive literature research and reciprocal blast alignments. The results can be seen in the next section. We also changed some inconsistencies of reversibility and cofactor usage for several reactions. Even after all such efforts, four DEMs still remained: TTP, hexulose-6-phosphate, deoxyinosine and xanthosine-5-phosphate. After the final addition of 101 exchange reactions, we ran FBA tests to check the consistency of all models. If a reaction was essential for biomass growth and no homolog gene was found in the genome, an orphan reaction was added to the reconstruction for modeling reasons only. The resulting refined models had a total of 457 reactions and 258 GPR associations (depending on the model) and were compared similarly as seen previously in Figure 2.4. The results indicate that all strains from all species are indeed metabolically similar (Figure 2.6). In Figure 2.7 we show the complete Venn diagram for all strains of M. hyopneumoniae. Venn diagrams with more than 4 datasets are complex to understand, however, we wanted to elucidate that even though we only showed 4 strains in the previous figure, all strains from each species are extremely similar. In this case, the only difference between the models is the absence of 3 reactions in the network of MHP232, due to a missing gene coding for the alcohol dehydrogenase, present in all other strains.


Figure 2.6: Venn diagrams representing the comparison of refined networks (A) between species and (B) between strains of M. hyopneumoniae. This analysis shows that most of the metabolism is common to all organisms. MHR: M. hyorhinis; MHP: M. hyopneumoniae; MFL: M. flocculare.

The overall characteristics of each reconstruction can be seen in Table 2.15, and a list of all reactions added to the models along with the corresponding genes (when available) can be seen in Appendix A.


Figure 2.7: Venn diagrams representing the comparison of refined networks from all strains of M. hyopneumoniae.

Table 2.15: Characteristics of the reconstructed models.

| Model |  | Genes |  | Reactions |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Organism | Name | Total | $\operatorname{GPR}^{* 1}$ | Enzymatic ${ }^{* 2}$ | Transport ${ }^{*}$ | Spontaneous ${ }^{* 3}$ | $\text { Total }{ }^{*} 4$ |
| MHP232 | iMF170 | 170 | 233 | 208 | 111 | 9 | 426 |
| MHP168 | iMF172a | 172 | 234 | 209 |  |  | 427 |
| MHP168L | iMF172b | 172 |  |  |  |  |  |
| MHP7422 | iMF168 | 168 |  |  |  |  |  |
| MHP7448 | iMF171 | 171 |  |  |  |  |  |
| MHPJ | iMF172c | 172 |  |  |  |  |  |
| MHP | iMFmhp | NA*5 |  |  |  |  |  |
| MHRHUB1 | iMF177 | 177 | 239 | 209 | 111 | 6 | 423 |
| MHRGDL1 | iMF175 | 175 | 242 | 210 | 113 |  | 426 |
| MHRSK76 | iMF181 | 181 | 243 | 211 |  |  | 427 |
| MHR17981 | iMF182 | 182 |  |  |  |  |  |
| MHR | iMFmhr | $N A^{*} 5$ |  |  |  |  |  |
| MFL27399 | iMF159 | 159 | 217 | 196 | 105 | 9 | 401 |
| MFL27716 | iMF157 | 157 |  |  |  |  |  |
| MFL | iMFmfl | $N A^{* 5}$ |  |  |  |  |  |
| Pan-Network | iMFpan | $N A^{* 5}$ | 258 | 230 | 117 | 9 | 457 |

Notes:
*1: GPRs are gene-proteins-reaction associations present in each genome. For iMFmhp, iMFmhr, iMFmfl and iMFpan that account for more than one species, we added a GPR to the species model when any of the strains harbored a gene responsible for a specific activity.
*2: Reactions were considered as transport reactions even if the transporter was capable of performing a concomitant enzymatic activity.
*3: Spontaneous reactions included diffusion of small molecules and spontaneous conversions.
*4: The total number of reactions included also all exchange reactions needed for the mathematical modeling.
*5: Genes in the pan-network and species models were added synthetically (one per reaction), to enable a reaction essentiality analysis.

### 2.3.3 Characteristics of the models

In a way similar to most mycoplasma species studied so far [266, 314, 307], all the reconstructed networks in our study have low connectivity due to the simplicity of the biological model. Out of 457 reactions in the final pan-network, 258 had in at least one species a gene coding for the corresponding enzyme. From these, 212 were common to all species (Figure 2.6). The overall metabolism from the models reconstructed consisted of 11 distinct subsystems: amino sugar metabolism, amino acid metabolism, carbohydrate metabolism (further broken down into: glycolysis, pentose phosphate pathway, ascorbate degradation, myo-inositol degradation, general carbohydrate metabolism and pyruvate metabolism), cofactor metabolism, lipid metabolism, and nucleotide metabolism (Figure 2.8 for iMFpan; the GPR association in each species by subsystem can be seen in Table 2.16).

While all enzymes are present in the glycolytic pathway, most metabolic pathways have major enzyme gaps. For the pyruvate and cofactor metabolism, for instance, gaps accounted for up to $50 \%$ of the reactions (Figure 2.9, for iMFpan).


Figure 2.8: Distribution of the model reactions in the subsystems and types. The 457 reactions present in the model iMFpan were separated into (A) biological subsystems and (B) further into reaction types, with the exclusion of exchange reactions.

Table 2.16: All GPR associations for pan and species networks distributed in subsystems.

| Number of GPRs |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Subsystem | iMFmhp | iMFmhr | iMFmfl | iMFpan |  |
| Amino acid metabolism | 63 | 64 | 63 | 64 |  |
| Amino sugar metabolism | 4 | 7 | 4 | 8 |  |
| Glycolysis | 13 | 13 | 13 | 13 |  |
| Pentose Phosphate Pathway | 7 | 7 | 7 | 7 |  |
| Ascorbate degradation | 7 | 6 | 7 | 7 |  |
| Carbohydrate Metabolism | 14 | 27 | 12 | 27 |  |
| Myo-inositol degradation | 8 | 0 | 1 | 8 |  |
| Pyruvate Metabolism | 9 | 7 | 7 | 9 |  |
| Lipid metabolism | 17 | 16 | 11 | 19 |  |
| Nucleotide Metabolism | 59 | 60 | 59 | 60 |  |
| Cofactor metabolism | 33 | 36 | 33 | 36 |  |
| Total | $\mathbf{2 3 4}$ | $\mathbf{2 4 3}$ | $\mathbf{2 1 7}$ | $\mathbf{2 5 8}$ |  |



Figure 2.9: Distribution of the GPR associations by subsystems in iMFpan. This analysis accounted for 347 reactions (only enzymatic and transport reactions), from which 258 had GPR associations. TRUE: GPR associations present; FALSE: GPR associations missing.

Table 2.17: GPR exclusive associations for species networks distributed in subsystems.

| Number of Exclusive GPRs |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: |
| Subsystem | iMFmhp | iMFmhr | iMFmfl |  |
| Aminoacid metabolism |  | 5 |  |  |
| Amino sugar metabolism |  |  |  |  |
| Glycolysis |  |  |  |  |
| Pentose Phosphate Pathway |  |  |  |  |
| Ascorbate degradation |  | $\mathbf{1 3}$ |  |  |
| Carbohydrate Metabolism |  |  |  |  |
| Myo-inositol degradation | $\mathbf{7}$ |  |  |  |
| Pyruvate Metabolism | $\mathbf{2}$ | $\mathbf{2}$ |  |  |
| Lipid metabolism | $\mathbf{1}$ | $\mathbf{1}$ |  |  |
| Nucleotide Metabolism |  | $\mathbf{3}$ |  |  |
| Cofactor metabolism |  | 24 | $\mathbf{0}$ |  |
| Total | 10 |  |  |  |

M. flocculare was the only species that did not show any exclusive metabolic activities in the models. M. hyopneumoniae has 10 exclusive reactions, linked to myo-inositol metabolism and alcohol dehydrogenase activity. M. hyorhinis had 24 exclusive reactions; most of them correspond to carbohydrate metabolism (Table 2.17).

A global model comparing each species enzymatic capabilities can be seen in expanded Figures 2.35 and 2.36, at the end of this Chapter. Detailed versions of each subsystem can be found in the next section, along with zoomed images.

### 2.3.4 Subsystems Refinement in Detail

## Transporters

It is essential to any type of metabolic reconstruction to predict which metabolites may enter and may exit the cells in silico. For this, a set of transport assumptions is usually made and extensive searches take place to mimic as closely as possible the actual environment and capabilities of each species.

In general, transport can take place in two forms: passive or active transport (Figure 2.10). Passive Transport is the movement of a solute down its electrochemical gradient (A, B, C and D in Figure 2.10). This type of transport can take place with the aid of a facilitator protein (A, C and D) - permeases - or through simple diffusion (B). Molecules that diffuse freely across membranes are small and nonpolar. Examples include carbon dioxide, molecular oxygen and nitrogen. Weak acids also cross the membrane in the uncharged form [42]. Small, polar and uncharged molecules (such as water, ammonia, hydrogen peroxide and urea) can move across the plasma membrane through hydrophilic apertures (D) [47]. Active Transport, on the other hand, is the movement of a solute against its electrochemical gradient, with the aid of transporter proteins. Primary active transport (E, Figure 2.10) uses ATP hydrolysis or a high energy coupling molecule to drive the solute against the gradient, whereas secondary active transport (F, Figure 2.10) of a solute is enabled by another substrate moving down its gradient (an ion gradient, for instance).


Figure 2.10: Summary of transport types (Source [179])

Before the genome sequencing era, it was anticipated that because of their small genomes, reduced metabolism and complex nutritional demands, the Mollicutes should have a relatively larger fraction of membrane transporters than large genome bacteria [305]. However, such expectations were not confirmed after the sequencing of several genomes; the number of transporters seemed to be proportionally similar to the number present in the genomes of other bacteria [84, 105, 196, 203].

As a general rule, enzymes in reduced genomes seem to gain more functions than their homologs in large genomes [125]. This may be the case for several transporters with broad specificity in $M$. hyorhinis, M. hyopneumoniae and M. flocculare.

In this way, an extensive search for transporters throughout all species studied here was performed. Substrate assignment was based only on homology to characterized transporters in other species and must be experimentally confirmed. We tried to specify them as much as possible, although no experimental data were available for these species. Closely related species were also used to sustain our assumptions.

As mycoplasmas in general lack both electron transport and a functional TCA cycle, they use ATP as primary energy source. For instance, even though uncharged solutes, such as sugars and amino acids, may cross the membrane coupled with protons (secondary active transport), the proton motor force (pmf) detected in another Mollicutes species - Acholeplasma laidlawii - was proposed to arise from proton pumps primarily from ATP hydrolysis [42]. Indeed, these organisms have a great number of ATP-dependent transport systems (ATP binding cassette or ABC transport systems). Also, in fermentative species of mycoplasmas, sugar is thought to be transported through another active transport system, called group translocation transport systems (PEP:Pyruvate phosphotransferase systems or PTS).

ABC transporters are generally involved in the transport of a variety of substrates such as amino
acids, sugars, peptides and toxins. ABC transporters are integral membrane proteins that enable active transport across the lipid membrane against a concentration gradient of substrates, by using energy of ATP hydrolysis[174]. ABC transporters are composed of two transmembrane permeases (TMDs) and two nucleotide binding domains (NBDs), which are cytoplasmic proteins able to hydrolize a nucleotide triphosphate and a substrate binding domain, usually a lipoprotein and not necessarily needed if the substrate is delivered by the lipid bilayer. The results from the search for ABC transporters are summarized in Table 2.18, for one member of each species: MHRHUB1, MHR7448 and MFL27399. Complete systems had at least one TMD and one NBD that were either in the same gene or in separate genes. However, as proposed in other bacteria, several of the incomplete ABC systems may be functional, using components from the complete systems [272]. ABC incomplete components are listed in Table 2.19. We also found a great number of lipoproteins with no assigned function in the neighborhood of some of these ABC cassettes. These conserved but not characterized proteins were proposed as possible substrate binding proteins whenever a SBP was missing from the cassette (two examples can be seen in Figure 2.12.

Import through PTS, on the other hand, results in the phosphorylation of carbohydrate substrates whereas ABC transport does not modify the substrate (Figure 2.11), which means that ABC transport of carbohydrates requires more energy than PTS transport [28]. Phospho-transferase transport systems (PTSs) are widely spread among bacteria. They consist of two cytoplasmic energy-coupling proteins (enzyme I and phosphocarrier, Fig 2.11) and a range of substrate-specific enzymes II which simultaneously catalyze phosphorylation and translocation into the cell. The phosphorylation status of each component is also an indicator of carbohydrate availability and can be used as a signal to environmental conditions [138].


Figure 2.11: Schematic representation of an ABC transport system and a PTS. Components that are common to other transport systems are depicted in yellow and specific-substrate proteins are depicted in purple. Substrate (MET) is depicted in green and cofactors in blue (ATP, ADP, PEP, PYR). (a) ABC import systems are composed of 3 separate portions, a substrate binding protein (SBP), two transmembrane domains (TMD, usually heterodimers) and two nucleotide binding domains (NBD, usually homodimers). (b) PTS is composed by two general proteins, phophocarrier (HPr) and enzyme I (EI), common to all systems; Enzymes IIA (EIIA), IIB (EIIB) are cytoplasmic phosphocarriers and enzyme IIC (EIIC) forms a membrane channel.


Figure 2.12: Gene context and components of two ABC transport systems in M. hyorhinis (MHR), M. hyopneumoniae (MHP) and M. flocculare (MFL). Only one strain from each species was chosen (MHRHUB1, MHP7448, MFL27399). The nonspecific sugar ABC transport system has three conserved lipoproteins upstream the ATP-binding protein, whereas the specific glycerol ABC transport system has only one substrate binding protein. In blue, annotated genes coding for transmembrane domain proteins; in red, annotated genes coding for nucleotide-binding proteins; and in green, annotated genes coding for substrate binding protein. In dark grey, annotated genes coding for lipoproteins with unknown function. In this work, these lipoproteins were proposed to act as substrate binding proteins based on gene context.

Table 2.18: ABC systems present in M. hyorhinis, M. flocculare and M. hyopneumoniae. Only three strains are presented here: MHRHUB1, MHP7448 and MFL27399.

| ABC-Systems |  |  | ABC Components |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Substrate | Abbr | Organism | NBD |  | TMD |  | SBP |  |
| Sugar | ABC-Sga | MHRHUB1 | MHR_0064 |  | MHR_0065 | MHR_0066 | MHR_0061 | MHR_0062 <br> MHR_0063 |
|  |  | MHP7448 | MHP7448_0369 |  | MHP7448_0370 | MHP7448_0371 | MHP7448_0366 | MHP7448_0367\| <br> MHP7448_0368 |
|  |  | MFL27399 | MYF_01550 |  | MYF_01540 | MYF_01545 | MYF_01555 | MYF_01560\| MYF_01565 |
| Fructose/Arabinose/Galactose/Xylose | ABC-Fru | MHRHUB1 | MHR_0163 |  | MHR_0164 |  | MHR_0162 |  |
|  |  | MHP7448 | MHP7448_0514 |  | MHP7448_0515 |  | MHP7448_0513 |  |
|  |  | MFL27399 | MYF_00865 |  | MYF_00860 |  | MYF_00870 |  |
| Maltose/ Maltodextrin | ABC-Mal | MHRHUB1 | MHR_0194 |  | MHR_0195 | MHR_0196 | MHR_0487 |  |
|  |  | MHP7448 |  |  |  |  |  |  |
|  |  | MFL27399 |  |  |  |  |  |  |
| Myo-inositol | ABC-Myo | MHRHUB1 |  |  |  |  |  |  |
|  |  | MHP7448 | MHP7448_0231 |  | MHP7448_0233 |  | MHP7448_0234 |  |
|  |  | MFL27399 |  |  |  |  |  |  |
| Glycerol | ABC-Gly | MHRHUB1 | MHR_0123 |  | MHR_0121 | MHR_0122 | MHR_0276 |  |
|  |  | MHP7448 | MHP7448_0379 |  | MHP7448_0380 | MHP7448_0381 | MHP7448_0378 |  |
|  |  | MFL27399 | MYF_01485 |  | MYF_01475 | MYF_01480 | MYF_01490 |  |
| Peptides | ABC-Pep | MHRHUB1 | MHR_0319 |  | MHR_0318 |  | MHR_0318 |  |
|  |  | MHP7448 | MHP7448_0452 |  | MHP7448_0453 |  | MHP7448_0453 |  |
|  |  | MFL27399 | MYF_01280 |  | MYF_01275 |  | MYF_01275 |  |
| Oligopeptides 1 | ABC-Opp1 | MHRHUB1 | MHR_0359 | MHR_0360 | MHR_0361 | MHR_0362 | MHR_0357 |  |
|  |  | MHP7448 | MHP7448_0215 | MHP7448_0214 | MHP7448_0213 | MHP7448_0212 | MHP7448_0217 |  |
|  |  | MFL27399 | MYF_02620 | MYF_ 02615 | MYF_ 02610 | MYF_ 02605 | MYF 02595 | MYF_ 02630 |
| Oligopeptides 2 | ABC-Opp2 | MHRHUB1 | MHR_0635 | MHR_0636 | MHR_0637 | MHR_0638 | MHR_0639 |  |
|  |  | MHP7448 | MHP7448_0501 | MHP7448_0502 | MHP7448_0503 | MHP7448_0504 | MHP7448_0505 |  |
|  |  | MFL27399 | MYF_00935 | MYF_00930 | MYF_00925 | MYF_00920 | MYF_00915 |  |
| Nucleosides | ABC-Nucl | MHRHUB1 | MHR_0073 |  | MHR_0074 | MHR_0075 | MHR_0076 |  |
|  |  | MHP7448 | MHP7448_0605 |  | MHP7448_0606 | MHP7448_0607 | MHP7448_0604 |  |
|  |  | MFL27399 | MYF_02910 |  | MYF 02915 | MYF_ 02920 | MYF 02905 |  |
| Thiamine/ Phosphate/ Phosphonate | ABC-Pi | MHRHUB1 | MHR_0624 |  | MHR_0623 |  | MHR_0625 |  |
|  |  | MHP7448 | MHP7448_0361 |  | MHP7448_0362 |  | MHP7448_0360 |  |
|  |  | MFL27399 | MYF_01410 |  | MYF_ 01415 |  | MYF_01405 |  |
| Cobalt | ABC-Co | MHRHUB1 | MHR_0108 | MHR_0109 | MHR_0110 |  |  |  |
|  |  | MHP7448 | MHP7448_0263 | MHP7448_0264 | MHP7448_0265 |  |  |  |
|  |  | MFL27399 | MYF_01890 | MYF_01895 | MYF_01900 |  |  |  |
| Putrescine/ Sperimidine | $\begin{gathered} \text { ABC-Spd/ } \\ \text { Put } \end{gathered}$ | MHRHUB1 | MHR_0466 |  | MHR_0467 | MHR_0468 |  |  |
|  |  | MHP7448 | MHP7448_0540 |  | MHP7448_0540 | MHP7448_0541 |  |  |
|  |  | MFL27399 | MYF_00720 |  | MYF_00715 | MYF_00710 | MYF_00725 |  |
| Multidrug/Mn/Zn | ABC-Mn/Zn | MHRHUB1 | MHR_0020 |  | MHR_0019 |  |  |  |
|  |  | MHP7448 | MHP7448_0469 |  | MHP7448_0470 |  |  |  |
|  |  | MFL27399 | MYF_01155 |  | MYF 01150 |  | MYF_01160 | MYF_01165 |
| Toxin Secretion | ABC-Detox 1 | MHRHUB1 | MHR_0310 |  | MHR_0310 |  |  |  |
|  |  | MHP7448 | MHP7448_0160 |  | MHP7448_0160 |  |  |  |
|  |  | MFL27399 | MYF_02330 |  | MYF_02330 |  |  |  |
| Multidrug Efflux | ABC-Detox2 | MHRHUB1 | MHP7448_0664 | MHP7448_0665 | MHP7448_0664 | MHP7448_0665 |  |  |
|  |  | MHP7448 | MHR_0149 | MHR_0150 | MHR_0149 | MHR_0150 |  |  |
|  |  | MFL27399 | MYF_03280 | MYF_03285 | MYF_03280 | MYF_03285 |  |  |

Notes:
In yellow: Genes that had not been annotated as part of an ABC transport system; In blue: The gene was already associated to an $A B C$ transport system, but a new activity was proposed.

We were able to assign in all species, based on sequence homology, complete putative ABC transporters for multidrug/toxin efflux (ABC-Detox1 and ABC-Detox2) and for import of sugars (ABCSga, ABC-Fru), oligopeptides (ABC-Opp1, ABC-Opp2), peptides (ABC-Pep), spermidine/putrescine (ABC-Spd/Put), phosphonate/phosphate/thiamine (ABC-Pi), cobalt (ABC-Co), manganese/zinc (ABC-

Table 2.19: Incomplete ABC systems present in M. hyorhinis, M. flocculare and M. hyopneumoniae. Only three strains are presented here: MHRHUB1, MHP7448 and MFL27399.

| Incomplete ABC-Systems |  | ABC Components |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Substrate | Organism | NBD |  | TMD | SBP |
| Amino Acids | MHP7448 | MHP7448_0019 | MHP7448_0020 |  |  |
|  |  | MHP7448_0021 |  |  |  |
|  |  | MHP7448_0023 | MHP7448_0024 |  |  |
|  |  | MHP7448_0305 | MHP7448_0306 |  |  |
|  |  | MHP7448_0314 | MHP7448_0315 |  |  |
|  |  | MHP7448_0340 | MHP7448_0383 |  |  |
| Unknown | MHRHUB1 | MHR_0024 | MHR_0025 |  |  |
|  | MHP7448 | MHP7448_0210 |  |  |  |
|  | MFL27399 | MYF_01465 |  |  |  |
| Multidrug Efflux | MHRHUB1 | MHR_0041 | MHR_0370 |  |  |
|  |  | MHR_0651 | MHR_0643 |  |  |
|  | MHP7448 | MHP7448_0623 | MHP7448_0624 |  |  |
|  |  | MHP7448_0627 | MHP7448_0628 |  |  |
|  |  | MHP7448_0657 |  |  |  |
|  | MFL27399 | MYF_03225 | MYF_03230 |  |  |

$\mathrm{Mn} / \mathrm{Zn}$ ) and glycerol (ABC-Gly). In the search for a possible nucleotide transporter, we came across with a work from Nakhyung [177] in which a system previously annotated as sugar ABC transport was experimentally validated as involved in nucleoside uptake in Mycoplasma bovis. We found similar genes in the genomes of all species (ABC-Nucl). M. hyopneumoniae has an extra ABC transporter proposed for myo-inositol (ABC-Myo) and M. hyorhinis has a unique ABC transport for maltose/maltodextrins (ABC-Mal).

Regarding PTSs, genes coding for enzyme I (EI) and for the phosphocarrier (HPr) were found in all strains from M. hyorhinis, M. hyopneumoniae and M. flocculare. Although the gene was not annotated in MHRHUB1, the genomic sequence is present and there is no other gene overlapping the region (Table 2.20). Enzymes II (IIA, IIB and IIC) are usually specific to one or a few given substrates and although most of them were encoded as separate genes, several EII fusions exist [138] (one example is the PTS-Fru in all species studied here, Table 2.21). A summarized list of all PTSs found in one member of each species is given in Table 2.21. Three complete PTSs were common to all species: one non specific for sugar (PTS-Sga), one with specificity for fructose (PTS-Fru) and another for mannitol (PTS-Mtl). M. hyopneumoniae and M. flocculare also shared two extra complete systems: one for Ascorbate (PTS-Asc) and one for N-acetylglucosamine (PTS-GNAc). M. hyorhinis and M. hyopneumoniae seem to have an extra IIB component specific for glucose and along with the other components of PTS-Sga were proposed to form a complete glucose PTS (PTS-Glc). Specificity was also based on sequence homology and must be validated by experimental data.

Other transport systems with passive transports (permeases, facilitators and ion channels) and unknown mechanisms (and might be related to active or passive transports) were found throughout the genomes and can be seen in Table 2.22.

Table 2.20: Genes coding for the PTS general proteins phosphocarrier (HPr) and Enzyme I (EI) in M. hyorhinis, M. flocculare and M. hyopneumoniae.

| Organism | HPr | Enzyme I |
| :---: | :---: | :---: |
| MHP168 | MHP168_619 | MHP168_482 |
| MHP168L | MHP168L_619 | MHP168L_482 |
| MHP232 | mhp_628 | mhp_470 |
| MHP7422 | MHL_2667 | MHL_1734 |
| MHP7448 | MHP7448_0609 | MHP7448_0472 |
| MHPJ | MHJ_0611 | MHJ_0469 |
| MHRHUB1 | NC_014448[746585..746857] | MHR_0477 |
| MHRGDL1 | MYM_0652 | MYM_0503 |
| MHRSK76 | MOS_701 | MOS_540 |
| MRH17981 | VBIMycHyo71610_0382 | VBIMycHyo71610_0229 |
| MFL27716 | MFC_00378 | MFC_01092 |
| MFL27399 | MYF_02935 | MYF_01135 |

Notes: The HPr gene was not annotated in the genome of $M$. hyorhinis strain HUB1 (MHRHUB1), but the genomic sequence is present and conserved.

Table 2.21: Genes coding for the PTS specific enzymes II in M. hyorhinis, M. flocculare and M. hyopneumoniae.

| PTS-Systems |  |  | PTS Components |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Substrate | Abbr | Organism | IIA | IIB | IIC |
| Sugar | PTS-Sga | MHRHUB1 | MHR_0457 | MHR_0458 | MHR_0459 |
|  |  | MHP7448 | MHR_0194 | MHR_0195 | MHR_0487 |
|  |  | MFL27399 | MYF_01515 | MYF_01510 | MYF_01505 |
| Fructose | PTS-Fru | MHEHUB1 | MHR_0231 | MHR_0231 | MHR_0231 |
|  |  | MHP7448 | MHP7448_0492 | MHP7448_0492 | MHP7448_0492 |
|  |  | MFL27399 | MYF_02570 | MYF_02570 | MYF_02570 |
| Mannitol | PTS-MtI | MHRHUB1 | MHR_0169 |  |  |
|  |  | MHP7448 | MHP7448_0548 | MHP7448_0550 | MHP7448_0550 |
|  |  | MFL.27399 |  | MYF_02645 | MYF_02645 |
| N-Acetylglucosamine | PTS-GIcNAc | MHRHUB1 |  |  |  |
|  |  | MHP7448 | MHP7448_0574 | MHP7448_0574 | MHP7448_0574 |
|  |  | MFL27399 |  | MYF_02765 | MYF_02765 |
| Ascorbate | PTS-Asc | MHRHUB1 |  |  |  |
|  |  | MHP7448 | MHP7448_0554 | MHP7448_0553 | MHP7448_0552 |
|  |  | MFL27399 | MYF_02665 | MYF_02660 | MYF_02655 |
| Glucose | PTS-GIc | MHRHUB1 |  | MHR_0602 |  |
|  |  | MHP7448 |  | MHP7448_0591 |  |
|  |  | MFL27399 |  |  |  |

The protein GlpU, coded by gene MPN241 in M. pneumoniae, was recently described to act on the uptake of glycerophosphodiesters [95]. We found homologs of this gene in all strains of $M$. hyopneumoniae and M. hyorhinis adjacent to the gene responsible for the metabolization of these substrates $(g l p Q)$ [244]. In MHRHUB1, the gene was annotated as a pseudo gene.

All species also possess several unspecific amino acid permeases (Aa-F), a glycerol facilitator

Table 2.22: Genes coding for passive transporters or transport systems with unknown mechanisms in M. hyorhinis, M. flocculare and M. hyopneumoniae.

| Transport Systems |  |  | Transport Components |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Substrate | Abbr | Organism | Gene 1 | Gene 2 | Gene 3 |
| Glycerol | Gly-F | MHRHUB1 |  |  |  |
|  |  | MHP7448 | MHP7448_0358 |  |  |
|  |  | MFL27399 | MYF_01395 |  |  |
| Glycerophosphodiesters | glpU | MHRHUB1 | pseudo MHR_0261 |  |  |
|  |  | MHP7448 | MHP7448_0302 |  |  |
|  |  | MFL27399 |  |  |  |
| Nonspecific | MIF | MHRHUB1 | MHR_0432 |  |  |
|  |  | MHP7448 | MHP7448_0136 | MHP7448_0302 |  |
|  |  | MFL27399 | MYF_02200 |  |  |
| Aminoacids | Aa-F | MHRHUB1 | MHR_0146 | MHR_0529 | MHR_0586 |
|  |  | MHP7448 | MHP7448_0081 | MHP7448_0113 | MHP7448_0357 |
|  |  | MFL27399 |  | MYF_00625 | MYF_00390 |
| CobaltMagnesium | Corc | MHRHUB1 | MHR_0236 |  |  |
|  |  | MHP7448 | MHP7448_0643 |  |  |
|  |  | MFL27399 | MYF_03085 |  |  |
| Sodium/Phosphate | Trans-Na/Pi | MHRHUB1 | MHR_0250 |  |  |
|  |  | MHP7448 |  |  |  |
|  |  | MFL27399 |  |  |  |
| Cation ATPase | Trans-Cation | MHRHUB1 | MHR_0257 |  |  |
|  |  | MHP7448 | MHP7448_0268 |  |  |
|  |  | MFL27399 | MYF_01915 |  |  |
| Chromate | Trans-Chr | MHRHUB1 | MHR_0326 | MHR_0327 |  |
|  |  | MHP7448 | MHP7448_0145 | MHP7448_0146 |  |
|  |  | MFL27399 | MYF_02255 | MYF_02260 |  |
| Magnesium | Trans-Mg | MHRHUB1 | MHR_0433 |  |  |
|  |  | MHP7448 | MHP7448_0487 |  |  |
|  |  | MFL27399 | MYF_00995 |  |  |
| Sialic Acid | Trans-SA | MHRHUB1 | MHR_0451 | MHR_0579 |  |
|  |  | MHP7448 |  |  |  |
|  |  | MFL27399 |  |  |  |
| Zync | Trans-Zn | MHRHUB1 | MHR_0598 |  |  |
|  |  | MHP7448 | MHP7448_0090 |  |  |
|  |  | MFL27399 | MYF_00480 |  |  |
| Potassium | Trans-K | MHRHUB1 | MHR_0650 | MHR_0649 |  |
|  |  | MHP7448 | MHP7448_0546 | MHP7448_0545 |  |
|  |  | MFL27399 | MYF_00685 | MYF_00690 |  |

protein (Gly-F, which is less efficient than ABC-Gly [99]) and a major facilitator protein with unknown specificity (MIF). We could also find common transporters for cobalt and magnesium (CorC), chromate (Trans-Chr), magnesium (Trans-Mg), potassium (Trans-K), zinc (Trans-Zn) and a cation ATPase (Trans-Cation). M. hyorhinis had two extra transport systems: one for Sialic Acid (also known as N-Acetyl Neuraminate, Trans-SA) and one sodium phosphate co-transporter (Trans-Na/Pi).

## Carbohydrate and Energy Metabolism

## Glycolysis and Gluconeogenesis

As previously described [257], all genes for glycolysis exist in all strains of all species (Figure 2.13). M. hyorhinis may be able to convert dihydroxyacetone (DHA) directly to dihydroxyacetonephosphate (DHAP) with the action of DHA kinase (EC 2.7.1.29), but the mechanism of DHA uptake is not clear. None of the species can perform gluconeogenesis due to the absence of the enzymes phosphoenolpyruvate synthase (EC 2.7.9.2), fructose-1,6-bisphosphatase (EC 3.1.3.11) and glucose-6phosphatase (EC 3.1.3.9).


Figure 2.13: Glycolysis in M. hyorhinis, M. hyopneumoniae and M. flocculare. GLC: glucose; G6P: glucose-6-phosphate; F6P: fructose-6-phosphate; FBP: fructose-1,6-biphosphate; GAP: glyceraldehyde-3-phosphate; DHAP: dihydroxyacetone-phosphate; DHA: dihydroxyacetone; DPG: 1,3-biphospho-D-glycerate; 3PG: 3-phospho-D-glycerate; 23PG: 2,3-diphospho-D-glycerate; 2PG: 2-phospho-D-glycerate; PEP: phosphoenolpyruvate; PYR: pyruvate; EC 5.3.1.9: glucose-6-phosphate isomerase; EC 2.7.1.11: 6-phosphofructokinase; EC: 4.1.2.13: fructose-biphosphate aldolase; EC 5.3.1.1: triose-phosphate isomerase; EC 2.7.1.121: phosphoenolpyruvate-glycerone phosphotransferase; EC 2.7.1.29: glycerone kinase; EC: 1.2.1.12: glyceraldehyde-3-phosphate dehydrogenase; EC 2.7.2.3: phosphoglycerate kinase; EC 5.4.2.4: bisphosphoglycerate mutase; EC 3.1.3.13: bisphosphoglycerate phosphatase; EC: 5.4.2.12: phosphoglycerate mutase; EC 4.2.1.11: phosphopyruvate hydratase; EC 2.7.1.40: pyruvate kinase.

## Pentose Phosphate

In agreement with the literature [289, 8, 257], we found no enzymes for the oxidative branch of the pentose phophate pathway in the studied species, making of the ribulose-5-phosphate metabolite a dead-end. This problem was overcome by the assumption that reaction ribulose-phosphate-3epimerase (EC 5.1.3.1) could be reversible, as reported in Lactobacillus casei [22]. In Mycoplasma gallisepticum (and in two other Mollicute species, A. laidlawii and Spiroplasma melliferum), metabolomic analyses detected the presence of sedoheptulose-7-phosphate, a pentose-phosphate intermediate metabolite. The connection between glycolysis and a functional pentose phosphate pathway was also demon-
strated in M. pneumoniae, with the detection of ribose-5-phosphate (R5P) through mass spectrometry [314]. This gives us strong experimental evidence that the non-oxidative branch of the pentosephosphate pathway indeed exists in Mollicutes.


Figure 2.14: Pathway present in methylotrophs for the uptake of formaldehyde. The enzyme activity 3-hexulose-6-phosphate synthase (EC 4.1.2.43) was proposed in the gap filling step as the moonlight function of the enzyme 3-dehydro-L-gulonate-6-phosphate decarboxylase from the ascorbate metabolism (EC 4.1.1.85). Activity of the enzyme 6 -phospho-3-hexuloisomerase (EC 5.3.1.27, missing in all species) was also proposed by gap filling to reconnect hexulose-6-phosphate ( H 6 P ) to the rest of the network. Since the uptake of formaldehyde is not meant to be present in mycoplasmas, we did not add this orphan reaction (EC 5.3.1.27) to the networks and hexulose-6-phosphate (H6P) became a DEM. Rul5P: Ribulose-5-phosphate; F6P: fructose-6-phosphate; FCOH: formaldehyde.

Out of 11 reactions present in the final models, we found homologs for 9 in all species (Figure 2.15). The enzyme 6-phospho-3-hexuloisomerase (EC 5.3.1.27, enzyme missing in all species) in combination with enzyme 3 -hexulose-6-phosphate synthase (EC 4.1.2.43) is specific to methylotrophs, organisms that can obtain energy by oxidizing one carbon compound (such as formaldehyde) (Figure 2.14). Since this was not the case for any of the species, we did not add the orphan reaction of enzyme 6-phospho-3-hexuloisomerase to the models, and hexulose-6-phosphate (H6P) became a dead-end metabolite (Figure 2.15).

Although a gene coding for transaldolase (EC 2.2.1.2) is missing in all species, we included this reaction in the model to prevent both sedoheptulose-7-phosphate and erithrose-4-phosphate from becoming a DEM. We also found three inputs for the pentose phosphate pathway: (i) import of ribose (ABC-Sga) and subsequential phosphorylation to ribose-5-phosphate by the action of ribokinase (missing EC 2.7.1.15); (ii) degradation of ribonucleosides into ribose-1-phosphate by the action of PNPase (EC 2.4.2.1) and conversion of ribose-1-phosphate to ribose-5-phosphate by the action of phosphopentomutase (EC 5.4.2.7) ; (iii) ascorbate degradation into xylulose-5-phosphate.

## Ascorbate Metabolism

Ascorbate metabolism could feed the pentose phosphate pathway successfully. We found only one enzyme missing from the typical pathwyay: the L-ascorbate-6-phosphate-lactonase (EC 3.1.1.-, also called UlaG). This enzyme is responsible for the turnover of L-ascorbate-6-phosphate (A6P) to 3-keto-gulonate-6-phosphate (3KG6P). From the gene context, MHP7448_0377 from MHP7448 and its homologs from all other organisms/strains may code for the missing enzyme (Figure 2.16). The gene was annotated as a conserved hypothetical protein with a possible phosphotriesterase activity.

Since the activity of UlaG was not validated in vivo in Mycoplasma synoviae 53, we suggested a new set of reactions (one transport reaction, three spontaneous reactions, one enzymatic reaction and one orphan reaction) that could take place in M. hyopneumoniae and M. flocculare to feed the ascorbate metabolism. This was due to the use of the gap filling software, which proposed the phosphorylation of 3-keto-L-gulonate (3KG) to 3KG6P (R205) by the moonlight activity of glycerol kinase in M. hyopneumoniae and M. flocculare. In this way, M. hyopneumoniae and M. flocculare may have a secondary pathway for the uptake of ascorbate if compared to the genomes of M. hyorhinis.

## Uptake of other carbohydrates

All species can import glucose (ABC-Sga) and convert it to glucose-6-phosphate (G6P). While the uptake of fructose, mannitol and mannose are similar in all species (Figure 2.16) and they all


Figure 2.15: Pentose phosphate metabolism in M. hyorhinis, M. hyopneumoniae and M. flocculare. F6P: fructose-6-phosphate; E4P: erithrose-4-phosphate; GAP: glyceraldehyde-3-phosphate; S7P: sedoheptulose-7-phosphate; H6P: hexulose-6-phosphate; Xyl5P: xylulose-5phosphate; R5P: ribose-5-phosphate; Rul5P: ribulose-5-phosphate; PRPP: 5-phospho-alpha-D-Ribose 1-diphosphate; R1P: ribose-1-phosphate; Rib: ribose; FCOH: formaldehyde; EC 2.2.1.2: transaldolase; EC: 2.2.1.1: transketolase; EC 4.1.2.43: 3-hexulose-6-phosphate synthase; EC 5.1.3.3: aldose 1-epimerase; EC 5.3.1.6: ribose-5-phosphate isomerase; EC 2.7.6.1: PRPP synthetase; EC 5.4.2.7: phosphopentomutase; EC 2.4.2.1: PNPase; EC 2.7.1.15: ribokinase.
can be fed into glycolysis as fructose-6-phosphate (F6P) and DHAP, M. hyorhinis has an extra set of enzymes enabling the conversion of fructose-1-phosphate (F1P) into fructose-1,6-biphosphate (FBP) by the action of 1-phosphofructokinase (EC 2.7.1.56). The glyceraldehyde (GAld) produced by the activity of the moonlight enzyme fructose-biphosphate aldolase from glycolysis (Fba, EC 4.1.2.13) was proposed to exit the cells by an unknown mechanism. Only in M. hyopneumoniae the conversion of GAld into glycerol (glyceraldehyde reductase, EC 1.1.1.372) may exist through the moonlight activity of alcohol dehydrogenase, although up to date no experimental evidence can sustain that assumption.

Carbohydrate metabolism for other carbohydrates and oligosaccharides was proposed and can be seen in Figure 2.17. The presence of a unique transcriptional unit (TU) with carbohydrate metabolismrelated genes in M. hyorhinis (Appendix A) probably enables the uptake and metabolization of starch (alpha-amylase, EC 3.2.1.1), isomaltose (isomaltase, EC 3.2.1.10), maltose (maltose phosphorylase, EC 2.4.1.8), trehalose (trehalase, EC 2.4.1.64 and phosphotrehalase, EC 3.2.1.93) and sucrose (sucrase isomaltase, EC 3.2.1.48) into either G6P or F6P (Figure 2.17).


Figure 2.16: Ascorbate, Fructose, mannose and mannitol metabolism in M. hyorhinis, M. hyopneumoniae and M. flocculare. Ascorbate metabolism. ASC: ascorbate; A6P: ascorbate-6-phosphate; 3KG6P: 3-keto-gulonate-6-phosphate; LXyl5P: L-xylulose-5-phosphate; LRul5P: L-Ribulose-5-phosphate; Xyl5P: D-Xylulose-5-phosphate; LDAsc: L-dehydroascorbate; DAsc: Dehydroascorbate; 23DGul: 2,3-D-gulonate; 3KG: 3-keto-L-gulonate; EC 3.1.1.-: L-ascorbate-6-phosphate-lactonase; EC:4.1.1.85: 3-dehydro-L-gulonate-6-phosphate decarboxylase ; EC 5.1.3.22: L-ribulose-5-phosphate 3-epimerase; EC 5.1.3.4: L-ribulose-5-phosphate 4-epimerase; EC 1.1.1.130: 3-dehydro-L-gulonate 2-dehydrogenase. Fructose, Mannose and Mannitol Metabolism. MTL: mannitol; MTL1P: mannitol-1-phosphate; MAN: mannose; MAN6P: mannose-6-phosphate; FRU: fructose; F1P: fructose-1-phosphate; F6P: fructose-6-phosphate; FBP: fructose-1,6-biphosphate; DHAP: dihydroxyacetone-phosphate; GAld: glyceraldehyde; EC 1.1.1.17: mannitol-1-phosphate 5dehydrogenase; EC 5.3.1.8: mannose-6-phosphate isomerase; EC 2.7.1.56: 1-phosphofructokinase; EC 4.1.2.13: fructose-bisphosphate aldolase; EC 1.1.1.372: glyceraldehyde reductase.


Figure 2.17: Carbohydrate metabolism in M. hyorhinis, M. hyopneumoniae and M. flocculare. Simpler version on the left. STRCH: starch; ISOMAL: isomaltose; MAL: maltose; TRE: trehalose; TRE6P: trehalose-6-phosphate; GLC: glucose; SUCR: sucrose; GAL: galactose; G1P: glucose-1-phosphate; G6P: glucose-6-phosphate; FRU: fructose; F6P: fructose-6-phosphate; UDP-Glc: UDPglucose; UDP-Gal: UDP-galactose; EC 3.2.1.1: alpha-amylase; EC 3.2.1.10: oligo-1,6-glucosidase; EC 2.4.1.8: maltose phosphorylase; EC 2.4.1.64: trehalase; EC 3.2.1.93: phosphotrehalase; 3.2.1.108: lactase; 3.2.1.48: sucrase isomaltase; EC 2.7.1.4: fructokinase ; EC 2.7.1.2: glucokinase; EC 5.3.1.9: glucose-6-phosphate isomerase; EC 5.4.2.6: beta-phosphoglucomutase; EC 2.7.7.9: UTP-glucose-1phosphate uridylyltransferase; EC 5.1.3.2: UDP-glucose 4-epimerase.

Out of 4 genes in this TU, only one homolog was found in M. hyopneumoniae and M. flocculare: the membrane-bound $\alpha$-amylase/pullulanase enzyme acting on the endohydrolysis of large polysaccharides (EC 3.2.1.1). This enzyme was proposed in the models to act extracellularly on the degradation of starch into glucose and maltose.

Although we found no homologs for the $\beta$-phosphoglucomutase enzyme (EC 5.4.2.6) in M. hyopneumoniae and M. flocculare, we added the orphan reaction to the models to enable production of G1P. This metabolite is converted to UDP-glucose (UDP-Glc) by the action of UDP glucose pyrophosphorylase (EC 2.7.7.9) and UDP-galactose (UDP-Gal) by the action of UDP-galactose 4-epimerase (EC 5.1.3.2), which are essential for glycolipid metabolism and biomass growth.

## Myo-inositol metabolism

M. hyopneumoniae is the only species among Mollicutes which 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 2.18). A TU for the myo-inositol catabolism is present in all M. hyopneumoniae strains, with the exception of the enzyme 5-dehydro-2-deoxyphosphogluconate aldolase (IolJ, EC 4.1.2.29).


Figure 2.18: Transcriptional unit and myo-inositol catabolism in all M. hyopneumoniae strains.

The gene encoding this enzyme in other organisms is similar to the fructose-biphosphate aldolase (Fba) enzyme from glycolysis (EC 4.1.2.13). Since there are two copies annotated for Fba in M. hyopneumoniae (Fba and Fba-1, Table 2.23), we performed sequence alignments, synteny analyses and 3D modeling to check if either one would be a suitable candidate for this activity.

Table 2.23: Genes for myo-inositol catabolism in M. hyopneumoniae strains.

| Gene | EC | MHP168 | MHP168L | mHP232 | MHP7422 | MHP7448 | MHPJ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| ioIA | 1.2.1.- | MHP168_244 | MHP168L_244 | mhp153 | MHL_2920 | MHP7448_0225 | MHJ_0219 |
| ioIC | 2.7 .1 .92 | MHP168_245 | MHP168L_245 | mhp152 | MHL_2933 | MHP7448_0226 | MHJ_0220 |
| ioIB | 5.3.1.30 | MHP168_246 | MHP168L_246 | mhp151 | MHL_2944 | MHP7448_0227 | MHJ_0221 |
| ioIG-1 | 1.1.1.18 | MHP168_247 | MHP168L_247 | mhp150 | MHL_0860 | MHP7448_0228 | MHJ_0222 |
| ioID | 3.7 .1 .22 | MHP168_2448 | MHP168L_244 | mhp149 | MHL_0861 | MHP7448_0229 | MHJ_0223 |
| ioIE | 4.2.1.44 | MHP168_249 | MHP168L_249 | mhp148 | MHL_2983 | MHP7448_0230 | MHJ_0224 |
| mgIA |  | MHP168_250 | MHP168L_250 | mhp147 | MHL_3363 | MHP7448_0231 | MHJ_0225 |
| rbsC | 3.6 .3 .17 | MHP168_251 | MHP168L_251 | mhp146 | MHL_3008 | MHP7448_0233 | MHJ_0226 |
| rbsB |  | MHP168_252 | MHP168L_252 | mhp145 | MHL_0882 | MHP7448_0234 | MHJ_0227 |
| iolG-2 | 1.1.1.18 | MHP168_253 | MHP168L_253 | mhp144 | MHL_3371 | MHP7448_0235 | MHJ_0228 |
| fba | 4.2.1.13 | MHP168_014 | MHP168L_014 | mhp014 | MHL_0073 | MHP7448_0014 | MHJ_0014 |
| fba-1 | 4.2.1.29? | MHP168_581 | MHP168L_581 | mhp589 | MHL_3403 | MHP7448_0573 | MHJ_0574 |

We compared both predicted proteins to all Mycoplasma sequences in the NCBI non-redundant
protein sequences (nr) database. We found 192 homologs of Fba and 185 of Fba-1. Since both proteins were extremely similar, we only took into consideration extremely conserved and reciprocal hits (with e-values smaller than $10^{-50}$ ). In this way, we were able to reduce this number to 96 possible orthologs of Fba and only 22 of Fba-1). While the genome context for most of the Fba homologs were somewhat similar, the synteny analysis of the 22 homologs of Fba- 1 showed a similar context in only half (10) of the sequences; the other half corresponded instead to a tagatose/fructose biphosphate aldolase gene context. A phylogenetic analysis clustered the same ten gene products together into a Fba-1 group and the remaining 12 were included into the Fba group. In order to assign Fba-1 as a candidate for IolJ, we also conducted 3D analyses (with the Swiss PDB viewer [97, 246, 98]) based on the already characterized IolJ and Fba from Bacillus subtilis [312]. Figure 2.19 shows the differences in cavity analysis in IolJ (pink) and Fba (yellow) from B. subtilis.


Figure 2.19: Superposed 3D models of the iolJ and fba gene products from B. subtilis. We see 4 cavities for both proteins, but the internal cavities are slightly different.


Figure 2.20: Superposed 3D models of the Fba and Fba-1 from M. hyopneumoniae strain 7448. Cavities from Fba in yellow and cavities for the Fba-1 in pink.

The biggest difference in cavity sites for Fba and Fba-1 from $M$. hyopneumoniae lies in the internal portion of both predicted proteins (Fig 2.20). A detailed image of these cavities may be seen in Figure 2.21.


Figure 2.21: Superposed internal cavity prediction for Fba and Fba-1 from M. hyopneumoniae strain 7448. Cavities from the Fba in yellow and cavities for the Fba-1 in pink; important residues for these different cavities in green (for Fba) and red (for Fba-1).

Since Fba is an extremely important protein from glycolysis, it is highly likely that the most conserved between the two copies, Fba, is the one responsible for the enzyme activity of fructosebiphosphate aldolase (EC 4.1.2.13). The extra copy present in only 10 of all the Mycoplasma genomes may have diverged to a new function. Based on a tridimensional analysis, we concluded that Fba and Fba-1 may indeed code for different proteins with different functions. All steps to propose Fba-1 as the responsible enzyme for the IolJ activity (EC 4.1.2.29) are summarized in Table 2.24. The complete catabolism of myo-inositol may be seen in Figure 2.22.

Table 2.24: Analyses to determine an IolJ candidate.

| Step |  | fba | fba-1 |
| :---: | :---: | :---: | :---: |
|  |  | Annotated EC: 4.1.2.13 |  |
|  |  | \# of hits |  |
| Homolog Analysis | Homolog Search (E-value < 10e-5) | 192 | 185 |
|  | Conservation Analysis (E-value < 10e-50) | 149 | 20 |
| Ortholog Analysis | Reciprocal Hits | 96 | 22 |
|  | Synteny Analysis | 96 | 10 |
| 3D Analysis | Comparison to fba and iol/ from B. subtilis | fba | iolJ |
| Final EC |  | 4.1.2.13 | 4.1.2.29 |



Figure 2.22: Myo-inositol catabolism in M. hyorhinis, M. hyopneumoniae and M. flocculare.

## Amino sugar metabolism

All species can convert glucosamine (GLCN) to glucosamine-6-phosphate (GlcN6P). M. hyopneumoniae and M. flocculare can uptake and convert N-acetyl-glucosamine (GlcNAc, PTS-GNAc) to N-acetyl-glucosamine-6-phosphate (GlcNAc6P); M. hyorhinis lacks this transport system and instead, imports Sialic Acid (NeuNAc, TRANS-SA) and converts it to GlcNAc6P in three steps, unique in the M. hyorhinis models: N -acylglucosamine-6-phosphate 2-epimerase, N -acylmannosamine kinase and N -acetylneuraminate lyase (ECs 5.1.3.9, 2.7.1.60 and 5.1.3.9, respectively). At first, the genes coding for these three enzymes were absent in MHRHUB1 and MHRGDL1. However, based on a chromosome alignment, we were able to find that the regions are indeed present in these two strains, but were either annotated as pseudo genes or not annotated at all. The products of this metabolism are F6P, pyruvate and acetate, and directly enter the metabolism of glycolysis or pyruvate (Figure 2.23).


Figure 2.23: Amino sugar metabolism in M. hyorhinis, M. hyopneumoniae and M. flocculare. GlcN: glucosamine; GlcN6P: glucosamine-6-phosphate; NAcGlc: N-acetyl-glucosamine; NAcGlc6P: N-acetyl-glucosamine-6-phosphate; NAcNeu: N-acetyl-neuraminate (or sialic acid); NAcMa: N-acetyl-mannosamine; NAcMa6P: N-acetyl-mannosamine-6-phosphate; F6P: fructose-6phosphate; ACET: acetate; PYR: pyruvate; EC: 3.5.99.6: glucosamine-6-phosphate deaminase; EC 3.5.1.25: N -acetylglucosamine-6-phosphate deacetylase; EC 5.1.3.9: N -acylglucosamine-6-phosphate 2-epimerase; EC 2.7.1.60: N -acylmannosamine kinase; EC 4.1.3.3: N -acetylneuraminate lyase.

## Pyruvate metabolism

Out of fifteen enzymatic reactions in this pathway, only three were not found in any organism (Figure 2.25). Conversion of pyruvate to lactate by the action of lactate dehydrogenase (EC 1.1.1.27) and pyruvate to acetate by the action of pyruvate dehydrogenase (EC 1.2.1.-), phosphate acetyltransferase (EC 2.3.1.8) and acetate kinase (EC 2.7.2.1) were possible in all species. The first step of the conversion of pyruvate into acetate (EC 1.2.1.-) is catalyzed by the pyruvate dehydrogenase complex. Three enzymes usually compose this complex: E1, E2 and E3, coded by the genes pdhA, pdhB and pdhC. In the three Mycoplasma species studied, pdhC has no functional lipoyl-binding domain in the N-terminus [163]. Matic and collaborators [163] proposed that the lipoyl-binding domain of pdhD may be performing the enzymatic function normally attributed to pdhC in other organisms. It is also
interesting to note that an extra copy of pdhD was found in all species (pdhD-1). They were annotated as pdhD (or dihydrolipoamide dehydrogenase) in all genomes analysed except for MFL27399. The protein encoded by pdhD-1 is distant phylogenetically from pdhD, but whether this extra copy has activity (or even a different activity from pdhD) is not known. In this way, we proposed that only pdhD would be part of the active complex.

From experimental data, we knew that $M$. hyopneumoniae was able to produce oxaloacetate (OXAc) by the action of pyruvate carboxylase (EC 6.4.1.1), malate (MAL) by the action of malate dehydrogenase EC 1.1.1.37) and 2-oxoglutarate ( 2 KG ) by the action of aspartate transaminase (EC 2.6.1.1) [159, 281]. We also found experimental data on the decarboxylation of 2 KG to succinyl-coenzyme-A (SucCoA) by the action of oxoglutarate dehydrogenase (EC 1.2.4.2) in M. hyorhinis extracts [46].

Although we did not find any candidate for the activity of aspartate transaminase (EC 2.6.1.1), gap-filling proposed that this activity is performed by the pyruvate dehydrogenase complex. Moreover, Cordwell and collaborators [49] suggested that in Mollicutes lactate dehydrogenase (ldh, EC 1.1.1.27) could also function as malate dehydrogenase (EC 1.1.1.37). There is indeed a duplication of the $l d h$ gene in all strains: it is thus possible that the function of one of them diverged to malate dehydrogenase. Since we found no experimental evidence, we kept both genes separately for both reactions.
M. hyopneumoniae is the only organism among the three to have a gene coding for an alcohol dehydrogenase (EC 1.1.1.1), responsible for the turnover of acetaldehyde (AcAld) to ethanol (EtOH). This probably enables the turnover of acetyl-coenzyme-A (AcCoA) to AcAld by moonlight activity of the same enzyme (EC 1.2.1.10). While EtOH is the final product, AcAld and GAP can be converted to deoxy-ribose-5-phosphate (dR5P) in all organisms by the action of enzyme deoxyribose-phosphate aldolase (EC 4.1.2.4).

Conversion of pyruvate to formate by pyruvate formate-lyase (EC 2.3.1.54), malate to fumarate by fumarate hydratase (EC 4.2.1.2) and SucCoA to succinate succinyl-CoA synthetase (EC 6.2.1.5) were added to the models in a final step to accommodate the results from the experimental data (Chapter $3)$.

## Lipid Metabolism

The lipid metabolism in the models of the three species consisted mainly in the uptake of glycerol, choline, glycerophosphodiesters and fatty acids, and the production of cardiolipin (CL), 1,2-diacyl-sn-glycerol (DAG), phospho-choline (PC) and galactosyl-diacylglycerols (MGDG and DGDG) (Figure 2.26). Extracellular cholesterol and sphyngomyelin were incorporated unmodified directly into biomass.

While M. hyopneumoniae seems to have four different ways to uptake and metabolize glycerol, M. hyorhinis has three and M. flocculare only two. The three species are able to import glycerol-3-phosphate (G3P) unchanged (ABC-Gly); M. hyopneumoniae and M. flocculare are able to import glycerol (GlyF) and convert it to G3P by the action of glycerol kinase (EC 2.7.1.30); M. hyopneumoniae and $M$. hyorhinis can also uptake glycerol-phosphoglycerol (GPG) and glycerol-phosphocholine (GPC) and convert it to glycerol and G3P by the action of glycerophosphodiester phosphodiesterase (EC 3.1.4.46) or choline and G3P by the action of glycerophosphocholine phosphodiesterase (EC 3.1.4.2).

The turnover of glycerol-3-phosphate into DHAP by the action of glycerol-3-phosphate oxidase (GlpO, EC 1.1.3.21) allows the usage of glycerol as the sole carbon source, with the production of highly toxic hydrogen peroxide in the presence of molecular oxygen. This was only possible in the $M$. hyorhinis and M. hyopneumoniae models.

Although glycerol-3-phosphate 1-O-acyltransferase (EC 2.3.1.15), responsible for the turnover of G3P into acyl-sn-glycerol-3-phosphate (Acyl-G3P) is present only in M. hyorhinis, we added the reaction to the other species models for modeling reasons only. No homologs for the gene coding the enzyme phosphatidylglycerophosphatase (EC 3.1.3.27) were found; however, all Mollicutes definitely synthesize phosphatidylglycerol (PG), and hence must use a hitherto undetected enzyme for this step [305]. An orphan reaction for diacylglycerol cholinephosphotransferase (EC 2.7.8.2) was added to all networks to allow production of the biomass precursor phosphatidylcholine (PC). The ACP protein is present only in the $M$. hyorhinis models and may act as fatty acid and coenzyme- $\mathrm{A}(\mathrm{CoA})$ donor.

Orphan reactions for the production of monogalactosyl-diacyl-sn-glycerol (MGDG) by the action of monogalactosyldiacylglycerol synthase (EC 2.4.1.46) and digalactosyl-diacyl-sn-glycerol (DGDG) by the action of digalactosyldiacylglycerol synthase (EC 2.4.1.241) were added to the model based on experimental data [38].

Glycerol Metabolism and Pathogenicity? The metabolism of glycerol and the subsequent production of hydrogen peroxide by the action of GlpO are essential for the cytotoxicity of M. pneumoniae [99] and M. mycoides subsp. mycoides [294]. Particularly, the cytotoxicity of M. mycoides subsp. mycoides is due to the translocation of the hydrogen peroxide into the host cells. This is only possible because of the close proximity to the host cells along with the membrane-bound enzyme. Highly conserved homolog genes to this enzyme were only found in the genomes of the pathogenic M. hyopneumoniae and M. hyorhinis. Whether or not these enzymes from M. hyopneumoniae and M. hyorhinis are indeed capable of the same activities as GlpO is not yet confirmed; however, the high similarity between M. hyopneumoniae and M. hyorhinis proteins and GlpO from M. mycoides subsp. mycoides (Figure 2.24) may be an indication that this trait is essential for the pathogenicity of these two species. In addition, the predicted proteins from both $M$. hyopneumoniae and $M$. hyorhinis may have transmembrane portions as predicted by the software TMHMM (available at http://www.cbs.dtu.dk/services/TMHMM/).

A


B


Figure 2.24: (A) Alignment and (B) transmembrane prediction of GlpO proteins from M. mycoides subsp. mycoides (mycmycITA_00286|GlpO|Mmm), M. pneumoniae (MPN051|GlpD), M. hyopneumoniae (MHP7448_0588|GlpD) and M. hyorhinis (MHR_0259|GlpD). Alignments were made with the use of BLASTp alignment tool and transmembrane prediction was made with online software TMHMM).


Figure 2.25: Pyruvate metabolism in M. hyorhinis, M. hyopneumoniae and M. flocculare. PYR: pyruvate; GAP: glyceraldehyde-3-phosphate; LAC: lactate; OXAc: oxaloacetate; 2KG: 2-ketoglutarate or oxoglutarate; SucCoA: succinyl-coenzyme-A; SUC: succinate; MAL: malate; FUM: fumarate; FOR: formate; AcCoA: acetyl-coenzyme-A; AcetylP: acetyl-phosphate; ACET: acetate; AcALD: acetaldehyde; EtOH: ethanol; dR5P: deoxy-ribose-5-phosphate; dR1P: deoxy-ribose-1-phosphate; EC 1.1.1.27: lactate dehydrogenase; EC 6.4.1.1: pyruvate carboxylase; EC 2.6.1.1: aspartate transaminase; EC 1.2.4.2: oxoglutarate dehydrogenase; EC 6.2.1.5: succinyl-CoA synthetase; EC 1.1.1.37: malate dehydrogenase; EC 4.2.1.2: fumarate hydratase; EC 2.3.1.54: pyruvate formatelyase; EC 1.2.1.-: pyrvate dehydrogenase; EC 2.3.1.8: phosphate acetyltransferase; EC 2.7.2.1: acetate kinase; EC 1.2.1.10: acetaldehyde dehydrogenase; EC 1.1.1.1: alcohol dehydrogenase; EC 4.1.2.4: deoxyribose-phosphate aldolase; EC 5.4.2.7: phosphopentomutase; EC 2.4.2.1: PNPase.


Figure 2.26: Lipid metabolism in $M$. hyorhinis, $M$. hyopneumoniae and M. flocculare. Glycerol3P: glycerol-3-phosphate; GPG: glycero-phospho-glycerol; GPC: glycero-phospho-choline; LCAcylCoA: long-chain-acyl-coenzyme-A; Acyl-G3P: 1-acyl-sn-glycerol-3phosphate; 12DAG3P: 1,2-diacyl-sn-glycerol-3-phosphate; CDPDAG: CDP-1,2-diacyl-sn-glycerol-3phosphate; PGP: phosphatidyl-glycerol-phosphate; PG: phosphatidyl-glycerol; DAG: 1,2-diacyl-snglycerol; PhosphoC: phosphocholine; CDP-Cho: CDP-choline; PC: phosphatidyl-choline; UDPGal: UDP-galactose; MGDG: monogalactosyl-diacylglycerol; DGDG: digalactosyl-diacylglycerol; EC 2.7.1.30: glycerol kinase; EC 1.1.3.21: glycerol-3-phosphate oxidase; EC 6.2.1.3: acyl-CoA synthetase; EC 2.3.1.15: glycerol-3-phosphate 1-O-acyltransferase; EC 2.3.1.51: 1-acylglycerol-3-phosphate Oacyltransferase; EC 2.7.7.41: phosphatidate cytidylyltransferase; EC 2.7.8.5: CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase; EC 2.7.8.-: cardiolipin synthase; EC 2.7.8.41: cardiolipin synthase (CMP-forming); EC 3.1.3.4: phosphatidate phosphatase; EC 2.1.4.46: glycerophosphodiester phosphodiesterase; EC 3.1.4.2: glycerophosphocholine phosphodiesterase; EC 2.7.1.32: choline kinase; EC 2.7.7.15: choline-phosphate cytidylyltransferase; EC 2.7.8.2: diacylglycerol cholinephosphotransferase; EC 2.4.1.46: monogalactosyldiacylglycerol synthase; EC 2.4.1.241: digalactosyldiacylglycerol synthase.

## Amino acid Metabolism

The import of amino acids was added to the models in two forms: oligopeptide import and cleavage (ABC-Opp1/ABC-Opp2 and EC 3.4.24.-), and single amino acid import (Figure 2.27). Most reactions from this pathway account for amino acid transport and tRNA charging (Table 2.25). Glutamine (GLN) is the only amino acid that does not have a specific tRNA-ligase enzyme; instead, glutamate (GLT) is charged into a GLN-specific tRNA by the action of glutamate-tRNAGln ligase (EC 6.1.1.24) and in a second step, GLT is exchanged for GLN by the action of glutaminyl-tRNA synthase (glutamine-hydrolysing) (EC 6.3.5.7) (Figure 2.29). A similar charging (EC 6.1.1.23) and exchange steps (EC 6.3.5.8) can take place for asparagine and aspartate. Apart from tRNA-charging (23 reactions), according to the models, all species can convert cysteine into alanine (cysteine desulfurase , EC 2.8.1.7), glutamine into glutamate (CTP synthase glutamine-hydrolysing, EC 6.3.4.2) and interconvert glycine and serine ( glycine hydroxymethyltransferase, which is part of the folate metabolism, EC 2.1.2.1). The M. hyorhinis and M. hyopneumoniae models are able to convert aspartate into asparagine ( aspartate-ammonia ligase, EC 6.3.1.1) (Figure 2.28).


Figure 2.27: Import of amino acids and general tRNA charging in M. hyorhinis, M. hyopneumoniae and M. flocculare. Oligopeptides or dipeptides (Aa-Aa) are imported through the ABC-Opp transport system and degraded by the action of several peptidases (EC 3.4.24.-). There may exist also unknown mechanisms of transport for single aminoacids (Aa), but since we found no experimental data up to date, we added it as an unknown mechanism. tRNAs are specific for each amino acid and each tRNA charging reaction has a specific enzyme (EC 6.1.1.-). EC numbers for each tRNA and amino acid pair can be found in Table 2.25.


Figure 2.28: Interconversion of amino acids in M. hyorhinis, M. hyopneumoniae and M. flocculare. GLN: glutamine; GLT: glutamate; ASP: aspartate; GLY: glycine; SER: serine; CYS: cysteine; ALA: alanine; EC 6.3.4.2: CTP synthase (glutamine-hydrolysing); EC 6.3.1.1: aspartateammonia ligase; EC 2.1.2.1: glycine hydroxymethyltransferase; EC 2.8.1.7: cysteine desulfurase

Table 2.25: Amino acid tRNA charging reactions present in the models.

| Amino Acid tRNA Charging | EC Number | Reaction |
| :---: | :---: | :---: |
| Alanine | 6.1.1.7 | R032 |
| Arginine | 6.1.1.19 | R036 |
| Asparagine | 6.1.1.22 | R039 |
| Aspartic Acid | 6.1.1.12 | R040 |
| Cysteine | 6.1.1.16 | R054 |
| Glutamic Acid | 6.1.1.7 | R090 |
| Glutamine | 6.1.1.18 | Not Present |
| Glycine | 6.1.1.14 | R095 |
| Histidine | 6.1.1.21 | R100 |
| Isoleucine | 6.1.1.5 | R107 |
| Leucine | 6.1.1.4 | R109 |
| Lysine | 6.1.1.6 | R111 |
| Methionine | 6.1.1.10 | R116 |
| Phenylalanine | 6.1.1.20 | R156 |
| Proline | 6.1.1.15 | R162 |
| Serine | 6.1.1.11 | R209 |
| Threonine | 6.1.1.3 | R213 |
| Tryptophan | 6.1.1.2 | R220 |
| Tyrosine | 6.1.1.1 | R221 |
| Valine | 6.1.1.9 | R228 |



Figure 2.29: Specific amino acid charging for glutamine and asparagine in M. hyorhinis, M. hyopneumoniae and M. flocculare. GLT: glutamate; GLN: glutamine; ASP: aspartate; ASN: asparagine; EC 6.1.1.17: glutamyl-tRNA synthetase; 6.1.1.24: glutamate-tRNAGln ligase; EC 6.3.5.7: glutaminyl-tRNA synthase (glutamine-hydrolysing); EC: 6.1.1.12: aspartate-tRNA ligase; EC 6.1.1.23: aspartate-tRNAAsn ligase ; EC 6.3.5.6: asparaginyl-tRNA synthase (glutamine-hydrolysing); EC 6.1.1.22: asparagine-tRNA ligase.

Production of the biomass precursor S-adenosyl-L-methionine (SAM) from methionine is possible in all strains ( methionine adenosyltransferase, EC 2.5.1.6). The refinement process of this subpathway can be seen in Figure 2.30. The enzyme adenosylhomocysteine nucleosidase (EC 3.2.2.9), responsible for the conversion of S-adenosyl-L-homocysteine (SAH) into S-ribosyl-L-homocysteine (SRH), was found in all species. To restore connectivity between SAM and SAH, we used a gap filling software which proposed an orphan reaction that converted SAM into SAH by the action of a DNA methyltransferase (EC 2.1.1.37). This conversion produced a methylated glutamine (5MGLN) which was recycled into glutamine through a synthetic drain reaction with no additional costs (DRAIN). These synthetic reactions are unlikely to occur in vivo, and thus, we tried to find a better way of
connecting both metabolites. Indeed, we were able to add two reactions to restore connectivity: one that converted the DEM SRH into L-homocysteine (HCys) by S-ribosylhomocysteine lyase (EC 4.4.1.21), and another reaction that produced methionine from HCys with the concomitant conversion of SAM into SAH. The addition of these reactions also resulted in the production of autoinducer-2 (AI-2), that together with SRH are molecules known to act in cell communication and quorum sensing (discussed more in detail in Chapter 4).


Figure 2.30: Refinement process of the SAM production pathway in M. hyorhinis, M. hyopneumoniae and M. flocculare. MET: methionine; SAM: S-adenosyl-methionine; SAH: S-adenosyl-homocysteine; SRH: S-ribosyl-L-homocysteine; HCys: homocysteine; AI-2: autoinducer2; EC 2.5.1.6: methionine adenosyltransferase; EC 3.2.2.9: adenosylhomocysteine nucleosidase; EC 2.1.1.37: DNA methyltransferase; EC 4.4.1.21: S-ribosylhomocysteine lyase

## Nucleotide Metabolism

The mycoplasmas in this study cannot synthesize de novo purines and pyrimidines; therefore, they have only salvage pathways and interconversions to supply the cell with the nucleic acid precursors (Figures 2.31 and 2.32). The three species had the same enzymatic capabilities, except for the presence of thymidylate synthase (EC 2.1.1.46) in M. hyorhinis, which is also important for cofactor metabolism and is responsible for the conversion of dUMP and 5,10-methylene-tetrahydrofolate (MeTHF) to dTMP and dihydrofolate (DHF). Overall, the nucleotide metabolism consists in the uptake of guanine, adenine, uracil, thymine and cytidine and production of all deoxy-ribonucleotides (dATP, dCTP, dGTP, TTP) and ribonucleotides (ATP, CTP, GTP and UTP).


Figure 2.31: Purine metabolism in M. hyorhinis, M. hyopneumoniae and M. flocculare. IMP: inosine-monophosphate; XMP: xanthosine-monophosphate; EC 2.4.2.1: PNPase or purinenucleoside phosphorylase; EC 2.4.2.8: IMP-GMP pyrophosphorylase; EC 3.1.3.5: 5'-nucleotidase; EC 1.1.1.205: IMP dehydrogenase; EC 2.7.1.113: deoxyguanosine kinase; EC 2.7.4.13: (deoxy)nucleosidephosphate kinase; EC 2.7.4.6: nucleoside-diphosphate kinase; EC 1.17.4.1: ribonucleoside-diphosphate reductase; EC 2.7.4.8: guanylate kinase; EC 3.6.5.3: protein-synthesizing GTPase; EC 2.7.1.76: 2'deoxyadenosine kinase; EC 2.7.4.11: (deoxy)adenylate kinase; EC 2.4.2.7: adenine phosphoribosyltransferase; EC 2.7.4.3: adenylate kinase; EC 2.7.4.4: nucleoside-phosphate kinase; EC 2.7.1.40: pyruvate kinase.


Figure 2.32: Pyrimidine metabolism in M. hyorhinis, M. hyopneumoniae and M. flocculare. EC 3.1.3.5: 5'-nucleotidase; EC 2.4.2.2: pyrimidine-nucleoside phosphorylase; EC 2.4.2.9: uracil phosphoribosyltransferase; EC 2.7.4.22: UMP kinase; EC 2.7.4.6: nucleoside-diphosphate kinase; EC 3.5.4.5: cytidine deaminase; EC 6.3.4.2: CTP synthase (glutamine hydrolysing); EC 2.7.4.25: (d)CMP kinase; EC 1.17.4.1: ribonucleoside-diphosphate reductase; EC 3.6.1.6: nucleoside diphosphate phosphatase; EC 3.1.3.89: 5'-deoxynucleotidase; EC 2.7.1.74: deoxycytidine kinase; EC 3.5.4.1: cytosine deaminase; EC 3.5.4.12: dCMP deaminase; EC 3.5.4.13: dCTP deaminase; EC 3.6.1.23: dUTP diphosphatase; EC 2.7.4.13: (deoxy)nucleoside-phosphate kinase; 2.7.1.21: thymidine kinase; EC 2.1.1.45: thymidylate synthase; EC 2.7.4.9: dTMP kinase.

## Cofactor Metabolism

Around $60 \%$ of the reactions that comprised the cofactor metabolism in the models were orphan reactions (Figure 2.8).


Figure 2.33: Cofactor metabolism in M. hyorhinis, M. hyopneumoniae and M. flocculare. Pyridoxal metabolism. PYX: pyridoxal; PYP: pyridoxal-phosphate; EC 2.7.1.35: pyridoxal kinase. Thiamine metabolism. ThiPP: thiamine-pyrophosphate. Riboflavin metabolism. RBF: riboflavin; FMN: flavin mononucleotide; FAD: flavin adenine dinucleotide; EC 2.7.1.26: riboflavin kinase; EC 2.7.7.2: FAD synthetase. Pantothenate/CoA metabolism. RPAN: R-Panthothenate; R4PP: R-4-phospho-pantothenate; R4PPCys: R-4-phosphopathotenoyl-cysteine; (cont. next page).

Figure 2.33 (cont. previous page) 4PPAN: 4-phosphopantetheine; DPCoA: dephospho-coenzymeA; CoA: coenzyme-A; Holo-ACP: holo-[acyl-carrier-protein]; 35ADP: 3,5-adenosine-biphosphate; EC 2.7.1.33: pantothenate kinase; EC 6.3.2.5: phosphopantothenate-cysteine ligase; EC 4.1.1.36: phosphopantothenoylcysteine decarboxylase; EC 2.7.7.3: pantetheine-phosphate adenylyltransferase; EC 2.7.1.24: dephospho-CoA kinase; EC 2.7.8.7: holo-[acyl-carrier-protein] synthase; EC 3.1.3.7: $3^{\prime}\left(2^{\prime}\right), 5^{\prime}$ bisphosphate nucleotidase; EC 3.1.3.4: phosphatidate phosphatase. Folate metabolism. FOL: folate; DHF: dihydrofolate; THF: 5,6,7,8-tetrahydrofolate; MeTHF: 5,10-methylene-tetrahydrofolate; 10FTHF: 10-formyl-tetrahydrofolate; EC 1.5.1.3: dihydrofolate reductase; EC 2.1.1.45: thymidylate synthase; EC 2.1.2.1: glycine hydroxymethyltransferase; EC 6.3.4.3: formate-tetrahydrofolate ligase. Nicotinate metabolism. NIA: nicotinate or niacine; NACD: nicotinamide or niacinamide; NICNUC: nicotinate-D-ribonucleotide; NAD+/NADH: Nicotinamide adenine dinucleotide; dNAD: deamido-NAD ; NADP + /NADPH: Nicotinamide adenine dinucleotide phosphate; EC 3.5.1.19: nicotinamidase; EC 6.3.4.21: nicotinate phosphoribosyltransferase; EC 2.7.7.18: nicotinate-mononucleotide adenylyltransferase; EC 6.3.1.5: NAD+ synthetase; EC 6.3.5.1: NAD + synthase (glutamine-hydrolysing); EC 3.1.3.-: NADP phosphatase; EC 2.7.1.23: NAD kinase; EC 1.6.99.3: NADH oxidase (water forming); EC 2.7.1.86: NADP kinase; EC 1.8.1.9: thioredoxin disulfide-reductase.

As previously mentioned in the overview section of this chapter, the protein p37 was described in MHRHUB1 as an extracytoplasmic thiamine-pyrophosphate binding lipoprotein, responsible for the uptake of this cofactor into the cells [253]. In this way, thiamine-pyrophosphate (ThiPP) is imported unchanged and we propose it is incorporated directly into biomass (ABC-Pi). Pyridoxal (PYX) was proposed to be imported and converted to the biomass precursor pyridoxal-phosphate (PYP) by the action of pyridoxal kinase (EC 2.7.1.35 missing in all species). Riboflavin was proposed to be imported by an unknown mechanism and to be further phosphorylated by riboflavin kinase to flavin mononucleotide (FMN) (EC 2.7.1.26) and flavin adenine dinucleotide (FAD) (FAD synthetase, EC 2.7.7.2).

From the pantothenate/coenzyme-A metabolism, we found only homologs for the conversion of 4'-phosphopantetheine (4PPAN) by pantetheine-phosphate adenylyltransferase (EC 2.7.7.3) into dephospho-coenzyme-A (DPCoA). 4PPAN was proposed to either be imported into the cells or to be produced by sequential reactions from pantothenate (PAN): pantothenate kinase, phosphopantothenatecysteine ligase and phosphopantothenoylcysteine decarboxylase (EC 2.7.1.33, EC 6.3.2.5, EC 4.1.1.36), missing in all organisms. DPCoA can be further converted into CoA ( dephospho-CoA kinase, EC 2.7.1.24), and although at first this reaction was not present in the networks for M. flocculare, we were able to find homologs in both strains. In the lipid metabolism, CoA can be used either directly (in M. hyopneumoniae and M. flocculare) or to activate the ACP protein in M. hyorhinis, by holo-[acyl-carrier-protein] synthase(EC 2.7.8.7). The subproduct of this activation, adenosine-3;5-biphosphate (35ADP), can be converted to AMP by the action of $3^{\prime}\left(2^{\prime}\right), 5^{\prime}$-bisphosphate nucleotidase (EC 3.1.3.7) in all species.

Three forms of folate are incorporated to the biomass: MeTHF, 5,6,7,8-tetrahydrofolate (THF) and 10 -formyl-tetrahydrofolate (10FTHF). We assumed that folate is imported and converted to these three biomass precursors by dihydrofolate reductase (EC 1.5.1.3, only present in M. hyorhinis), serine hydroxymethyltransferase (EC 2.1.2.1, present in all species) and formate-tetrahydrofolate ligase (EC 6.3.4.3, missing in all species). M. hyorhinis shows also the capability of turning MeTHF back to DHF, as previously mentioned (in all species EC 2.1.1.45).

Most enzymes from the nicotinate metabolism are present in all species. Nicotinate (niacin, NIA) or nicotinamide (niacinamide, NACD) enter the cells by unknown mechanisms. Both are converted to NAD + by the action of four enzymes: nicotinamidase (EC 3.5.1.19), nicotinate phosphoribosyltransferase (EC 6.3.4.21), nicotinate-mononucleotide adenylyltransferase (EC 2.7.7.18) and NAD+ synthetase (EC 6.3.5.1). Although no homologs for NAD kinase were found, two reactions were added (EC 2.7.1.23 and EC 2.7.1.86) to allow the presence of NADP and NADPH, which are cofactors for several essential reactions. The NADPH and NADH produced from degradation of glucose and other carbohydrates are recycled by the action of two enzymes: NADH oxidase (water forming, EC 1.6.99.3) and thioredoxin disulfide-reductase (EC 1.8.1.9).

### 2.4 Discussion

### 2.4.1 Model Reconstruction and Refinement

In this work, we created a metabolism as realistic as possible for the three known mycoplasmas present in the respiratory tract of swines. It is essential to point out that, although we had to include $30 \%$ of orphan reactions in order to allow growth, we only used about half of the genomes of these organisms. The other half consists either of hypothetical or of conserved hypothetical proteins [257], which may in part fill the missing gaps of the models. As for most of the Mycoplasma species studied [265, 314, 307], all reconstructed networks exhibit low connectivity due to the simplicity of the biological model. We were able to show in this work that the three swine mycoplasmas have similar metabolic capabilities, except for the metabolism of myo-inositol, amino sugar, carbohydrates and the uptake of glycerol (Figure 2.34).

Figure 2.34: Differential metabolism of M. hyorhinis, M. hyopneumoniae and M. flocculare.

Overall, the methods used here enabled us to address some of the main problems caused by most automatic reconstruction methods which are the permissive inclusion of pathways and over prediction of capabilities [266].

Annotation errors arise with the attribution of ambiguous or partial EC numbers [60, 93]. Propagation of these errors may then lead to many other ones. Indeed, overprediction and redundancy caused by the Pathologic method (based only in EC numbers and gene ontology terms) were detected in the networks of Lactococcus lactis IL1403 [185] and Lactococcus plantarum [273], for example. These errors were manually removed during the refinement process, in a similar way we had to remove them in this work. Thus, it is clear that prediction of GPR associations based only on name-matching and EC-codes is not sufficient to add confidence to a model [93]. We showed that comparing the draft reconstructions, even for extremely similar organisms, may lead us to wrong assumptions (Figure 2.4 vs Figure 2.6). For instance, at first, we could have concluded that among the strains of M. hyopneumoniae, there were $5-10 \%$ of metabolic differences when in fact that number converges to approximately zero in the curated networks.

Thus, these draft-networks are comparable only if sequence alignments and manual curation are performed. When we decided to simultaneously refine the 12 models, we enhanced the confidence of each GPR association by adding information on the synteny between the genomes, predicted protein sequence alignments, and phylogenetic distance between orthologs, among others.

### 2.4.2 Metabolism and Pathogenicity

In this work, our main objective was not only to reconstruct the metabolic models for M. hyorhinis, M. hyopneumoniae and M. flocculare. We further wanted to compare the metabolism of the three species and find possible links to pathogenicity.
M. hyorhinis is not only a pathogen, but also a common contaminant in many mammalian cell cultures [66]. This may be explained by the wide range of carbohydrates which this organism can uptake and metabolize. This may be a crucial factor to the ability of M. hyorhinis to grow in diverse sites inside the host [134] and even to invade other hosts and be potentially involved with cancer in humans $[107,136]$. Different sites and different hosts might harbour distinct carbon sources and the fact that only $M$. hyorhinis is able to grow in these divergent conditions supports our hypothesis that one of the reasons for this might be their ability to uptake such a wide range of carbohydrates. To corroborate with this idea comes the fact that $M$. hyopneumoniae has been detected in other sites such as brain, liver and spleen [85, 161], but M. flocculare has never been detected outside of the respiratory tract of swines.
M. hyopneumoniae is the only Mycoplasma species with sequenced genome that has the genes for the catabolism of myo-inositol. Myo-inositol is readily abundant in the bloodstream of mammalian hosts, and can be used as a secondary carbon source for energy production [223]. Mycoplasma iguanae
has been described to produce acid from inositol [25]. Unfortunately, there is no available complete genome sequence for this organism, making impossible any comparison of the genes involved in this pathway. However, given these results, the ability of M. hyopneumoniae to grow in diverse sites if compared with $M$. flocculare might come from the fact that $M$. hyopneumoniae may be able to uptake and process myo-inositol. Whether this pathway is functional in M. hyopneumoniae is not yet known, and further investigation should take place to support this hypothesis. Furthermore, myo-inositol is a compound wide-spread in the blood stream of mammalians, which would make it a suitable carbon source for bacteria in the extremely vascularized respiratory system.

Furthermore, 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 [135]. 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 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 IolJ activity; instead we proposed that the activity of the missing enzyme IolJ 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 Sinorhizobium meliloti 1021 (SM_b21192 and SM_b20199, both annotated as fructose-biphosphate-aldolase, EC 4.1.2.13). This means that in at least one other pathogen that has the myo-inositol catabolism genes, the predicted IolJ enzyme is not close to the myo-inositol cluster, just as we proposed here.

Myo-inositol has also been extensively described in several organisms also as a signaling molecule [65, 89] that moreover is important for pathogenicity [223]. Altogether, these evidences show it is possible that the myo-inositol catabolism in M. hyopneumoniae is one of the reasons for its high pathogenicity when compared to M. flocculare.

Tajima and Yagihashi [268] reported that capsular polysaccharides from M. hyopneumoniae may play a key role in the interaction between pathogen and host. Recent reports have shown the capacity of species from the mycoides cluster to build a capsule using only phosphorilated glucose [14, 13]. However, no homolog proteins related to these activities were found in none of the species studied in this work. Although we were not able to introduce the capsule production itself in the models, we tried to relate its existence with the possible substrates used to produce it. The composition in capsular polysaccharides is also related to the level of pathogenicity in other bacteria. For instance, highly virulent $E$. coli strains seem to have practically nonimmunigenic capsular material, due to the fact that these antigens are similar or identical to the ones found in the host [21]. The structure of the K5 antigen from Escherichia coli capsules is basically N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) in a molar ratio of 1:1 [287]. Erlinger and collaborators [71] identified heparan sulfate (HS) as the predominant glycosaminoglycan in the porcine respiratory tract and the most common disaccharide unit within HS is GlcNAc linked to GlcA. Since M. hyopneumoniae and M. flocculare can import GlcNAc directly, they could possibly use it for the composition of its capsule. M. hyorhinis, on the other hand, imports sialic acid, and possibly uses it directly to produce its capsule, because in order to convert it to GlcNAc, it must perform a three reaction pathway. Of course this is highly speculative, and we are not sure if all these hypotheses indeed happen in vivo.

The metabolism of glycerol is the last pathway that is different among the three species. While M. hyopneumoniae has five different ways of uptaking glycerol (from dehydrogenation of glyceraldehyde and import of glycerol, glycerol-phosphate, glycerophosphoglycerol or glycerophosphocholine), the other two species lack at least two reactions (two for M. hyorhinis and three for M. flocculare). The metabolism of glycerol and the formation of hydrogen peroxide are essential for the cytotoxicity of lung pathogens M. mycoides subsp. mycoides [294] and M. pneumoniae [99]. M. flocculare cannot convert glycerol into DHAP with hydrogen peroxide formation. 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 results in tissue damage [313]. 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 a carbon source.

The reconstructed models from this chapter have also served as a basis for all the hypotheses
formulated with the experimental data that will be presented in Chapter 3. We were able to show in this chapter slight differences in the reconstructed metabolic networks that can partially explain the incapacity of M. flocculare to cause disease or the ability of M. hyorhinis to grow faster than the other two species. As a result of multiple factors, M. hyopneumoniae is the most pathogenic of the three species, but more experiments should be made in order to relate the peculiarities of this particular organism to pathogenicity. This will be further explored in the next two chapters.


Figure 2.35: Global energy and carbohydrate model showing the enzymatic activities for M. hyorhinis, M. hyopneumoniae and M. flocculare.


Figure 2.36: Lipid, amino acid, nucleotide and cofactor metabolism of M. hyorhinis, M. hyopneumoniae and M. flocculare.

## Chapter 3

## Metabolomics and Flux Balance Analysis

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This chapter describes the experimental approach for the identification of the metabolic differences among M. hyorhinis, M. hyopneumoniae and M. flocculare. Since these organisms lack most catabolic pathways, it is possible to relate to some extent the external metabolite measurements to the intracellular metabolic fluxes. At first, we analyzed the metabolites formed during growth in complex media. Since this gave us only a partial information due to the complex mixture of unassigned metabolites, we tried to cultivate the three mycoplasmas in previously described defined media. Although none of the three species was able to multiply in the media, all maintained viability. We were thus able to retrieve more information on metabolite exchange in a stationary culture. We tested all models through FBA analysis and compared the results with theoretical and experimental data. We then modified the models in order to accommodate all the metabolomics results. At the end of the chapter, we discuss how these differences in growth and metabolite uptake may influence the life-style of each species.

### 3.1 Overview

Metabolic analysis, or metabolomics, is the study of small molecules (molecular weight $<1,000 \mathrm{Da}$ ) in a biological system [80]. The metabolism of an organism results from the interaction between genotype, environment and homeostatic state [248]. Homeostasis is described as the property of a system to remain stable and relatively constant, and is usually related to the survival rate of organisms in an ecosystem [179]. Genetic and environment perturbations in this system induce cells to adapt and respond accordingly. This response can be quantified by the change in concentration of metabolites. These metabolic changes can be more informative than sequence or gene expression analysis as they represent the end product of gene regulation [264].

As previously mentioned (see Chapter 1), metabolomics can be divided into targeted and nontargeted analysis. Targeted analysis tries to identify a subset of previously defined metabolites and thus cannot detect any novel compounds in a sample [3]. Non-targeted analyses can be subdivided into metabolic profiling and metabolic fingerprinting. Metabolic fingerprinting is a global analysis that provides a snapshot of the sample without precise identification or quantification of the metabolites; metabolic profiling is an unbiased comprehensive analysis of all the metabolites in the sample [248].

Usually, the biological system is disturbed and the abundance of all metabolites is compared between different stages. It is clear that this is more difficult than a targeted analysis since the number (and classes) of metabolites present in the samples is usually not known beforehand. As a result, metabolic profiling experiments tend to be noisy [80].

The techniques most widely used for the identification and quantification of cellular components are nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS). MS is known for its greater sensitivity; however, its cost, especially when heavy labelled internal standards are needed (for absolute quantification) is a setback for the use of MS with high-throughput approaches. On the other hand, sample preparation in NMR is generally faster and the analysis time is shorter, resulting in lower costs. NMR is a robust, reproducible technique that can provide absolute quantification of many metabolites. [212] However, NMR can only measure medium to high abundance metabolites. MS, on the other hand, has high selectivity and sensitivity, showing the need to use parallel methods in order to obtain a better range of metabolites with higher confidence [57].

Since the number of detected variables (metabolites) in the sample is directly proportional to the number of dimensions of the dataset, the efficient retrieval of biologically relevant information from a metabolomics dataset requires specialized forms of data analysis [309]. In this way, multivariate analyses (MVA) based on principal components are used to reduce the spectral information and thus, to identify biologically relevant spectral features [309]. The two most popular methods are principal component analysis (PCA) and partial least squares projection to latent structures (PLS). In this work, we have used the metabolic profiling approach through NMR spectroscopy to identify the metabolites used by each species in both complex and defined media with exponential and stationary phase cells.

### 3.1.1 Principle Component Analysis (PCA)

Principle Component Analysis (PCA) is the most widely used MVA method for metabolomics. PCA aims at finding a linear transformation of the dataset into a lower dimension that preserves as much variance from the original high dimensional data as possible [118]. Principal components (PCs) are orthogonal and ordered according to the variance explained. The first PC explains the largest possible variance and each succeeding component has the highest variance possible under the constraint that it is orthogonal to the preceding components.

### 3.1.2 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear Magnetic Resonance (NMR) is a non-destructive analytical method that can determine the molecular structure along with the quantity of metabolites. NMR has the key advantage of having a simple sample preparation, being fast, non-destructive, capable of relatively high-throughput and automated analyses; the largest disadvantage is undoubtedly its relatively poor sensitivity [92, 248]. The basic principle underlying NMR is that the nuclei of atoms have magnetic properties that can be utilized to yield chemical information. Different compounds, composed by different nuclei, react
differently to NMR stimuli and generate distinct chemical shifts if compared to a standard. A simple hydrogen homonuclear 1-dimensional NMR (1H 1D NMR) experiment, for instance, takes into account only the chemical shifts of hydrogen atoms inside a mixture. This can be easily read if one measures a single compound, such as ethanol for instance (Figure 3.1).


Figure 3.1: Example of a 1D 1H NMR spectrum of ethanol plotted as signal intensity vs. chemical shift. This is merely an example of what we should expect when reading an NMR spectrum from an ethanol solution in deuterated chloroform $(90 \mathrm{MHz})$. There are three different types of H atoms in ethanol as concerns NMR. The hydrogen (H) in the -OH group is not coupled with the other H atoms and appears as a singlet, but the CH3- and - $\mathrm{CH} 2-$ hydrogens are coupled with each other, resulting in a triplet and quartet respectively (Figure source: [288]; Data available at: http://sdbs.db.aist.go.jp/)

However, complex mixture spectra may become unreadable through this simple technique (Figure 3.2). Furthermore, while NMR spectroscopy is established as a key technique for metabolomic approaches, the identification of individual metabolites present in complex biological samples still constitutes a primary obstacle due to a severe spectral overlap of resonances [293]. Moreover, the presence of high molecular weight molecules is usually associated with a broad resonance. This may be reduced with the aid of a 1D 1H CPMG approach. And although this technique yields fewer baseline distortions, a large number of overlapping signals remain [293] (an example can be seen in region 3 to 4.5 ppm from a spectrum of Friis medium in Figure 3.2). These convoluted regions may be resolved with the help of higher dimension NMR to correctly assign each peak [205].

### 3.2 Methods

### 3.2.1 Cultivation Methods

Strains were tested according to their availability either in France or Brazil (Figure 3.3). Selected strains for experimental testing were: M. hyopneumoniae strains 7448 (MHP7448) and J (ATCC 25934, MHPJ), M. flocculare strain ATCC 27716 (MFL27716), and M. hyorhinis strain ATCC 17981 (MHR17981). They were cultivated in both complex and defined media.


Figure 3.2: Example of spectra of Friis medium vs defined Yus+ medium. The convoluted region of spectrum from the Friis medium ( 3 to 4.5 ppm ) is more clear in the defined Yus+ medium spectrum.


Figure 3.3: Experimental availability of selected strains.

Complex media comprised (i) Friis media [86] (available in Brazil), and (ii) a commercial mycoplasma broth (provided by Indicia Biotechnology, available in France).

Defined media cultivation tests were performed in France and comprised (i) a medium described for M. pneumoniae strain 129 by Yus and collaborators (2009), and (ii) commercial media CMRL with no glutathione (Invitrogen). Since we had no information on metabolism of swine mycoplasmas, we decided to supplement the defined Yus medium with all amino acids (from now on the supplemented version will be referred to as Yus+). We also supplemented the CMRL-1066 medium with other
peptone and/or other cofactors (from now on these will be referred to as CMRL+ and CMRL + /Pep). Information on the composition of all defined media is available in Table 3.1.

Cells were cultivated at $37^{\circ} \mathrm{C}$ for different time periods, under gentle agitation ( 100 rpm ).

Table 3.1: Defined media composition used for the cultivation of M. hyopneumoniae, M. hyorhinis and M. flocculare.


### 3.2.2 Cell concentration estimation and viability by color changing units (CCU) measurement

Cell growth and viability were measured with triplicate time-matched samples of cells in culture media for color changing units (CCUs) as described by Stemke and Robertson [262]. Viability of cells is visible by a change in medium color from red to yellow. For the cell concentration measurements, the cultures were subjected to a series of 10 -fold dilutions in complex medium and $1 \mathrm{CCU} / \mathrm{mL}$ was defined as the highest dilution of cells able to change the color of the medium (Figure 3.4).


Figure 3.4: Growth estimation based on color changing units (CCU). Each time point growth is measured based on serial dilutions in plates; an aliquot from the original growth media is inoculated into a 96 -well plate and diluted by a factor of 10 . The number of wells at the end of two weeks that were able to acidify media (from red to yellow) are the number of CCUs in the original time point.

To estimate the doubling time and the growth rates, we used the following formula:

$$
\mu=\ln (2) / t_{d}
$$

where $\mu$ is the growth rate and $t_{d}$ is the doubling time (or generation time). The doubling time can also be calculated as follows:

$$
N=N_{0} * 2^{\left(t / t_{d}\right)}
$$

where $N$ is the final cell concentration, $N_{0}$ is the initial cell concentration and $t$ is the time between the two measurements.

### 3.2.3 Samples for NMR spectroscopy

NMR was performed with (i) complex Friis medium (with strains MHP7448, MHPJ, MHR19781 and MFL27716), and (ii) defined Yus+ medium [?] (with strains MHPJ, MHR19781 and MFL27716). For the Friis medium, cells were collected at the following time intervals: $0 \mathrm{~h}, 8 \mathrm{~h}, 10 \mathrm{~h}, 24 \mathrm{~h}, 32 \mathrm{~h}$ and 48 h . For the defined Yus + medium, cells were collected at the following time intervals: $0 \mathrm{~h}, 8 \mathrm{~h}, 24 \mathrm{~h}, 32 \mathrm{~h}$, $48 \mathrm{~h}, 56 \mathrm{~h}$ and 72 h . Cells were separated from growth media through sedimentation at 3360 g for the NMR analysis. Samples consisted of biological triplicates in complex medium and biological duplicates in defined medium.

### 3.2.4 NMR Analysis

Sample preparation
$60 \mu \mathrm{~L}$ of a mixture containing $1.25 \mathrm{M}_{\mathrm{K}}^{2}$ PO $\mathrm{PO}_{4}$ phosphate buffer $(\mathrm{pH}=7,4)$ in $\mathrm{D}_{2} \mathrm{O}$ with 2 mM NaN and $0.1 \%$ trimethylsilyl propionate (TMSP) was added to $540 \mu \mathrm{~L}$ supernatant samples. Both solutions were mixed thoroughly and 550 L were then transferred to 5 mm NMR tubes and sorted in 96 -tubes racks. All NMR experiments were carried out on a Bruker 800 MHz NMR spectrometer equipped with a 5 mm TXI probe and a SampleJet autosampler, enabling high-throughput data acquisition for large collections of samples. The temperature was controlled at $27^{\circ} \mathrm{C}$ throughout the experiments, and the samples were kept refrigerated at $4^{\circ} \mathrm{C}$ during a waiting time of less than 24 h in the autosampler, before the NMR analysis. Standard 1H 1D NMR pulse sequence nuclear Overhauser effect spectroscopy (NOESY) with z-gradient and Carr-Purcell-Meiboom-Gill (CPMG) with water presaturation (Bruker pulse program noesygppr1d and cpmgpr1d) were applied on each sample to obtain the corresponding metabolic profiles. A total of 128 transient free induction decays (FID) were collected for each experiment with a spectral width of 20 ppm . The relaxation delay was set to 4 s . The NOESY mixing time was set to 10 ms . The total acquisition time of each sample was 12 min 34 sec . The CPMG spin-echo delay was set to 300 ms , for a total filter of 77 ms , allowing an efficient attenuation of the lipid and protein NMR signals. The $90^{\circ}$ pulse length was automatically calibrated for each sample at around $10 \mu \mathrm{~s}$.

## Data processing

All FIDs were multiplied by an exponential function corresponding to a 0.3 Hz line-broadening factor, prior to Fourier transformation. 1H-NMR spectra were manually phased and referenced to the TSP signal ( $=-0.016 \mathrm{ppm}$ at pH 7.4 ) using Topspin 3.1 (Bruker GmbH, Rheinstetten, Germany). Extraction of a data matrix for multivariate statistical analysis from the 1 H NMR profiles was done using the Statistics toolbox of AMIX (Bruker Biospin). Spectra were integrated from 0.3 to 10 ppm at a step of 0.01 ppm but excluding the regions of residual water at $4.68-4.88 \mathrm{ppm}$. No normalization of the intensity was performed. The resulting data matrix contained 947 NMR variables.

## Multivariate data analysis

Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were performed using SIMCA-P 13 (Umetrics, Umea, Sweden) with scaling based on the Pareto method.

## Metabolites identification and quantification

Metabolite identification was achieved by comparing spectra with databases such as HMDB. Identification of the metabolites was further verified with homonuclear and heteronuclear 2D NMR experiments such as $1 \mathrm{H}-13 \mathrm{C}$ HSQC, $1 \mathrm{H}-1 \mathrm{H}$ TOCSY and J-resolved experiments. There was no numerical value for the confidence of the assigned peaks. Assignment was based on the heteronuclear 2D experiments and in comparison to databases. Absolute quantification of these validated metabolites was performed using Chenomx NMR Suite (Chenomx Inc., Edmonton, Canada).

### 3.2.5 FBA analysis

The metabolic networks were exported as mathematical Systems Biology Markup Language (SBML) models [108]. They were uploaded to the COBRA-toolbox v2.0 Matlab extension [241] for Flux Balance Analysis (FBA) testing. Minimum-maximum flux constraints were imposed based on literature information [75, 307]. Growth simulations were achieved using biomass production or a selected product as the FBA objective function.

Reaction essentiality analysis Since we have no information on gene essentiality for any species, we checked instead reaction essentiality in the pan-network. This was made by deleting individual reactions from the model and testing by FBA analysis if the in silico mutant was viable or not, similarly as tested for in silico knockouts. We checked all transport, enzymatic and spontaneous reactions from the pan-network.

### 3.3 Results

### 3.3.1 Mycoplasma cultivation

## Cultivation in Complex and Defined Media

We tested the cultivation of the three species of Mycoplasma in complex and defined media to gather experimental information and compare to in silico growth (Figures 3.5 and 3.6). In the CMRL media supplemented with cofactors (CMRL+), M. hyopneumoniae and M. flocculare remained viable for more time when peptone was present (CMRL+/Pep). Supplementation of peptone appeared to have a negative effect on the growth of M. hyorhinis, but more tests should be performed to verify this hypothesis. Defined Yus+ medium did not allow proliferation in any species, but maintained cell concentration and viability even after 5 days of culture (if inoculated afterwards in complex media). These results are in agreement with a previous work performed by Bertin and colleagues (2013) for M. mycoides subsp. mycoides that shows that CMRL-1066 contains all components to support cellular metabolism but not growth. Here, the supplemented versions CMRL+ or CMRL+/Pep allowed proliferation only for $M$. hyorhinis during the first 24 hours of growth. It seems that after this period of time, one or more essential metabolites initially present were no longer available.

## New defined media for Swine Mycoplasmas

By comparing the reconstructed models and the media composition, we propose that key cofactors were not delivered to the mycoplasmas in the correct form. For instance, thiamine pyrophosphate should be directly delivered to all species instead of its precursor thiamine, and pyridoxal-5-phosphate should be the cofactor of choice in place of pyridoxal. A list of correct precursors for the models versus the actual cofactors found in the defined Yus+ medium can be seen in Table 3.2. Based on these new cofactors, we also propose a new defined medium (Table 3.3) for M. hyorhinis, M. hyopneumoniae and M. flocculare.


Figure 3.5: Cultivation curves in defined and complex media by species. Cell concentrations were estimated by the CCU method and error bars were calculated as the standard deviation between triplicate time-matched samples. As expected, the three species had better growth rates in the complex medium than in defined media.


Figure 3.6: Cultivation curves in defined and complex media by media type. Cell concentrations were estimated by the CCU method and error bars were calculated as the standard deviation between triplicate time-matched samples. While the growth rates were different for complex medium and CMRL media, the defined Yus + medium was able to maintain a similar level of viability for the three species up to 5 days after inoculation.

Table 3.2: Correct precursors for biomass production for M. hyopneumoniae, M. hyorhinis and M. flocculare.


Table 3.3: New defined media proposed for the cultivation of swine mycoplasmas.


### 3.3.2 NMR Analysis

## Compounds detected in complex media

NMR metabolomics of the complex Friis medium was performed in order to gain information on which substrates are available for growth. All detected metabolites are listed in Table 3.4. Measurement of
the metabolites from the growth in complex medium was done as a ratio of the area of each metabolite from each spectra divided by the area in the initial Friis medium.

Table 3.4: Metabolites detected through NMR in complex Friis and defined Yus+ media.


## Metabolic profiling of mycoplasmas in exponential growth

NMR analysis of the complex culture medium was performed in order to detect possible differences in the metabolism of the three species during exponential growth. All data measured in complex media can be seen in Appendix B. A multivariance analysis was able to show distinct trajectories for each species, indicating that indeed in this dataset, there are sets of metabolites that can differentiate one species from another in the course of time (Figure 3.7).

Metabolites ratios (between initial and final concentrations) can be seen in Figure 3.8. No major differences in the metabolism were detected for carbohydrate, cofactor or nucleotide uptake. Trehalose is possibly degraded into glucose outside of the cells for the three species. This can be seen because glucose levels drop significantly only for the growth of M. hyopneumoniae strain J, which does not seem to degrade trehalose with the same efficiency as the other 3 organisms. Nucleic acid uptake of


Figure 3.7: Multivariance analysis of NMR datasets of complex media. Four separate trajectories over time indicate distinct features in each species. The analysis was made in two series: (A) one for M. hyopneumoniae strain 7448 and $M$. flocculare strain ATCC 27716, and one from (A) M. hyorhinis ATCC 17981 and M. hyopneumoniae strain J, since the sampling and lectures were independent from one another.
cytidine and inosine is high; uridine is proposed to be uptaken, based on the metabolic networks and on a peak that was superposed with cytidine and methylhistidine.


Figure 3.8: Ratios between initial and final concentrations of selected metabolites. Samples consisted of biological triplicates. Error bars not shown. Observe that these are the result of single peaks of NMR data. Overall, if a metabolite had more than one peak, no major differences were detected between them. We plotted also the ratio of uridine but only as an indication given that this peak was superposed with two other metabolites.

The major differences were related to the metabolism of pyruvate (Figure 3.9): M. hyorhinis produced higher quantities of pyruvate at the end of 48 hours in complex medium. As a result, pyruvate conversion to acetate was detected in low quantities for this species. M. hyopneumoniae and M. flocculare, on the other hand, produced higher amounts of acetate (this was even more pronounced for the growth of $M$. hyopneumoniae). Low amounts of formate, fumarate and succinate were also detected, and this indicates the presence of genes encoding the enzymes EC 2.3.1.54, EC 4.2.1.2, EC 6.2.1.5 in these genomes. All models were thus modified to accommodate these activities in a final refinement step.

The presence of myo-inositol in swine serum [102] may be directly related to the higher production of acetate in the growth of M. hyopneumoniae in complex medium if compared to M. flocculare. Since


Figure 3.9: Distinct products of the metabolism of pyruvate of MHP7448, MHPJ, MFL27716 and MHRHUB1 in complex Friis medium. Error bars were calculated as the standard deviation between duplicate time-matched samples. In complex medium, both MHP and MFL27716 can produce high amounts of acetate, with higher concentrations in MHP; MHRHUB1, on the other hand, produces low concentrations of acetate in this medium. Instead, the final glycolysis product for MHRHUB1 is pyruvate. The three species can produce low amounts of formate. For simplicity reasons, error bars not shown. For acetate, pyruvate and formate see Figure 3.14
the myo-inositol pathway produces AcCoA , the recovery of a molecule of CoA from myo-inositol is possible and is directly linked to the production of acetate. Since the myo-inositol catabolic pathway is one of the few distinctions between the metabolic models of $M$. hyopneumoniae and M. flocculare, it is plausible to assume that the differences in acetate concentration in vitro may indeed arise from the ability of M. hyopneumoniae to uptake myo-inositol. Acetate production seems to be independent from formate production in M. hyopneumoniae and M. flocculare (Figure 3.10. However, the similar low concentrations of acetate and formate in the growth of $M$. hyorhinis might be an indication that both are produced concomitantly by the action of the enzymes EC 2.3.1.54 (turnover of pyruvate to formate and AcCoA ), EC 2.3.1.8 (AcCoA to Acetyl-P) and EC 2.7.2.1 (acetyl-P to acetate).

To check if the lower concentrations of acetate were a result of an impaired activity of the pyruvate dehydrogenase complex in M. hyorhinis, we compared the predicted proteins PdhA, PdhB, PdhC and PdhD from all species. Known active sites of ODP2 (homolog of PdhC) and OLDH1 (homologs to PdhC and PdhD respectively) from Bacillus subtilis were analyzed in search for a possible mutation. No active sites have been described for PdhA and PdhB so far, but the enzymes for all three swine mycoplasmas do not present significant differences. Although not all active sites from PdhD seem to exist in mycoplasmas, all predicted proteins from the species analyzed (including M. genitalium and M. pneumoniae) are similar in terms of active sites, when these are present. The only difference between M. hyorhinis and the other two species was found in PdhC, in the protein portion related to the binding to PdhA and PdhD. This portion is the most variable between the species. An enzyme characterization should be made in order to verify if this complex is not active in this species in these cultivation conditions, or if it is responsible for another unforeseen activity.

## Compounds detected in Mycoplasma Defined Medium

NMR analysis of the defined Yus+ medium was performed in order to identify which metabolites present in the media were actually detectable by this technique and are listed in Table 3.4. Compounds in defined medium had their concentrations detected and were not measured as a ratio of the initial concentration, as was done in the previous experiment. Also, we had time point measurements to check the stability of the medium throughout the entire experiment (mentioned in the pictures as


## mer Metabolite detected by NMR

Figure 3.10: Two possible ways for the production of acetate in swine mycoplasmas. (A) Acetate production be independent on the formation of a formate molecule by the action of the pyruvate dehydrogenase complex or (B) dependent on the formation of a formate molecule by the action of pyruvate formate-lyase (EC 2.3.1.54)
negative control, CTRL-). Most components do not seem change concentration over time, as can be seen in Figure 3.11.


Figure 3.11: Stability of most metabolites throughout the time of the experiments in defined media. Samples consisted of duplicate time-matched samples. Error bars were calculated as the standard deviation between replicates. It is important to notice also that samples from each time point were incubated separately, so small fluctuations may occur.

Although we added only glutamate and cysteine to the medium, we were able to detect pyrogluta-
mate and cystine. This is probably due to a spontaneous turnover of L-glutamate into pyroglutamate and cysteine rapid oxidation to cystine [310].

## Metabolic Profiling of Mycoplasmas in Stationary Culture

NMR analysis of the culture in defined Yus + medium was performed in order to detect possible differences in the metabolism of the three species during a stationary phase of the culture. All the data measured in defined Yus + medium can be seen in Appendix B. A multivariance analysis was able to show distinct trajectories for each species, indicating that indeed this dataset, has different sets of metabolites that can differentiate one species from another. No differences in the course of time are visible, maybe because the cells were not growing (Figure 3.12).


Figure 3.12: Multivariance analysis of the NMR datasets in defined Yus+ medium. Three clusters indicate distinct features in each species.

Defined medium cultivation also showed some differences among the species (Figure 3.13). Differences in the metabolism of pyruvate were in accordance with our previous complex medium experiment, in which acetate was not produced in high levels for M. hyorhinis, while the three species produced low concentrations of formate (Figure 3.14). Even though we detect a high difference in the ratio of initial and final formate among the three species (Figure 3.13), when we plot the actual time concentrations (Figure 3.14), we see that this difference is less pronounced once we consider the actual variability between samples. We also detected similar final concentrations of acetate for $M$. hyopneumoniae and M. flocculare. This result corroborates with our previous assumption that when no source of myo-inositol is present, no difference in the concentration of acetate is seen between these two species.

As concerns carbon source uptake, the presence of trehalose is actually a residue of complex medium components. This is due to the difference in inoculum volume, which, for M. hyopneumoniae and $M$. flocculare was up to 10 times higher than for $M$. hyorhinis. The inoculum volume for $M$. hyopneumoniae and M. flocculare was in accordance to approx. $10 \%$ of final medium volume; however, since the cells of M. hyorhinis were more concentrated, we added less volume of this species inoculum to the defined Yus+ medium. The presence of trehalose also resulted in a difference of glucose concentration: since trehalose is probably degraded outside the cells into two molecules of glucose, whenever trehalose was present in higher concentration (M. hyopneumoniae and M. flocculare), we see a decrease in the ratio of trehalose accompanied by an increase in the ratio of glucose.

A variable amount of glycerol already in the initial time points may be in part explained by the inoculum volume; Inoculum consisted of 5 to $15 \%$ of glycerol for cryoprotection. Even with these initial differences, we detected a high ratio between final and initial concentrations of this metabolite, indicating production of glycerol in M. hyopneumoniae. This seems extremely odd if we consider our previous results and the reconstructed networks. Glycerol production has never been described for any Mollicutes, which is also why we did not assume this activity was present in the M. hyopneumoniae


Figure 3.13: Ratio between initial and final concentrations of metabolites in the growth of the three species in defined Yus+ medium. Samples consisted of duplicate time-matched samples. Error bars not shown.
models used in this work. Moreover, since the levels of glycerol in our defined Yus+ medium were higher than the one described by Yus + and collaborators (2009), we did not make any assumptions on this particular metabolite, as more standardized experiments should be made in order to validate these results. As these experiments were done at the end of this PhD , we did not have time to repeat them. For future experiments, we intend to wash the cells prior to inoculation to avoid carry-over from the inoculum.

The analysis of amino acid uptake was not trivial since the defined medium contained peptone. This metabolite was at first not measured through the CPMG experiment but, in the course of time,


Figure 3.14: Distinct products of the metabolism of pyruvate from growth in defined Yus + medium of MHPJ, MFL27716 and MHR17981. Error bars were calculated as the standard deviation between duplicate time-matched samples. These results corroborate with the previous experiment in complex medium, in which little ammounts of acetate were produced by MHRHUB1. In defined medium, MFL27716 and MHPJ produce similar amounts of acetate. The three species can produce low amounts of formate in both media.
was degraded into single amino acids, and thus changed the overall signal of the NMR spectra. Despite this issue, Figure 3.13 shows that we detected lower concentrations of most amino acids at the end of the growth curve for $M$. hyorhinis, if compared either to the control medium or to the other species. This may be a result of several factors, such as lower rates (in comparison to the other two species) of peptone degradation by membrane proteases or higher uptake rates of amino acids by M. hyorhinis. At this point, we could no longer verify which hypothesis is more suitable for this particular distinction; however, since peptone was not necessary for the maintenance of the viability of M. hyorhinis in the defined medium, it is possible that this species may harbor transporters with higher specificity or effectiveness for single amino acids. On the other hand, we detected higher levels of amino acids for M. hyopneumoniae. Inverse explanations are possible here: either M. hyopneumoniae harbors more effective proteases in its membrane, which is plausible, given the number of studies concerning peptidases in this species [216, 267]; or it has a lower capacity of uptaking amino acids if compared to both M. hyorhinis and M. flocculare. Even if the last hypothesis has not been investigated up to this point, we consider it highly unlikely, given the enhanced ability of M. hyopneumoniae to survive in peptone-depleted medium (CMRL+) when compared to M. flocculare.

### 3.3.3 FBA Analysis

We tested all models to verify if they could indeed produce or consume the detected metabolites in the NMR analyses. Acetate, pyruvate and lactate were obtained during FBA growth on glucose with biomass as the objective function. Lactate was obtained in higher concentrations when oxygen uptake was constrained to low rates. At first, succinate, formate and fumarate were not produced in silico during model refinement. To accommodate growth in complex media results, we added reactions EC 2.3.1.54, EC 6.2.1.15 and EC 4.2.1.12 to allow, respectively, the production of formate, succinate and fumarate.

We then tested the pan-network to arrive at an acceptable in silico growth rate, close to the ones in vivo. For comparison, the usual growth rate of the E. coli core model in aerobic growth is approximately $1.7 h^{-} 1$ [190]. Since our organisms have experimental slower growth, we wanted to arrive at lower in silico growth rates than the ones reported in E. coli.

In an unconstrained model, we arrived at a high growth rate of $65.3657 h^{-} 1$. After we added
only three constraints related to in vivo exchange rates for glycerol (based on the network of $M$. pneumoniae), glucose and oxygen (based on the assumption of maximum uptake rates reported in other organisms), we were able to reduce the initial high growth rate to $1.0587 h^{-1}$, much closer to the actual in vivo one (Table 3.5).

Growth rates for Mycoplasma species are somewhat controversial. Since the time of growth in complex commercial media (in France) did not give us the same results as the growth in Friis medium (in Brazil), we estimated the doubling time based on our experiments in France and compared it to the literature: for M. hyopneumoniae, the doubling time was calculated between 4.8 and 6.5 hours. This was in accordance with a previous work that estimated the doubling time of this species to be between 4.8 and 7.5 hours [32]. These doubling times give us a range from $0.11 h^{-} 1$ to $0.14 h^{-} 1$. We had no information for $M$. flocculare, but from our experiments, the doubling time and in vivo growth rate were approximately 6 hours and $0.11 h^{-} 1$, respectively. We also calculated the doubling time for M. hyorhinis and they ranged from 1.2 to 2.5 hours, resulting in an in vivo growth rate from 0,27 to $0.57 h^{-}$, respectively.

Table 3.5: Changes in the upper (UB) and lower boundaries (LB) in the pan-network to reduce the initially high in silico growth rate.

| Boundaries |  |  | Initial Growth Rate $\left(\mathbf{h}^{\mathbf{- 1}}\right)$ |
| :---: | :---: | :---: | :---: |
|  | Unconstrained | 65.3657 |  |
| Reaction | Metabolite | Constraint $(\mathbf{m m o l} / \mathbf{g D W . h})$ | Growth Rate $\left(\mathbf{h}^{\mathbf{- 1}}\right)$ |
| EX_036 | Glycerol | $\mathrm{LB}=-0.1278$ | 48.4547 |
| EX_032 | Glucose | $\mathrm{LB}=-5$ | 13.0959 |
| EX_068 | Oxygen | $\mathrm{LB}=-5$ | 1.0587 |

We also tested ATP and NADH maximum yields by fixing the glucose uptake rate at -1 mmol $g D W^{-} 1 h^{-} 1$, adding synthetic drain reactions for ATP and NADH and maximizing them as objective functions.

ATP_drain: ATP $\rightarrow$ ADP +Pi
NAD $\bar{H}$ _drain: $\mathrm{NADH} \rightarrow \mathrm{NAD}+H^{+}$
At first, we checked the maximum amount of ATP the models could produce (ATP_drain was set as the FBA objective function). At first, the tests yielded an extremely high production of ATP, not related to in vivo rates. Thus, we mapped the network in the search of which ATP precursors were causing this unusual behaviour (Figure 3.15). The two major precursors of ATP in these conditions were cytidine and glucose. The extremely high production rate was generated because of an extremely high uptake of cytidine. To address this issue, we decided to constraint the uptake of this particular nucleotide to a rate closer to the ones considered for other models [75]. After this, we arrived at a growth rate of $0.6343 h^{-} 1$, closer to the in vivo reported rates.

Also, in order to compare the energy metabolism of our models to the core metabolic network of $E$. coli, we blocked the reaction responsible for the link between nucleotide metabolism and carbohydrate metabolism (EC 5.4.2.7). This particular reaction converts ribose-1-phosphate (R1P) into ribose-5phosphate (R5P). The addition of these two constraints, in turn, made the production of ATP only possible from extracellular glucose. In this way we proceeded to cofactor yield, robustness, carbon source and topology analysis.

The maximum ATP and NADH yields were calculated at $9.7444 \mathrm{mmol} g D W^{-} 1 h^{-} 1$ and 4.2556 mmol $g D W^{-} 1 h^{-} 1$, respectively. These two numbers are fairly in agreement with the calculated yields from E. coli, of 17.5 and $10 \mathrm{mmol} g D W^{-} 1 h^{-} 1$ for aerobic growth.

## Robustness Analysis

FBA was performed to maximize biomass production with varying rates of oxygen and glucose. As expected, growth rates are directly proportional to the uptake rates of both glucose and oxygen (Figure 3.16). Both metabolites seem to be limiting for the uptake of each other, since higher fixed uptake rates ( $-100 \mathrm{mmol} g D W^{-} 1 h^{-} 1$ ) yielded higher growth rates. A phenotypic phase plane ( PhPP ) is a constraint-based method that provides a global view of how optimal growth rates are affected by


Figure 3.15: Precursors and reaction rates for the production of ATP in the pan-network: (a) before and (b) after the constraint of cytidine uptake rate..
changes in two environmental variables (for instance, carbon and oxygen uptake rates). In a PhPP analysis, FBA and linear programming are used to identify phases with distinct metabolic pathway utilization patterns. We calculated a PhPP by varying both uptake rates simultaneously to find a line of optimality for growth, which represents the relationship between the glucose and oxygen uptake rates that results in optimal growth rate (Figure 3.17).


Figure 3.16: Growth rates of the pan-network with variable uptake rates of glucose and oxygen. The uptake rate of glucose was varied with a fixed oxygen rate at -5 and $-100 \mathrm{mmol} g D W^{-} 1$ $h^{-} 1$ and no other fixed constraints. The uptake rate of oxygen was varied (from -1 to -20 ) with a glucose uptake rate fixed at -5 and $-100 \mathrm{mmol} g D W^{-} 1 h^{-} 1$.


Figure 3.17: Phenotypic phase planes for growth with varying glucose and oxygen uptake rates. The line of optimal growth (as a white arrow) can be seen between phase planes 2 and 3 .

## Growth on Alternate Carbon Sources

For simplicity reasons and as a default, the carbohydrate present in the biomass composition was glucose. However, there may exist a broad set of carbohydrates that actually compose the biomass in vivo. Whenever we wanted to try the growth on different carbon sources and glucose was excluded from the in silico medium, glucose was exchanged in the biomass reaction by another suitable carbohydrate.

The pan-network was able to grow on glucose, sucrose, starch, trehalose, lactose and isomaltose (Table 3.6). Growth on fructose, mannose, mannitol, glucosamine, glycerol, amino sugars (GlcNAc and sialic acid), myo-inositol, dihydroxyacetone and ascorbate was possible if the carbohydrate of biomass was changed to a non-specified sugar and if we did not take into account the production of glycolipids (MGDG and DGDG) (Table 3.7).

Overall, all species were able to grow on glucose, starch, fructose, mannose, mannitol and glucosamine. Only M. hyorhinis models were capable of growing on maltose, isomaltose, trehalose, sucrose, dihydroxyacetone and sialic acid. Both M. hyorhinis and M. hyopneumoniae models could
grow in lactose and glycerol; M. hyopneumoniae and M. flocculare could grow in GlcNAc and only M. hyopneumoniae was able to grow in myo-inositol.

Table 3.6: Carbon sources and yield growth rates in the unmodified pan-network.

| Substrate | Growth Rate <br> $\mathbf{( h - 1 )}$ |
| :---: | :---: |
| Clucose | 0.4354 |
| Sucrose | 0.4371 |
| Starch | 0.5357 |
| Trehalose | 0.4864 |
| Lactose | 0.3866 |
| Isomaltose | 0.4371 |

Table 3.7: Growth on alternate carbon sources specific to species models.


## Topological features of the $S$ matrix

Determination of the number of reactions each metabolite occurs in can be analysed to compare the models reconstructed in this PhD thesis with the ones already described for other models. Figure 3.18 shows similar characteristics of the pan-network and of the E. coli model [190] regarding metabolite connectivity.


Figure 3.18: Connectivity of metabolites (loglog plot) in (A) the pan-network and (B) E. coli (extracted from [190]) show similar features for both networks..

## Reaction Essentiality Analysis

Since we have no information on gene essentiality for any species, we checked reaction essentiality in the pan-network. This means that we deleted individual reactions (all transport, enzymatic and spontaneous reactions) even if they did not have a GPR association. A total of 111 reactions ( 69 enzymatic, 37 transport, and 5 spontaneous reactions) were essential for growth in the pan-network (Appendix C). Gene essentiality accounts for more than metabolic enzymes, which means that proteins not included in our models and are related to protein synthesis, DNA polymerization or RNA turnover, are not accounted for in the essential reactions. If we added these proteins with non metabolic-related activities, we would arrive at numbers closer to those of M. genitalium (382, [90]) and M. pneumoniae (310, [109]). The lack of experimental information on gene essentiality is also a setback for the validation of the models created here. Further experiments on this matter should help us better refine and complete the networks.

### 3.4 Discussion

Even though the metabolic networks were quite similar, we chose to perform our own metabolomic experiments due to the lack of experimental information for the three species.

The models reconstructed in the previous chapter served as a basis for all the assumptions made for the experimental data. Metabolic profiling of both complex and defined media pointed to new differences that we were not able to identify based solely on the sequenced genomes. Growth tests allowed us to assign correct cofactors for biomass production in each species and may help formulate new defined media for the cultivation of swine mycoplasmas. By detecting some metabolites in the cultivated media, we could infer the presence of some enzymes that at first were not present in the models as can be seen in Figure 3.19.

Indeed, through NMR metabolic profiling, we could detect a few metabolites that enabled us to establish a distinction among the three species: the production of acetate, pyruvate and formate represent the major differences among the networks. Based on the results of this chapter and on the previous assumption from Chapter 2, we were able to complete our differential metabolism from the three species (Figure 3.20).

While M. hyorhinis seems to lack the turnover of pyruvate to acetate in the conditions of our experiments, making pyruvate the final product of glycolysis, M. hyopneumoniae and M. flocculare exhibit high concentrations of acetate at the end of the growth curve. This was a striking difference detected by NMR analysis and was not foreseen in the model reconstruction. However, the predicted protein sequences from the pyruvate dehydrogenase complex of the three species were distinct in relation to PdhC, more specifically in the region responsible for binding to other complex components. We could not verify at this point if this complex is not active in the particular growth conditions of


Figure 3.19: Reconstructed models (A) prior and (B) after NMR results were used to refinement.
our work in M. hyorhinis, neither if it is responsible for another unforeseen activity. However, a transcriptome profiling of $M$. hyorhinis has detected both pdhA and pdhB in the pool of genes with the highest number of transcript reads [255], indicating that the complex might be translated in vivo. Moreover, pyruvate dehydrogenase activity has been previously detected in M. hyorhinis extracts [46], and it seems to be correlated to oxygen availability. As previously described by Tourtellotte and Jacobs (1960), M. hyorhinis had also low capacity to produce acetate, which corroborates with our findings here. In our cultivations, the cells were not grown in a complete aerobic system, which may explain the differences between our findings and those previously published by Constantopoulos and


Figure 3.20: Differential metabolism of M. hyorhinis, M. hyopneumoniae and M. flocculare; updated from Figure 2.34 with NMR and growth experiments.

## McGarrity (1987).

M. hyopneumoniae is the only Mycoplasma species with sequenced genome that has the genes for the catabolism of myo-inositol. Myo-inositol is readily abundant in the bloodstream of mammalian hosts, and can be used as a secondary carbon source for energy production [223]. Mycoplasma iguanae has been described to produce acid from inositol [24]. However, there is no available complete genome sequence for this organism, making impossible any comparison of the genes involved in this pathway. Although at this point we cannot confirm that this pathway is functional in M. hyopneumoniae, the NMR results we obtained suggest that myo-inositol might be directly related to the higher production of acetate in the complex medium of $M$. hyopneumoniae as compared to M. flocculare. This assumption is based on two factors: first, a previous work has detected myo-inositol in the swine serum, which is a component of the complex medium [102]; and second, the only difference in the networks that could influence the acetate concentration is the myo-inositol catabolism. This pathway, if active, might act as an alternative production of AcCoA , precursor to CoA, an essential cofactor for growth in all species.

Experimental growth tests also showed that neither species was able to grow in the defined media. This is probably due to a lack of the correct cofactors. For instance, thiamine, pyridoxal/pyridoxine, pantothenate, spermine and folate were the actual media components tested, but the reconstructed models were not able to convert them to biomass precursors (thiamine to thiamine pyrophosphate, pyridoxal to pyridoxal-5-phosphate, pantothenate to 4 -phospho-pantheteine, or spermine into spermidine). Only M. hyorhinis seems to be able to uptake folate directly while the other two might need intermediate metabolites. This might also explain why M. hyorhinis has an enhanced grwoth capacity over the other species and is extensively found as a cell culture contaminant.

Even though we supplemented CMRL-1066 (CMRL+) with all missing components present in the Yus+ medium, cells maintained viability for longer periods in the latter. Cofactors present originally in both media existed in lower concentrations in the CMRL-1066. This means that possibly one or more cofactors with higher concentrations in the Yus+ medium are essential for the viability of the three species. Common cofactors with higher concentration in Yus+ medium included: choline, folate, pantothenate, pyridoxal, thiamine and spermine.

Other differences among the species seem to indicate that M. hyopneumoniae and M. flocculare lack one or more amino acid transporters. This may be due to two things: (i) both remained viable for longer periods whenever CMRL+ was supplemented with peptone, and (ii) we detected a higher amino acid accumulation in the defined media if compared to M. hyorhinis. M. hyorhinis on the other hand may have all the transporters for single amino acids, but this hypothesis needs to be further verified. From the sequence analyses, we did not find significant differences among the species that would explain this distinct behavior. Moreover, since M. hyorhinis seemed to overgrow in the first 24 hours of culture in both CMRL media (CMRL+ and CMRL+/Pep), it is possible that one or more compounds were missing in the Yus + medium in order to allow growth of this species. Possible candidates for such (present in CMRL-1066 and absent in the Yus+ medium) would be: CoA, nicotinamide, ascorbate and/or pyridoxine.

Whether the main differences among the species we reported here (summarized in Table 3.8) are related to virulence or pathogenicity have not yet been addressed experimentally, but it is tempting to speculate. The same factors that may enhance virulence of $M$. hyopneumoniae may help the commensal species $M$. flocculare to better survive inside the host.

All the metabolic differences we reported here, together with our previous findings and literature data have helped us better understand the differential metabolism of each species and, moreover, seem to point to the existence of a machinery for differential gene expression in these species.

Table 3.8: Differential metabolism that might result in enhanced virulence in the pathogenic species.

| Virulence Factor for pathogenic species | Metabolic Activity |  | Species |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: |
|  |  |  | M. hyopneumoniae | M. hyorhinis | M. flocculare* |
| Enhanced Growth Rate | Carbohydrate Metabolism | Myo-inositol uptake and catabolism | $\checkmark$ | $\square$ | $\square$ |
|  |  | Lactate degradation | $\checkmark$ | $\checkmark$ | $\square$ |
|  |  | Sucrose, maltose, isomaltose, trehalose uptake and metabolism | $\square$ | $\checkmark$ | $\square$ |
|  | Cofactor metabolism | Folate conversion to THF | $\square$ | $\checkmark$ | $\square$ |
|  |  | Coenzyme-A precursor production from myo-inositol | $\checkmark$ | $\square$ | $\square$ |
|  | Pyruvate Metabolism | Acetate production by pyruvate dehydrogenase complex | $\checkmark$ | $\square$ | $\checkmark$ |
|  | Amino Acid Metabolism | Enhanced uptake of single amino acids | $\square$ | $\checkmark$ | $\square$ |
| Host Evasion | Nonimmunogenic capsule production | GIcNAc uptake | $\checkmark$ | $\square$ | $\checkmark$ |
| Cytotoxicity | Glycerol metabolism | Hydrogen peroxide production | $\checkmark$ | $\checkmark$ | $\square$ |
|  |  | Enhanced glycerol uptake | $\checkmark$ | $\square$ | $\square$ |

Notes:
Although M. flocculare is commensal, it presents some metabolic similarities to M. hyopneumoniae that may help increase the virulence of the pathogenic species. In M. flocculare, the presence of these two metabolic activities may not be related to pathogenicity itself, but rather to enhanced survival mechanisms in the host.

## Chapter 4

## Dialogue Between Species

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### 4.1 Overview

From the previous chapter, we assessed that it may be possible that some cofactors or metabolites are missing from the defined medium. This is the reason why we have to remember that the metabolism of these organisms cannot be completely understood when isolated from their environment. The environmental context may explain the differences between the in silico models and the in vivo behavior. Our main objective in this chapter was to propose hypothetical links between the knowledge we have from experimental data and our predictions from the networks. Again, it is important to notice that we do not claim that the metabolism and the metabolic networks that we infer will be able to fully explain what happens in each species and the dialogue between these bacteria and the host. However, we tried as much as possible to relate the reconstructed networks to known virulence factors from these organisms. For instance, even if we could not model the polysaccharide capsule in the networks, we tried to relate their composition to the different levels of pathogenicity among the species. In the broad context of systems biology, we propose general models at the end of the chapter for the interaction of these mycoplasmas and the host. However, these are only hypothetical models, as they have not been tested up until now in vivo in any of these species.

### 4.1.1 Persistence in host and escaping the host immune system

Mycoplasmas are able to survive inside the host in both diseased and healthy states. The three species have their specific mechanisms of evading host immune system. Here we will discuss some of these mechanisms that not only help the survival of them but also allow them to undergo a harmful metabolism and cause diseases. Even though M. flocculare does not cause any illness, it may be present in both healthy and diseased pigs and may profit from the pathogenic metabolism from the other two pathogenic species. Most of all, it is essential to remember that all species co-exist in a complex community and any unbalance may turn a non-pathogenic system into a pathogenic one.

## Biofilm formation and regulation

Biofilms are sessile bacterial communities attached to each other, and/or to a surface, usually enclosed by a sugary polysaccharide extracellular matrix, called glycocalix or slime layer [165]. Because of this encasement of polysaccharides, biofilms can enhance the resistance of pathogens to immune system effectors and antimicrobial agents[50, 296]. Biofilms represent the predominant state of bacteria in nature; only a small fraction of bacteria in natural ecosystems are believed to exist planktonically [54]. Biofilm formation seems to be related to environmental persistence and survival of Mycoplasma species [166]. Even though mycoplasmas lack described factors important for biofilm formation in other species, the majority of them have the ability to form biofilms [166]. Biofilm formation usually requires complex processes as well as regulation of gene expression, and is likely to be an important step in disease persistence in the host [133]. On the other hand, there are no known regulatory systems or other recognizable global regulators in most species of mycoplasmas [165].

## Variable surface antigens

Many surface proteins have been identified in Mycoplasma species and some of these are of utter importance to biofilm formation and cyto-adherence; some of them will be discussed below. Simmons and collaborators have studied biofilm formation in Mycoplasma pulmonis [251] and reported that its ability to form biofilm is dependent of the variation in the number of variable surface antigen (Vsa) repeats. Size variation of Vsa is a random mechanism that occurs as a result of slipped-strand mispairing of tandem repeats during DNA replication [251]. The authors also found out that strains that produced short Vsa proteins formed biofilm on glass surfaces; and strains that produced long Vsa proteins formed microcolonies, or free-floating biofilms, instead of attached biofilms. In a previous work [252], they also correlated the number of tandem repeats with the ability of M. pulmonis to resist complement lysis from the host: short VsA proteins that adhered strongly to polystyrene made the organisms highly susceptible to complement killing.

Although there are many Vsa silent genes in their genomes, each M. pulmonis cell produces only one Vsa at a time. In a clonal population, it may occur that individual cells start expressing alternative Vsa proteins due to phase variation [250]. These Vsa phase variants in M. pulmonis arise only when the cells need an adaptative response to immune system effectors.

In conclusion, it seems that aggregates of short Vsa M. pulmonis cells are selected to attach to the host epithelium and establish tower structures composed mainly by long Vsa cells, shielding the biggest part of biofilm community from the innate immune system [250]. One hypothesis is that some of these long Vsa containing cells, embedded in towers, detach to establish new infection foci, as previously proposed as the life-cycle of a biofilm in [165] (Figure 4.1, adapted and completed from [165], page 259 ).

Phase and size variation of proteins may also lead to epitope masking or unmasking. This means that the switch of different versions of a protein may help the bacteria to shield themselves from the immune system. Indeed, elongated variants and switch of variant lipoproteins (Vlp) have been associated to immune evasion of $M$. hyorhinis during infection [230, 43, 44]. Populations of $M$. hyorhinis capable of escaping adaptative immune system revealed a strong selection for the long variant Vlp, even when susceptible variants (short Vlp) were inoculated [43].

Conversely, Mycoplasma arthritidis avirulent strain 158-1 has an elongated T-cell mitogen (mia ORF 619) if compared to the virulent strain 158 [283]. Whether the strain 158-1 is avirulent because it has this elongated variant surface antigen or because it has some other unidentified mutation in its genome is unknown; however, no differences in 2D-PAGE protein profiles between the two strains other than this mutation were found.

Phase variation was not observed for any deduced protein variants identified so far in different $M$. hyopneumoniae strains [79]. These finding suggest a selective pressure for the maintenance of integrity of these proteins, while allowing the occurrence of size variation. Variability of surface proteins that bind to host molecules, or adhesins, in M. hyopneumoniae, also seem to arise post-translationally with the action of kinases [79] or proteases that recognize distinct cleavage motifs [59]. These processes generate combinatorial complexity on the surface of this pathogen and resulting proteolytic fragments are known to bind epithelial cilia, extracellular matrix and host circulatory molecules [31, 58, 247, 216].

For instance, P102 from M. hyopneumoniae strain 232 (mhp182) was described as a plasmin(ogen)binding protein [247]. Plasminogen is a pro-enzyme involved in degradation of fibrin clots and extracellular matrix proteins. The ability to bind and recruit plasminogen has been described as a promoter of virulence in several bacterial species [141, 285, 224]. Another example, P97, is a highly expressed protein that binds swine epithelial cilia, heparin, fibronectin and a range of glycosaminoglycans in $M$. hyopneumoniae [317, 306, 106, 64, 116, 58] and its post-translational cleavage has been described to play an important role in disease process [64]. The Djordjevic group has also discovered that Himar mutants located in one of the copies of P97 (mhp271) were unable to form biofilms (unpublished data, available on [214]).

The proteolytic process of surface proteins must involve specific proteases, which up to date, remain unknown. However, precise cleavage sites have been determined for several adhesins [59, 19, 267].

Recently, recombinant leucine aminopeptidase and glutamyl aminopeptidase (MHJ_0461 and MHJ_0125) from M. hyopneumoniae strain J [225, 115] were described to moonlight as adhesins that bind and recruit plasmin(ogen) and heparin. This interaction promotes adhesion and facilitates the cleavage of plasminogen to plasmin [225]. Plasmin has been extensively characterized as an endoprotease with broad substrate specificity that cleaves extracellular matrix proteins [9]. Altogether, the resulting fragments from plasmin activity provide a pool of free peptide substrates for both MHJ_0125 and MHJ_0461 and a source for aminoacids and oligopeptides for growth of $M$. hyopneumoniae [225, 115].

## Capsular polysaccharides

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 [304]. Liu and collaborators (2013) 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
[268], as previously mentioned, reported that capsular polysaccharides from M. hyopneumoniae plays a key role in the interaction between pathogen and host. In several bacterial species, it has been reported that the amount of capsular polysaccharide is a major factor in their virulence [48] and it decreases significantly with in vitro passages [123].

It is still unknown whether capsules play an active role in adherence in Mycoplasma species, due to the fact that the two mycoplasmas with chemically defined capsules (M. mycoides subsp. mycoides and A. laidlawii) do not adhere to host cells [217]. Apart from these two species, the composition of all other Mycoplasma capsules remains largely unknown, but even if they are not important for adherence in these two particular species, they are important to pathogenicity [217].

Although genes related to polysaccharide production were described in the M. mycoides cluster [13], they were not found in the three species studied here. It is important to note that even though this could be highly speculative and there is indeed lack of information regarding the species studied here, it is still plausible to think that the in vitro-passaged strains of M. hyopneumoniae became attenuated due to the decrease in the amount of capsular material [268]. Therefore, these indications point to the conclusion that the amount of capsular polysaccharides on M. hyopneumoniae is related to its pathogenicity.

## A link between biofilms, surface protein size and capsules?

It might be possible that the incapability the two highly pathogenic species (M. mycoides subsp. mycoides and $A$. laidlawii) to adhere is in accordance with previously mentioned biofilm formation in M. pulmonis. The authors reported greater quantities of extracellular polysaccharides in biofilm microcolonies (with longer Vsa proteins) than in attached biofilms (short Vsa proteins) [251]. Thus, it seems that the expression of longer proteins are related to the secretion of larger quantities of polysaccharides and therefore, less susceptibility to the host immune system. A general and hypothetical model for biofilm formation in pulmonary mycoplasmas can be seen in Figure 4.1.


Figure 4.1: Biofilm model in M. pulmonis Aggregates of thin polysaccharide capsule mycoplasmas are selected to attach to the host epithelium (pink cells) and establish tower structures composed mainly of thick polysaccharide capsule-mycoplasmas. Thin-capsule mycoplasmas are more susceptible to immune system effectors, such as macrophages (in light orange) and are easily degraded. Thickcapsule mycoplasmas are more resistant to the immune system and detach to establish new infection foci (Adapted from [165].)

## Intra-species associations

Recently, a study also revealed that multiple strains of $M$. hyopneumoniae can infect the same host [35], but that this was not linked to the severity of lung lesions. Other studies have suggested that simultaneous or subsequent infections with different strains may result in more severe lung lesions [295]. While the occurrence of multiple strains may not be directly linked to the pathogenicity itself, it is possible that this may be a mechanism for host evasion and host persistence. If the presence of one single strain already results in the production of several surface antigens, the presence of multiple species exponentially rises the amount of different variable antigens and makes it more difficult for the immune system effectors to recognize these bacteria.

### 4.1.2 Overview of the composition of the swine respiratory tract lining fluid

Apart from containing proteins and being an amino acid source (as mentioned in the previous section), the fluid layer that covers the respiratory tract of mammalians is a complex mixture of water, electrolytes, lipids and carbohydrates [10]. This fluid layer, also known as airway surface liquid (ASL), promotes normal lung function and protects the host against pathogens and environmental stress [278]. The ASL consists of two components: a gel-like mucus layer and a deep periciliary liquid layer, the epithelial lining fluid (ELF); gel and liquid layers are separated from the airways by a surfactant layer [278], to prevent alveolar collapse at the end of expiration. Pulmonary surfactant is comprised of approximately $90 \%$ lipids and $10 \%$ proteins [290]. One of the most used techniques to assess the composition of ASL is the use of bronchoalveolar lavage (BAL) fluid, in which a saline solution is used to wash out the ASL.

Even though the porcine lungs share many similarities with human lungs in terms of size and structure, the ASL composition of pig airways is poorly characterized [227]. The components of the ASL and bronchoalveolar lavage fluid (BAL) for the healthy porcine lung are still largely unknown [10]. Here, we show an extensive review on ASL composition (from porcine, murine and human) and further on this chapter we propose available metabolites from the host to the bacteria based on the presented literature data.

## Surfactant composition and metabolism

Based on lipid composition from several mammalian models, lipids from pulmonary surfactant are comprised by $90 \%$ of phospholipids and $10 \%$ of neutral lipids [290]. Phosphatidylcholine (PC) and phosphatidylglycerol (PG) make up for almost $80 \%$ of phospholipids; Sphingomyelin (SPM), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE) and phosphatidic acid (PA) are present at an average of $10 \%$. Less information is available concerning the neutral lipid components of pulmonary surfactant. In mammalian species, apart from major component cholesterol ( 80 to $90 \%$ ), monoacylglycerol, diacylglycerol, and triacylglycerol are also present in most species [290]. Fatty acid composition analysis from PC detected higher levels of oleic and linoleic acid radicals in rat and minipig if compared to human or rabbit [242]. PI is also highly present in BAL composition from minipigs in contrast to a minor role in BAL from human [242]. Alveolar type II epithelial cells produce pulmonary surfactant with substrates from circulation (mainly glucose, choline, fatty acids and phosphate) (Figure 4.2). The synthesized surfactant phospholipids are packaged into a storage form, the lamellar bodies, and are secreted to the gel-like phase of ASL to produce large lipid aggregates along with cytoskeleton molecules. During respiration, small aggregates are formed and they can be recycled by type II cells or degraded by macrophages and type II cells [110, 2].

## Carbohydrate composition

High molecular weight glycoconjugates (HMG) are the major contributors to the characteristic high viscosity and stringy, gel-like properties of mucus [101]. The most abundant glycoproteins in mucus are a protein family called mucins, which can be either membrane-bound or secreted. They consist of a peptide chain that accounts for 10 to $20 \%$ of the molecule total weight [308] and 80 to $90 \%$


Figure 4.2: Synthesis and recycling of pulmonary surfactant. Surfactant is synthesized by lung alveolar type II epithelial cells by recycling of small vesicules or from substrates from circulation. The synthesized surfactant phospholipids are packaged into a storage form, lamellar bodies and secreted to form the air-aqueous interface. During respiration, small and physiologically active large aggregates are formed that can be recycled by type II cells or degraded by macrophages. Adapted from [110].
of oligosaccharide chains. The oligosaccharide portion of mucins has variable sugar residue lengths that are attached to serine or threonine residues by O-glycosidic linkages with N -acetylgalactosamine (NAcGal) [101].

Since measurements of the carbohydrate composition of mucins have not revealed significant differences among species [101], we based our model in experimental data from other mammalian species. Mucins consist of heterogeneous nonrepeating sugars that often form branched rather than linear chains. The overall composition revealed the presence of NAcGal, fucose, galactose, glucosamine, galactosamine and sialic acid [308].

Other studies suggest the abundant presence of proteoglycans in mucus. Proteoglycans are more often associated with structural components of tissues: hyaluronic acid, chondroitin sulfates, dermatan sulfate, and heparin. [15, 145].

## Protein composition

ASL has been proposed to have a redundant and polyfunctional pool of secreted peptides and proteins with host defense and immunomodulatory properties. The coordinated activity of all secreted proteins promote regular lung fuction and maintain lung health [278]. The proteome from both ASL and BAL were determined recently in newborn pigs [10]. The distribution of proteins by function was similar between BAL and ASL and most of them were related to metabolic and other cellular processes (40\%), cell communication and response to stimuli (15\%), transport (10\%) and immune system processes $(10 \%)$. Based on the proteome of BAL and ASL and along with experimental information from surfactant phospholipid metabolism and carbohydrate composition we reconstructed a model for the ASL from swines.

### 4.1.3 Inter-species dialogue

Mycoplasmas are not the only genus of bacteria present in lungs of swines. Several other bacteria have been reported to inhabit the respiratory tract but, most of them are prevalent only in diseased states. Besides mycoplasmal pneumonia, the Porcine Respiratory Disease Complex (PRDC) has emerged as an economically signigicant respiratory disorder characterized by the slow growth, fever, cough, loss of appetite, lethargy and dyspnea in pigs $[276,39]$. The term PRDC refers to a multifactorial respiratory disease in pigs, resulting from interactions between viruses, bacterial infections and environmental factors. Its primary causes include Porcine Reproductive and Respiratory Syndrome virus (PRRSV), porcine circoviruses (PCV), swine influenza virus (SIV), M. hyopneumoniae, Actinobacillus pleuropneumoniae, Bordetella bronchiseptica and Haemophilus parasuis. Secondary (or opportunistic) agents include Pasteurella multocida, Streptococcus suis and Actinobacillus suis [258]. PRDC must be seen as the result of a complexity of events, including pathogens, but not only limited to them. Environmental factors such as stress, nutrition, sanitation, overcrowding or genetic factors that result in different immune responses are also essential for the development of the disease [258]. Here, we tried to gather relevant information on both healthy and diseased states of this community in order to propose a model for the switch from a non-pathogenic to a pathogenic community.

## Quorum Sensing

An important mechanism for survival in a community of bacteria is quorum sensing (QS). QS is a cell-to-cell communication mechanism, by which bacteria coordinate social activities, such as biofilm formation, virulence factor secretion, antibiotic production, among others [11]. QS is generally achieved by secretion and sensing of small diffusible molecules called autoinducers (AIs) [301]. One of the products from S-adenosyl-L-methionine (SAM) cycle is indeed autoinducer-2 (AI-2), a widely recognized signal molecule for intra and interspecies communication in bacteria, used to control gene expression in response to cell density and metabolic potential of the environment [292]. AI-2 is synthesized by the S-ribosylhomocysteinase (LuxS) enzyme, and both have already been proposed to regulate capsular polysaccharide production in Streptococcus aureus [318]. Unfortunately, no homologs for this enzyme or for any known component from QS systems were found in any Mycoplasma species so far [165]. On the other hand, McAuliffe and collaborators (2010) showed that several putative ABC transporters are directly linked to biofilm formation in $M$. capricolum. These findings support the idea that, at least partially, bacterial communication is also important to biofilm formation. Even though mycoplasmas may not produce it, it does not mean that they cannot sense it in some way. Or vice-versa, they may be able to produce something that is sensed by other bacteria. Indeed, AI-2-like molecules have been experimentally detected in supernatant cultures from P. multocida, A. pleuropneumoniae and A. suis; B. bronchiseptica and H. parasuis have at least in part in their genomes the genes for the production of this metabolite.

## Inter-species associations

Little information is available concerning the prevalence of bacteria in healthy lungs, and it has been reported that both pathogens and associations between pathogens occur mostly in pigs with clinical signs than in healthy pigs [194]. However, bacteria from the Pasteurellaceae family (which comprise genera Actinobacillus, Pasteurella, Haemophilus) have been reported to be the dominant family in the core microbiome of healthy tonsils [150], which is close to the entrance of the respiratory tract. Indeed, associations between pathogens have been studied mostly for pigs showing clinical signs of pneumonia [275, 276, 41]. Even though many species are related to PRDC, it is essential to note that enzootic pneumonia caused by M. hyopneumoniae is by far the most costly disease in pig industry, and this bacteria is usually seen as an essential component to the successful establishment of a pathogenic community in the host [258]. Also, M. hyopneumoniae infections take longer to cause lesions and take longer to be successfully eliminated than infections from other pathogens [276]. However, M. hyopneumoniae itself does not cause a high mortality rate of pneumonia, and the association with different pathogens usually enhances the severity of lung lesions [275, 41]. Literature information of the association of M. hyopneumoniae with other bacteria and viruses are summarized in table 4.1.

Table 4.1: Association between M. hyopneumoniae and other organisms in the respiratory tract of swines (NF: Association not found).

| Association with M. hyopneumoniae |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: |
| Organism |  | Healthy pigs | Diseased pigs | References |
| Bacteria | M. hyorhinis | Yes | Yes | [Ciprian 1998] |
|  | M. flocculare | Yes | Yes | Personal communication [Maboni] |
|  | A. pleuropneumoniae | NF | Yes | [VanTTil1991,Zimmerman2012] |
|  | A. suis | NF | NF |  |
|  | B. bronchiseptica | NF | Yes | [Ciprian 1998] |
|  | H. parasuis | NF | NF | [Ciprian1998] |
|  | P. multocida | NF | Yes | [Amass 1994, Ciprian 1998] |
|  | S. suis | NF | Yes | [Zimmerman2012] |
| Virus | PRRSV | Yes | Yes | [Ciprian1998,Thacker 1999] |
|  | PCV | Yes | Yes | [Ciprian 1998] |
|  | SIV | NF | Yes | [Thacker2001] |

### 4.2 Results

### 4.2.1 Interaction with the host

## Surface antigens in swine mycoplasmas

In order to understand the different adherence capabilities of each species, we compared all genomes in search for known surface antigen homologs. The results are available in Table 4.2. It is interesting to remember that all surface proteins are potential adherence factors; and since they are in close proximity to host cells they can also be immunogenic antigens responsible for acquired immunity against diseases [300]. Furthermore, membrane lipoproteins are one of the most dominant antigens in Mollicutes, and many of them are known to be variable amongst different strains, undergoing size and antigenic variations [222].

While Vlp from $M$. hyorhinis are exclusive to this species, there are nine adhesins specific to $M$. hyopneumoniae and M. flocculare, and an extra copy of both P97 and P102 (and an additional frame shift) exclusive to M. hyopneumoniae. Besides adhesins and variable surface proteins, the existence of other antigenic proteins in the surface may be of great interest. Whether they act in the adhesion was not yet verified, but P65 for instance, which is present in all species, is a major antigenic surface lipoprotein with lipase activity [245]. This protein is one of the few in M. hyopneumoniae genome to present variably repeated nucleotide sequences in intergenic upstream flanking region. This may result in switching the gene on/off in subpopulations of $M$. hyopneumoniae to escape the immune system.

We cannot further discuss whether these surface proteins may play a direct role in pathogenicity due to lack of experimental data. Moreover, what we could assess up until now is that many surface proteins with distinct metabolic functions (peptidases, hydrolases, lipases) appear to moonlight as adhesins, which might indicate a direct link between metabolism and virulence, but for now we have no data supporting this hypothesis. And even though size and phase variations seem to appear randomly in subpopulations, the presence of similar sets of proteins in both pathogenic and attenuated $M$. hyopneumoniae strains and commensal $M$. flocculare made us question if any kind of gene regulation may influence directly which proteins are expressed in each strain. This subject will be further addressed in Chapter 5.

Table 4.2: Surface proteins in M. hyornhinis, M. hyopneumoniae and M. flocculare strains.


## Respiratory tract metabolism and uptake of metabolites

Metabolites readily available for bacteria in the respiratory tract of swines are listed in Table 4.3, based on surfactant composition and metabolism, and carbohydrate composition of mucus.

Table 4.3: Metabolites readily available in the respiratory tract of mammalians.


Apart from readily available metabolites, enzymes present in the ASL fluid can also convert some of these metabolites into essential precursors for survival in these bacteria (proteome data available from [10], as mentioned in the Methods section). From a total of 4018 proteins detected, we correalated 491 with a corresponding EC numbers. Overall, the respiratory tract of swines is enriched in metabolism of cofactors, vitamins, lipids, carbohydrates and glycans (Figure 4.3), and some of them will be discussed in detail below.

## Lipid metabolism in ASL

The main entrances of M. hyorhinis, M. hyopneumoniae and M. flocculare models are: glycerol, choline, glycerol-phosphate, glycerophosphodiesters and fatty acids (Figure 4.4). Cholesterol and sphingomyelin are incorporated directly into biomass. From the metabolites available in the respiratory tract (Table 4.3), only cholesterol and sphyngomyelin are readily available. However, phosphatidylglycerol (PG) and phosphatidylcholine (PC) can be degraded to produce glycerophosphoglycerol (GPG, EC 3.1.1-) and glycerophosphocholine (GPC, 3.1.1.5) and free fatty acids. GPC and GPG can also be degraded into sn-glycerol-3-phosphate (Glycerol3P) and either glycerol (EC 3.1.4.46) or choline (EC 3.1.4.2). Fatty acids are thought to be released from extracellular host lipids by lipases [305], several of which are encoded in the genomes from the three species. In this way, even though a gene for wide range lipase 3.1.1- was not detected in ASL, it is possible that these mycoplasmal lipases are able to perform missing activities related to lipid degradation (for instance, EC 3.1.4.-). It is also possible that sn-acyl-glycerol-3-phosphate (Acyl-G3P) or sn-1,2-diacylglycerol-3-phosphate (12DAGG3P) are imported through unknown transport systems to the mycoplasma cells.


Figure 4.3: Predicted metabolism from the porcine respiratory tract, from the KEGG database resource [119, 120]. We used available information from [10]. In red, we highlighted detected enzymes from ASL and BAL proteomes. Metabolism of cofactors, vitamins, lipids, carbohydrates and glycans seem to be enriched.


Figure 4.4: Lipid metabolism in ASL.
Another precursor for lipid metabolism in the three species of mycoplasmas is UDP-galactose (UDP-Gal). And even though the enzymes responsible for the production of UDP-glucose (UDP-Glc, EC 2.7.79) and its conversion to UDP-Gal (EC 5.1.3.2) were not found in any species, we added it to the models, based on experimental evidence for the presence of MGDG and DGDG in M. hyopneumoniae and M. flocculare (based on [38] as discussed in the previous chapter ). It seems that UDP-Glc or UDP-Gal may be present in the ASL after all, since three enzymes for the UDP-galactosyl metabolism were detected in the ASL proteome (Figure 4.5, EC 5.1.3.2, EC 1.1.1.22, EC 2.4.1.17).


Figure 4.5: UDP-Gal metabolism in ASL.

## Carbohydrate metabolism in ASL

Carbohydrate acquisition is essential for both colonization and pathogenesis in most respiratory tract pathogens [28]. Since free carbohydrates are usually scarce in these areas, generally bacteria acquire carbon through modification of complex glucans. M. hyorhinis is the species that grows faster from the three mycoplasmas, and this is probably a result of the completeness of its carbohydrate metabolism. Glycoproteins and proteoglycans are responsible for the major fraction of carbohydrates in ASL (Figure 4.6). Apart from the enzymes present in mycoplasma species for carbohydrate degradation, a large quantity of sugar hydrolases, mucinases, amylases, glycogen debranching enzymes, among others were present in the pool of enzymes of ASL; Thus, we considered that most monomers from these macromolecules may be present in this environment. In this way, NAcGal, fucose, galactose, glucosamine, galactosamine and sialic acid from mucins were considered readily available for mycoplasmas. The most common monomers from proteoglycans detected in mammalians (based on composition from Table 4.3) are: glucose (Glc), galactose (Gal), GalNAc, N-acetyl-glucosamine (NAcGlc), glucuronic acid (GlcA) and xylose (Xyl). Even though phosphatidylinositol is a phospholipid, it is possible that M. hyopneumoniae uses the product of its degradation (myo-inositol) to carbohydrate metabolism (Figure 4.7).


Figure 4.6: Metabolism of glycans in ASL. Mucins and proteoglycans are degraded into protein fraction (not shown) and mono or oligosaccharides before becoming available for Mycoplasma species.

## Cofactor and Vitamin Metabolism in ASL

There is a lack of experimental information on cofactor and vitamin metabolism in ASL, also because some of these metabolites are usually in under detectable concentrations. The only available information is on solute concentration of the murine respiratory tract [70], and it mentions the presence of sodium, calcium, choride and urea. Nevertheless, the presence of a high number of enzymes for this specific metabolism led us to believe that some of these metabolites may be present in ASL. In this way, we used proteome data from [10] to propose which cofactors may be readily available for mycoplasmas. No enzyme for the conversion of pyridoxal (PYX) into pyridoxal-5-phosphate (PYP) were found in M. hyorhinis, M. hyopneumoniae or M. flocculare. However, three enzymes for the production of PYP from pyridoxal (EC. 2.7.1.35), pyridoxine-5-phosphate (EC 1.4.3.5) and pyridoxamine-5-P (EC 1.4.3.5) were found in ASL (Figure 4.8). From the models, we proposed that thiamine pyrophosphate (ThiPP) was fed to mycoplasmas, and indeed, there is such an enzyme that produces ThiPP from Thiamine (EC 2.7.6.2).


Figure 4.7: Phosphatidylinositol degradation to myo-inositol in ASL metabolism. The product myo-inositol can be used by M. hyopneumoniae models as a carbon source.


Figure 4.8: Production of pyridoxal-5-phosphate and thiamine-pyrophosphate in ASL.

The enzymes for production of biomass precursors spermidine (EC 2.7.1.22) and putrescine (EC 2.7.1.16) were also detected (Figure 4.9). Although there is no direct link to the presence of enzyme that converts riboflavin to FMN in ASL (EC 2.7.1.26), we did infer its presence from reactions that use its subsequent conversion products FMN (EC 2.7.7.2) and FAD (EC 4.6.1.15).


Figure 4.9: Spermidine/putrescine, riboflavin, pantothenate/coenzyme-A and nicotinate metabolisms in ASL.

We found no direct link to pantothenate (RPAN) presence in ASL, only an to its intermediate product 4-phosphopantetheine (4PPAN). This metabolite is used for production of dephospho-coenzyme-A (DPCoA, EC 2.7.7.3) and cofactor coenzyme-A (CoA). ACP itself was also detected in the proteomes of both BAL and ASL. We have previously proposed that 4PPAN, instead of RPAN was the coenzyme A true precursor in the models. Niacinamide (NACD) instead of nicotinate (NIA) may be the true precursor for nicotinate metabolism, since only enzyme EC 2.4.2.12 was detected (instead of EC 6.3.4.21).

Missing EC 1.5.1.3 from M. hyopneumoniae and $M$. flocculare models was detected in ASL pro-
teome (Figure 4.10). These organisms may uptake tetrahydrofolate (THF) directly instead of biomass precursor folate (FOL).

## Toxic Products

Mycoplasmas also produce metabolites that are transported to ASL. It is interesting to note the presence of several enzymes that are capable of detoxification of the system from reactive oxygen species such as superoxide (O2-, EC 1.15.1.1) and hydrogen peroxide (H2O2, EC 2.7.1.16) (Figure 4.11). It is clear that the lung will have these mechanisms; but here we also proposed that they are important for the protection of the host against these pathogens.


Figure 4.10: Metabolism of folate in ASL.


Figure 4.11: Production of reactive oxygen species (ROS) by mycoplasmas and detoxification mechanisms in ASL.

### 4.2.2 Inter-species associations and enhanced pathogenicity

Healthy hosts harboring Mycoplasma species have only been reported whenever no other bacterial species were present. This may indicate that the presence of other bacterial types itself may be one of the switches to a pathogenic state. However, since M. hyopneumoniae can cause disease in the absence of other bacterial species, this is not the only factor involved in the modification from a non-pathogenic to a pathogenic state.

Indeed, if we take a look into it, the sole presence of other bacterial types may cause two specific differences in the system: (i) with more competition, comes less carbon sources; and (ii) the presence of other bacteria may increase the presence of lipopolysaccharides (LPS) in the environment. These macromolecules are the most potent inflammatory component of gram-negative bacteria [211], and given that mycoplasmas do not produce LPS, they are not direct targets of recruited effectors. 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 [167]. This means that, in vitro, the competition for a carbon source between fast and slow-growing bacteria usually ends with the overpopulation of the fast growing ones. Given the fact that mycoplasmas are found in vivo in great quantities even in competition states with other bacteria, we must assume that other factors exist and are usually not mimicked in cell culture.

From the models reconstructed, M. hyopneumoniae (and M. hyorhinis, with less efficiency) may use glycerol as a secondary carbon source in the absence of readily carbohydrates in the medium. One possible switch to a pathogenic state may be related to the overproduction of hydrogen peroxide resulting from less available carbohydrates in the system (Figure 4.12). This overproduction of hydrogen peroxide is also beneficial for the colonization of opportunistic bacteria in the respiratory tract, once it damages the cilia of the host. M. hyopneumoniae may also use the abundant myo-inositol present in the medium to generate energy, which may be a positive feature and possibly the reason why this is the most successful of the three species in diseased states.

## Quorum sensing

Even though M. hyorhinis, M. hyopneumoniae and M. flocculare do not have any of the known components of QS systems, this is not true for the other bacteria in this environment (Table 4.4).

Table 4.4: AI-2 synthesis and detection genes present (green) in the genomes of pathogens from the respiratory tract of swines.

| Al-2 synthesis and detection genes in E. coli, P. aeruginosa and $X$. campestris |  |  | Organism |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Gene | EC number | Accession Number | A. suis | B. bronchiseptica | H. parasuis | P. multocida | S. suis |
| sahH | 3.3.1.1 | pae:PA0432 |  | $\checkmark$ |  |  |  |
| luxS | 4.4.1.21 | eco:b2687 | $\checkmark$ |  | $\checkmark$ | $\checkmark$ | $\checkmark$ |
| pfs | 3.2.2.1613.2.2.9 | eco:b0159 | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ |
| lux0 |  | eco:b4004 | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ |
| luxU |  | xcc:XCC0483 | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ |
| luxQ | 2.7.13.3 | eco:b3210 | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ |
| luxP |  | eco:b3751 | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ |
| metK | 2.5.1.6 | eco:b2942 | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ |
| metE | 2.1.1.14 | eco:b3829 | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ | $\checkmark$ |
| metH | 2.1.1.13 | eco:b4019 |  | $\checkmark$ |  |  | $\checkmark$ |
| Abbreviations: IuxS, Al-2 synthetase/SRH cleavage enzyme; pfs, SAH-nucleosidase enzyme; sahH, SAH hydrolase; metH and metH, methionine synthetase; metK SAM synthetase; luxP, Al-2 binding protein; luxQ, membrane bound hybrid sensor kinase; IUxU, histidine phosphorelay protein; luxO response regulator. |  |  |  |  |  |  |  |

Besides intra-species communication, QS can also be important to sensing metabolites from competitors in the same environment. In this way, even if M. hyorhinis, M. hyopneumoniae and M. flocculare may not sense these molecules, it does not mean that the other bacteria present in the pulmonary tract do not sense molecules produced by mycoplasmas. For instance, we did not find an enzyme with the activity EC 4.4.1.21, which converts S-rybosyl-L-homocysteine (SRH) into Lhomosysteine (HCys) and autoinducer-2 (AI-2). But the enzyme for this activity was detected in ASL, and thus, it is possible that SRH produced by the three mycoplasmas is converted into HCys and AI-2 by the ASL metabolism (Figure 4.13). It is also important to say that it is possible that many other unknown enzymes (hypothetical proteins or moonlight enzymes) in mycoplasmas may act in a different and never described communication system. For now, we have no further information on the subject.


Figure 4.12: Difference in M. hyopneumoniae and M. hyorhinis models from high and low concentrations of carbohydrates sources. (A) High concentration of carbohydrates implicate that glycerol is used mainly for lipid metabolism and no hydrogen peroxide is produced. (B) Whenever concentration of carbohydrates is low, both species may convert glycerol to DHAP and produce toxic hydrogen peroxide.

### 4.3 Discussion

In this chapter, we were able to discuss the possible dialogues that can happen between host and pathogens and between pathogens themselves. We could indeed solve the main problems of metabolite availability (or gaps) of the reconstructed models from the previous chapters with some of the components (metabolites and enzymes) present in the swine lung airways. We also discussed some other important virulence factor from the pathogenic species, such as biofilm formation, surface antigens and polysaccharide capsules. Supporting the idea that the interaction between mycoplasmas and the other species occurs mainly during disease development, is the fact, reported in the literature,
that while the three Mycoplasma species are widespread in pig populations and can easily be found in healthy hosts [73, 200], other pathogenic species are predominantly prevalent in diseased animals [194]. In this way, we only proposed the presence of the other species in a simpler interaction model of diseased states. But the main question permeating this chapter is: what causes the switch from a nonpathogenic Mycoplasma community to a pathogenic one? And what makes some strains pathogenic while others inflict no harm in the host cells?


Figure 4.13: Autoinducer-2 metabolism between mycoplasmas and other species. Even though we did not find an enzyme with the activity S-ribosylhomocysteinase in the mycoplasmas models (as described in Chapter 2), this enzyme responsible for this activity was detected in ASL proteome. It is possible that AI-2 produced by ASL metabolism from SRH from mycoplasmas is sensed by other bacteria.

Mycoplasmas can survive for longer periods inside the host, which means that they may have more efficient mechanisms for evading host immune system than other species. Indeed, the production of highly toxic LPS, for instance, induces strong response in non-compromised immune systems. Given that mycoplasmas lack LPS, the recruited effectors from the innate immune system do not target them. Also, antibodies against lipid-A portion of LPS will not be effective against these mycoplasmas. This evasion is enhanced with the presence of different strains (in the case of M. hyopneumoniae) and the production of different types of variable surface antigens. In this way, subpopulations from each species are not targeted by the same effectors, at the same time. And this gives them more time to generate variablility and undergo selective pressure to further escape the host immune system. Several studies show that immunosuppressed animals experimentally infected with mycoplasma species develop less severe microscopic lesions of pneumonia if compared to normal animals [56, 271, 269]. Indeed, many pathogens are known to cause autoimmunity. This means its possible that whenever M. hyopneumoniae alone causes mild pneumonia (or M. hyorhinis causes polyserotisis), some kind of stress to the host immune system may be the primary cause of pathogenicity (Figure 4.14).

From current data, it is not possible to conclude whether the pathogenic states of M. hyopneumoniae and M. hyorhinis are indeed induced by the excess of immune response from the host. However, induction of autoimmunity has been described as one of the mechanisms of M. pneumoniae, the causative agent of pneumonia in humans [126, 122]. Since associations with other species have been reported only for M. hyopneumoniae, following hypotheses will discuss only this species.


Figure 4.14: Hypothesis for a possible switch from a healthy to a pathogenic state of $M$. hyopneumoniae without any external bacterial or viral agents.

Other bacterial species may enter the system, and this may come from direct contact with infected animals or even any disturbance in the immune system that allows the infection of normal bacteria from the oral cavity or tonsils. Our hypothesis, as already mentioned, is that the competition with fast-growing species results in a lower carbohydrate concentration (Figure 4.15). Mycoplasmas may have to overcome this environmental starvation with the uptake of glycerol (for M. hyopneumoniae and, possibly M. hyorhinis) or uptake of myo-inositol (for M. hyopneumoniae). Another speculation is that maybe the lack of information on the association of M. hyorhinis with other pathogenic bacteria may come from the fact that in the absence of carbohydrates (competition state), M. hyopneumoniae may overgrow M. hyorhinis populations. The uptake of glycerol as a carbon source might also 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 may be responsible for the recruitment of immune system effectors. Since Mycoplasmas may have more efficient mechanisms for evading host immune system, the newly introduced and fast-growing bacteria will be eliminated at a higher rate. This will increase the amount of carbohydrates available for M. hyopneumoniae and, consequently, will reduce the production of hydrogen peroxide and weaken the immune response. Selective pressure for highly resistant subpopulations will also decrease and less resistant variants will arise. At the end, either the balance will be restored or the immune system will have completely eliminated all bacteria from its respiratory tract, including mycoplasmas. It is important to note that even though this is a plausible hypothesis, no experimental data from the three species studied here support this model. More tests should be applied for validation.

Thus, while M. hyopneumoniae might cause no harm, depending mostly in the environment, the characteristics of the host and the composition of this dynamic lung microbiome, any unbalance in this system is 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 factors that include intra-species mechanisms, community composition, host susceptibility and environmental factors.


Figure 4.15: Hypothesis of an unbalance of a non-pathogenic community with the entry of a new fast growing bacteria in the system. At an early stage, the competition with fastgrowing bacteria generates a low carbohydrate concentration for M. hyopneumoniae, which starts to use glycerol as a carbon source. This results in production of highly toxic hydrogen peroxide. The presence of new bacteria also triggers a strong immune response that targets at first new toxic and fastly reproducing bacteria. Mycoplasmas have several ways of evading the host system, and highly resistant subpopulations continue to reproduce. At the end, balance is probably restored once carbohydrate levels return to normal, hydrogen peroxide production decreases and selective pressure over resistant strains is diminished.

## Chapter 5

## Ongoing work: Search for Promoters

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This chapter discusses some preliminary results we obtained during this PhD thesis in search for promoter sequences in mycoplasmas. We will first describe the methods used here, that combine a software developed by our team, the SMILE algorithm. This is an unbiased tool for the search of motifs among an input of sequences of interest. This software was also combined with a tool used for an easier visualization of the SMILE results, developed also during the course of the PhD thesis.

### 5.1 Overview

Control of transcription initiation is one of the major mechanisms to regulate gene expression in bacteria. The presence of different sigma subunits (from RNA polymerase), specific to different environmental conditions, allows bacteria to rapidly adapt to new conditions. Each sigma factor recognizes a particular promoter sequence, and thus turns on specific response genes accordingly. For instance, E. coli has at least six sigma factors [18] and Bacillus subtilis has at least 18 [140]. Mycoplasmas, on the other hand, seem to have only one type of sigma subunit (the primary sigma-70) in most genomes sequenced so far and thus their major control of gene expression is thought to be quantitative regulation rather than on/off switch for gene expression [176]. On the other hand, several findings point to the fact that differential gene expression, such as stringent response to amino acid starvation or heat shock response, occurs in some species [105, 91], even though the mechanisms are not yet elucidated [176]. In this way, with the use of a software developed by our team [162], we tried to gather more information about the patterns present in the upstream regions of transcription units in Mycoplasma species.

### 5.1.1 Genome organization in mycoplasmas

Several species of mycoplasmas have been described to transcribe polycistronic mRNAs; however, there is little experimental confirmation of the in silico transcriptional unit (TU) predictions for most species. To my knowledge, M. hyopneumoniae, M. hyorhinis, M. flocculare and M. pneumoniae have been, so far, the few species with genome-wide studies on this matter [96, 256, 302, 255, 254]. M. pneumoniae genes, for instance, seem to be arranged in long clusters with almost no intergenic regions (IRs) [96]. Siqueira and collaborators [255] have experimentally validated all predicted TUs from $M$. hyopneumoniae (strain 7448), M. hyorhinis (strain ATCC 17981) and M. flocculare (strain ATCC 27716), and have proposed that TUs are continuously transcribed in these species. Although it has been suggested that the IR sizes inside the TUs are usually similar in prokaryotes and have been used to predict TUs [172, 228, 207], this rule does not seem to apply to M. hyopneumoniae, M. hyorhinis and $M$. flocculare; instead, a high variability in TU size, IR size and number of genes present in each TU were reported [255, 254].

### 5.1.2 Promoters in mycoplasmas

Despite the number of sequenced genomes, very little information is available on promoter sequences and transcription mechanisms in mycoplasmas. M. hyopneumoniae, M. hyorhinis and M. flocculare, along with other mycoplasmas, were thought until recently to have only one sigma factor (sigma70) and few regulatory proteins [289]. However, recent studies show indications that other sigma factors may exist, more specifically in M. genitalium [280] and M. hyopneumoniae [51]. Despite the apparently reduced gene regulatory toolbox, and corroborating to the existence of other sigma factors, is the fact that environmental stresses induce complex and specific transcriptional responses in M. hyopneumoniae [154, 240, 188]. Sigma-70 is responsible for correctly placing the RNA polymerase in specific promoter elements situated usually 10 and 35 base-pairs (bp) upstream of the transcription start sites (TSS) (Figure 5.1). And it is often described that the capacity of RNA polymerase to bind the -10 and -35 boxes is optimal when they are separated by exactly 17 bases [176]. More distal promoter regions were shown to exist in M. hyopneumoniae up to 350 base pairs upstream the TSS [256].

Although a strong consensus -10 region was detected in M. pneumoniae, only a weak consensus in the - 35 region has been reported [303, 96]. In M. hyopneumoniae, a pattern similar to a sigma-70 - 10 element was found in association with $85 \%$ of the TUs, but no - 35 promoter element has been identified [302, 255]. The distance between a promoter and a start codon may vary from 13 to 348 bases in M. hyopneumoniae; however, most promoters were reported to be located at positions -1 to -25 upstream the start codon [254]. In the same study, the authors showed that $70 \%$ of all TUs have internal promoter-like sequences in IRs; this suggests alternative mechanisms for differential transcription for this specific species. It remains unknown if different promoter sequences indeed exist in M. hyopneumoniae or which proteins are related to the reported differential gene expression


Figure 5.1: Diagram showing the positioning of RNA polymerase on the TSS in bacteria. The sigma-70 factor recognizes conserved promoter regions (in yellow) usually at -10 and -35 upstream the TSS and correctly places the RNA polymerase to initiate transcription; other distal promoters may also exist up to 350 base pairs upstream the TSS.
in this species. Moreover, it is undefined whether these unknown mechanisms are specific to $M$. hyopneumoniae or if they are shared by other Mycoplasma species.

Complementarily, the variable size of IRs (described above) along with the presence of promoterlike sequences in the IRs inside TUs shows the complexity of gene regulation in M. hyopneumoniae. Furthermore, although there must exist differential expression between pathogenic and non-pathogenic strains of M. hyopneumoniae, the high synteny of genomes among strains indicates that gene organization is not directly related to pathogenicity [254].

### 5.1.3 Promoter prediction

Promoter prediction is a key computational challenge, necessary for characterizing the transcriptional units of bacterial cells, traditionally known as operons [149]. Mycoplasmas have a complexity of these units greater than what was expected before the genome-sequencing era, and may have multiple transcription start sites (TSS) and multiple associated promoters to a single gene [176].

Several algorithms employ different methods to search for promoters, but most of them are trained only for a small number of genomes. Moreover, most of these algorithms rely on the sequence motifs recognized by already described sequences recognized by characterized sigma factors; the current state-of-the-art in promoter prediction is biased toward housekeeping genes recognized by the general sigma-70 [1].

We will not enter in the detail of the different approaches for promoter prediction, it suffices to say that they rely on a small number of proteins that come from a small number of model genomes and most of this data is not inferrable for many organisms, especially for high AT genome content organisms such as mycoplasmas. Low GC content results in a genome-wide spread of promoter-like AT rich elements.

### 5.2 Methods

### 5.2.1 SMILE Algorithm

The algorithm SMILE [162] implements an exact algorithm to find motifs in a set of sequences. A motif is seen as a pattern throughout many sequences. This pattern may occur as a single word or it may be divided in separate or structured portions. SMILE simultaneously infers consensus motifs and locates the corresponding elements in each sequence. The software is available at http: //team.inria.fr/bamboo/en/softwares/smile. SMILE is an unbiased method, which depends only in the input sequences and a set of parameters that will vary the number of outputs depending on their stringency.

To run SMILE, one has to simply load a set of sequences of interest (input.fasta), along with several parameters in search for common features among the sequences (Figure 5.2). An alphabet file accounts for the type of molecule examined (alphabet_dna), a parameter quorum represents the percentage of sequences in the input file that must contain at least once the motif (in our case, we fixed that $50 \%$ of the sequences must have the same motif in order for the software to consider it for statistical validation). Total substitutions represents the number of positions (but not the position itself) that may vary in a motif and boxes are the numbers of parts a motif can have (single, $\mathrm{n}=1$; or structured, $\mathrm{n}>1$ ). Total min length and max length represent the total variable size of the motif (accounting for all boxes). If motifs with more than one box are sought, additional information must be provided, such as individual minimal and maximum sizes, number of substitutions in each box and an interval for the length of the distance between two boxes. Once the motifs are extracted according to these given parameters, a statistical validation checks whether they are significant or not. For this, a $\chi^{2}$ test is computed and compares the occurrence of each motif in the input sequences and in a shuffled version (hence the parameters k-mer and number of shufflings) of the input sequences (also representing the null hypothesis).

| 6 |
| ---: |
| 7 |
| 8 |
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| 12 |
| 13 |
| 14 |
| 15 |
| 16 |
| 17 |
| 18 |
| 19 |

FASTA file
FASTA file
Output file
Output file
Alphabet file
Alphabet file
Quorum
Quorum
Total min length
Total min length
Total max length
Total max length
Total substitutions
Total substitutions
Boxes
Boxes
Shufflings
Shufflings
100
Size k-mer
Size k-mer

Figure 5.2: SMILE parameter file (see text for explanation on arguments).

Depending on how permissive these parameters are, this input can be very large and difficult to interpret. Thus, we created also a visualization tool to help us distinguish between visual patterns of statistically significant motifs, mapped in the input sequences.

### 5.2.2 Processing visualization

Processing is an open source software suitable for visualizing all types of data (available at https: //processing.org/). The software is written in java and the user is able to write scripts to easily create complex images. In this work, we have developed several scripts to map the occurrence of the motifs in the original input sequences and check whether we could find visual patterns that stood out.

### 5.2.3 Selection of species and data collection

We tested several Mycoplasma species apart from M. hyopneumoniae, M. hyorhinis and M. flocculare (Table 5.1); to avoid bias towards a specific organism, we only selected one strain from each species. We took into consideration the 350 bp upstream a TU and did not take into consideration at this point internal promoters.

Table 5.1: Selected species from mycoplasmas for promoter analysis.

| Species | Abbreviation |
| :---: | :---: |
| M. pneumoniae M129 | MPN129 |
| M. genitalium G37 | MG37 |
| M. conjunctiviae | MCJ |
| M. agalactiae PG2 | MAPG2 |
| M. hyopneumoniae 7448 | MHP7448 |
| M. hyorhinis HUB1 | MHRHUB1 |
| M. capricolum ATCC 27343 | MCA |
| M. synoviae 53 | MS53 |
| M. gallisepticum R_low | MGA |

### 5.3 Preliminary Results and Discussion

As this subject was a project not at first related to this PhD thesis, we present here only initial and partial results and our perspectives for this ongoing work.

### 5.3.1 Pribnow box detection

Our first goal was to validate the SMILE algorithm in search for a - 10 motif, close to a Pribnow box. Thus, we set a SMILE search for one box from 5 to 10 nucleotides in the 350 nucleotides upstream the translation predicted site (first ATG), with variable parameters (Table 5.2).

The score is calculated based on the occurrences of the motif in the input file sequences versus the shuffled sequences (If evaluation $=$ Shuffling) or versus the input sequences that should not bear this motif (in this case, evaluation $=\mathrm{CDS}$ Sequences).

The score values between shuffling and CDS sequences cannot be compared, since they were calculated by different methods.

In the first test, we allowed no substitutions in the motif, which means that the motifs 1 to 5 are found exactly as the sequence indicated in the original input sequences. In test number 2 , one substitution is allowed in any position of the sequence, but the motif sequence indicated is a consensus showing which nucleotide is the most common in each position throughout the input sequences. The third test used a different type of validation; instead of using shuffled sequences, we gave to the software an input file containing sequences where we did not expect to find the motifs detected. In our case, we used all CDS from all the genomes analysed. Although the nucleotide occurrence for intergenic regions and from CDS regions is not the same, we wanted to find possible already detected motifs from tests 1 and 2 , to enhance confiability. Indeed, we were able to find in all tests similar sequences, and when we clustered the sequences, we were able to detect that most of the motifs (marked in yellow in Table 5.3) were related to a single extended pribnow box (Table 5.3). The consensus for the pribnow box is TATAAT in most species, and is clearly present in motifs $2,4,5,6,8,10,11,12,13$ and 14.

Motifs $6,8,11,12,13$ and 14 show an upstream region of the Pribnow box, with a high occurrence of a guanine immediately or two bases before the TATAAT. Furthermore, in motifs $1,3,5,7,10,11$

Table 5.2: SMILE tests for the search of promoters in mycoplasmas.

| Input Parameters |  |  |  |  |  |  | Results |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Test \# | Quorum | Min Length | Max Length | Substitutions | Evaluation | Motif | Motif Sequence | Input Original Sequences (\%) | $\begin{array}{\|c\|} \hline \text { Evaluation } \\ \text { Wrong } \\ \text { Sequences (\%) } \end{array}$ | Score* |
| 1 | 20 | 6 | 10 | 0 | Shuffling | 1 | AATTATA | 34.31 | 22.63 | 12.72 |
|  |  |  |  |  |  | 2 | TATAATT | 37.03 | 24.66 | 11.80 |
|  |  |  |  |  |  | 3 | ATTATA | 55.99 | 42.07 | 11.58 |
|  |  |  |  |  |  | 4 | TATAAT | 57.97 | 44.71 | 10.38 |
|  |  |  |  |  |  | 5 | TATAATTATA | 33.50 | 22.40 | 15.37 |
| 2 | 20 | 6 | 10 | 1 | Shuffling | 6 | GTATAATTA | 30.86 | 18.06 | 13.19 |
|  |  |  |  |  |  | 7 | ATAATTATA | 55.03 | 18.63 | 12.95 |
|  |  |  |  |  |  | 8 | TGATATAAT | 31.30 | 39.96 | 12.68 |
|  |  |  |  |  |  | 9 | AAAGGAG | 48.93 | 19.47 | 11.93 |
|  |  |  |  |  |  | 10 | TATAATTAT | 54.37 | 39.94 | 11.67 |
|  |  |  |  |  |  | 11 | GATATAATT | 32.18 | 21.75 | 10.88 |
|  |  |  |  |  |  | 12 | gGtatalt | 31.30 | 20.21 | 10.75 |
|  |  |  |  |  |  | 13 | GATATAAT | 52.24 | 40.16 | 10.29 |
|  |  |  |  |  |  | 14 | GTATAATT | 57.16 | 46.29 | 10.11 |
| 3 | 20 | 6 | 10 | 0 | CDS Sequences | 15 | TTTTTTT | 50.00 | 26.12 | 301.96 |
|  |  |  |  |  |  | 16 | TTTTTT | 80.96 | 57.97 | 250.28 |
|  |  |  |  |  |  | 17 | ATTTTTTT | 34.41 | 16.05 | 242.44 |
|  |  |  |  |  |  | 18 | TTITTTTA | 31.76 | 16.43 | 170.18 |
|  |  |  |  |  |  | 2 | TATAATT | 37.06 | 21.63 | 144.63 |
|  |  |  |  |  |  | 19 | TATTTTTT | 31.32 | 17.49 | 134.13 |
|  |  |  |  |  |  | 20 | TTTTTTA | 60.88 | 43.78 | 131.07 |
|  |  |  |  |  |  | 21 | ATTTTTT | 58.53 | 41.73 | 127.42 |
|  |  |  |  |  |  | 4 | tatant | 58.01 | 41.78 | 119.05 |
|  |  |  |  |  |  | 22 | TTTTTTAT | 0.30 | 18.13 | 104.69 |
|  |  |  |  |  |  | 3 | ATTATA | 0.56 | 41.14 | 100.55 |

and 14, a TATA extension of the Pribnow box was detected. In order to map these occurrences in the original input sequences, we used the software Processing, and indeed, an enrichment of occurrences in the region from - 15 to -60 upstream the ATG is seen (Figure 5.3).

### 5.3.2 Search for other promoter elements

Based on the previous searches, we tried to detect other conserved promoter elements in these genomes. We first tried to search two boxes of at least 6 nucleotides separated by a distance ranging between 8 to 40 nucleotides. The results are plotted in Figure 5.4. Although we detected AT-rich repetitive motifs, we could not find any particular consensus; however, the distances between any two motifs seemed to be conserved; this distance is more frequent from 12 to 15 base pairs. This is somewhat in agreement with the distance between a -10 and a -35 element, if we consider that the Pribnow box is extended in these species and would rather start at -12 or -13 , instead. Also, the presence of other conserved distances (from 21 to 29 and from 33 to 39 ), can be an indication that other important but distal elements may influence the regulation of these transcriptional units.

### 5.3.3 Detection of a Ribosome Binding Site

For these analyses, we only used the genomes of the swine mycoplasmas M. hyopneumoniae, M. hyorhinis and M. flocculare. When we studied more in particular the promoter regions of high transcription mRNAs (and not TUs), we were able to detect an extremely conserved ribosome binding

Table 5.3: Clustering of motifs validated by both methods.

| Motif | Sequence |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 1 |  |  |  |  |  | ATT | ATA |
| 2 |  |  | TAT | T | A | ATT |  |
| 3 |  |  |  |  |  | ATT | AtA |
| 4 |  |  | TAT | T | - | A $T$ |  |
| 5 |  |  | T $\mathrm{A}^{\text {T }}$ | T | - | ATT | A $\mathbf{T A}^{\text {a }}$ |
| 6 |  | G | TAT |  | A | ATT | A |
| 7 |  |  | A 1 |  | A | ATT | A $\mathbf{T A}^{\text {a }}$ |
| 8 | T G |  | TAT |  | A | A T |  |
| 10 |  |  | T ${ }^{\text {A }}$ | T | A | ATT | A T |
| 11 | G | G A | TAT |  | A | AT T |  |
| 12 | G | G G | TAT | T | A | A T |  |
| 13 | G | G A | TAT |  | A | A $T$ |  |
| 14 |  | G | TAT | T | A | AT $\mathbf{T}$ |  |
| Consensus? | $\mathbf{T} \mathbf{G}$ |  | $\mathbf{T A T}$ |  |  | ATT | A T A |



Figure 5.3: Mapping of each nucleotide position occurrence from the motifs detected in Test 1. We found an enriched region from -10 to -50 bases upstream the predicted translation site.
site (RBS, sequence AAAGGA), ranging from 2 to 12 bases of distance from the predicted translation initiation site (Figures 5.5 and 5.6 ). This RBS was usually preceded by an AT rich region, similar to the Pribnow box (TATAAT) in many cases.

### 5.4 Conclusion

Although this is the beginning of a study on promoters in mycoplasmas, we were already able to validate that the method used is indeed fit to address this question, even as concerns genomes with low


Figure 5.4: The variable distance between two boxes seems to occur in a certain pattern. Distances between 12 and 15 seem to be more conserved than larger distances. All ranges overlap with each other, for instance, range $8: 10$ contains motifs separated by distances of 8,9 and 10 base pairs.

| STATISTIC Model | ON THE \%right | NUMBER OF \#right | SEQUENC \%shfl. | $\begin{aligned} & \text { ES HAI } \\ & \text { \#sh } \end{aligned}$ | $\begin{aligned} & \text { G AT L } \\ & \text { Sigma } \end{aligned}$ | ST ONE Chi2 | $\begin{aligned} & \text { CCURRENCE } \\ & \text { Z-score } \end{aligned}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| AAAGGA | 67.74\% | 21 | 25.52\% | 7.91 | 2.50 | 11.11 | 5.24 |
| TATAAT | 58.06\% | 18 | 28.81\% | 8.93 | 2.34 | 5.40 | 3.88 |
| TAAAAAT | 58.06\% | 18 | 37.48\% | 11.62 | 2.49 | 2.63 | 2.57 |
| ATTTTT | 87.10\% | 27 | 74.55\% | 23.11 | 1.59 | 1.57 | 2.45 |
| ATAAAAA | 58.06\% | 18 | 41.16\% | 12.76 | 2.19 | 1.77 | 2.39 |

Figure 5.5: SMILE output file with the occurrence of motifs in the upstream regions of high transcription mRNAs in M. hyopneumoniae, M. hyorhinis and M. flocculare.

GC content. By using an unbiased method (SMILE algorithm) we attained similar results, regarding the detection of a - 10 element, as many other publications in mycoplasmas [96, 256, 302, 255, 254]. We were also able to find an extended Pribnow box, upstream with the presence of a guanine immediately before, and downstream with an extension of the AT-rich box. According to other studies, mainly in Gram-positive bacteria like B. subtilis, the lack of a fl35 motif can be compensated by the presence of an extended fll0 box (TG-N-Pribnow) [63]. The presence of this TG-N-Pribnow was already experimentally shown for M. hyopneumoniae [302].

However, having a well conserved Pribnow box is not enough to drive productive transcription [149], and many other elements must also help the transcription initiation. Indeed, we were able to find hints that point to other elements in these promoter regions. For now, we could only define that the distances between two motifs can be conserved. The most conserved distance in our study seem to be in accordance with a 17 distance between the -10 and -35 elements; however the existence of more distal elements may also be an indication that gene regulation in mycoplasmas is much more complex than it was anticipated. The variable size of intergenic regions along with the presence of promoter-like sequences inside TUs enhances even more the complexity of gene regulation in these mycoplasmas.

```
ATARAAGAAAGGATATTTGTAHATAATTTTAHATTARATAMATTAHAHAATTTACAAGGAAGAGATTY
AсTTTTTGCTATCATATGAATTTAATCGATATTAAAAAACAAAATTAGACACATTAAAGGAGTATAAA
CACTGICTAGGAAAGTAAGACAAAAAATTTTTAAAAAT TTAAAAAAATTACGAAAGGAMATCGARAAM
GGCTAAATITGTGITATAATTCTTATCGTTAAGAAATAAAAAATTTTTTGAA_AAAGGATAAATAAAGG
TITGTCTAGGAAAATAAGACAAAATAATACTCAAAATICAAAAARATTMGAGAAAGGATACCAAAAAA
MATTGTTTAATTATAAACAAATTMAAGATAATTTATCACAATTTATGGTAGTAATATAAAGGAGAAAAR
```



```
GAAATTTTTTCTTATATTITCICGAGTITTATAAAAA CCTCGAACAAAATATAAAAAAGGAGATAT
LACTAGGGTTTCTCTTCTCTTGCCTGTTTTTTAAAATTATTTTTTTAAATGTAGAAAACACAAATTATA
FTACAATAGGGGGTGAGATTAACCCCCCTTGTAATTAAATCTTAGAATTTTAAGAAATCATAAATTATA
                    GAATACTTTAATTAARTTTATTTACAAACAAAGGAGMCFAA%
                            AATTTATATTTAACTAMARAAAAAABAARAGARGGTACAA:
TTTGGITGGGACGATAATAAAAATTTGCCCAAATTTAAAAAACTAACAAAAAAGGAGAAATTTAATTTY
```



```
\TTTAATAACCCACACAATAAAACTTAAAAAAAACATATCATATTTATATTTTTGG AAAGGAGATAAAA
        TTPAACTAATTTTT TTAGTAGAATATAATCTACTMTTACTTTA AAAGGAGAAAMA 
```



```
GGITAAAAAAACCCTGGITTTTATACGTTAATCGATCTTTTTAGACTAAATTTTCGAAAGGA ARATAA
TATCGAATGAAGCTGCTATTAAAGAGTGTTTGTAATTTGTCCACCATGGTGGTCGGAGCATTGTCACC
TTTTTTCITTTTTGITITATAATAAAAAT TATATTATTAT TAAAAATTTTTGGAAAGGTTAGAAGTAAA)
ATGTAATGGINTTGTGTTAAMCCIGCCATMATTAAAAGGCATAAGCCTAARAGCAAAATAGGAGATMM
CATAATCAAAATGATTTATAATTTTTTITAACATCTTTGCAAATAGATGTTAATAAGAAGAAAGGACAAA
```



```
    ATTTTTTAAGTATAATTATTTTATATAAGGCCAATTTTTAGGCATATATTTATTAAAAAGGAGATTA
TAACAAAAA/ATAAAAAATTTTTCAAATTAAATTCAAAATTAACGAAAGGATAAATMTTATITAAAAAA
LAAAAAAAAAAAMATGCTITATTTTTGGTATTAGTGTATAATTGTGGGTGATAGTGGAGATAATAGGAARA
A &AATATTTTTTTGGITT1TTTTCTTTGTTAGNGTATAATTGNGGTTGATAGTGGAGACAAGCGGAAM
AGTTGICGGICTTGGCGGAGCAACTGGTTCTGGIGCAGCATTAACTGTTGCAAAAATCGCCAAAGAT
                                    AAATTGGAACCAAAAAAAGGTAGAAATACTR
TAAATTTATATTATAATTTATACAAAGAGACAAAAAGGAAAAATTITAGAAAAACCCGGAGGTTTTIR
ITATTCCGTTCAACATGCATGTATTAAGCACACAGCTAGCGTTTATCCTGAGCCAGGATCAAACTCTCR
```

Figure 5.6: Processing visualization of the SMILE results on the occurrences of RBSs in the upstream regions of high transcription mRNAs in M. hyopneumoniae, M. hyorhinis and M. flocculare.

Moreover, the high synteny of the gene organization between pathogenic and non-pathogenic strains of $M$. hyopneumoniae indicates that gene organization is not directly related to pathogenicity [254], which makes it even more interesting the study of differential expression in these organisms.

More tests will be made in order to detect the actual sequence of conserved motifs in the species studied here, and our objective in the future is try to analyze transcriptional units based on their biological functions. In this way, we might be able to find motifs related to specific biological functions. Since they appear in low numbers, most of them are not detected as being significant by looking at all the transcriptional units at once.

## Conclusion and Perspectives

In this PhD thesis, we aimed at studying the metabolism of M. hyorhinis, M. hyopneumoniae and $M$. flocculare to better understand what could influence their different life-styles and pathogenicity (or lack of thereof).

We presented in this work an overview of the differential metabolisms of M. hyopneumoniae, M. hyorhinis and M. flocculare using different approaches. The reconstructed models showed some distinctions among the species, namely the myo-inositol metabolism for $M$. hyopneumoniae, the uptake of carbohydrates for M. hyorhinis, and the usage of glycerol as a carbon source for the two pathogenic species.

We were able to partially explain that the incapacity of $M$. flocculare to cause disease is probably related to its inability of producing the toxic hydrogen peroxide. Conversely, the capacity of both M. hyopneumoniae and M. hyorhinis to damage the cilia might be related, but not limited to, this enzymatic activity. Both M. hyorhinis and M. hyopneumoniae seem to have enhanced growth rates and wider carbohydrate uptake ranges over $M$. flocculare. This can also be supported by the ability of the two pathogenic species to survive in diverse sites. For M. hyorhinis, this ability is even more pronounced, which is probably why this species is found as a common cell line contaminant.

However, it was only after we performed growth tests using a defined medium that hypothetically could lead to bacterial growth in silico, that we were able to assess that some cofactors or metabolites were probably missing from this medium and that some cofactors were not correctly assigned in the network. Identification of the correct cofactors for biomass production may help us formulate new defined media for the cultivation of swine mycoplasmas in the future. This in turn may be of great help for any further biochemical studies done on these species. The results obtained from growth tests in both complex and defined media also provided us hints that although the metabolic models are similar, and thus code for similar enzymes, the pool of active enzymes in each species seem to quite different.

Metabolic profiling of both complex and defined media pointed to new differences that we were not able to identify based solely on the sequenced genomes. Indeed, what we found in the NMR analysis also pointed to the existence of a machinery for differential gene expression in these species. The production of acetate represents one of the major differences among the networks. M. hyorhinis seems to lack the turnover of pyruvate to acetate, thus making of pyruvate the final product of glycolysis in the experiments performed in this work. M. hyopneumoniae and M. flocculare, on the other hand, exhibit high acetate concentrations at the end of the growth curve. Moreover, the myoinositol catabolism also seems to be linked to the capacity of $M$. hyopneumoniae to produce more acetate than $M$. flocculare. We intend to continue our search for the actual myo-inositol function in the metabolism of this species and to find out why this is the only Mycoplasma species identified so far that harbors in its genome the genes to completely degrade myo-inositol. We furthermore intend to check if the myo-inositol is really uptaken and what are the possible products from this pathway.

Moreover and as mentioned before, M. hyopneumoniae and M. flocculare are closely related genetically, and adhere to cilia in a similar way. This is probably due to the similar repertoire of adhesins in both species. Adhesion itself is not per se a virulence factor, but it surely helps these organisms to evade the immune system. Along with biofilm formation and polysaccharide capsules, these are the most studied evasion factors in mycoplasmas.

We always have to keep in mind that the metabolism of these organisms cannot be completely understood when isolated from their environment. The environmental context may explain the differ-
ences between the in silico models and the in vivo behavior. We were able to fill some of the gaps from the metabolic networks with the actual metabolites and enzymes present in the airway surface liquid from swines. This in turn gave us more indication that some of the defined Yus medium cofactors were indeed the incorrect ones for these specific swine mycoplasmas.

Metabolic exchanges are not the only possible dialogue between host and pathogens. Another ongoing project in our team is to try to understand the exchange of small RNAs from the pathogen to the host and (why not?) of microRNAs from the host to the pathogen. Of course this is a very initial work, but, based on many reports on the importance of these small RNAs, why not think that indeed they might target something else than the cell which transcribes them?

Based on literature data, we proposed the interaction between mycoplasmas and other species mainly in diseased states. Our metagenomic project so far has not revealed a great prevalence of bacteria other than the three mycoplasmas of this study (in both healthy and diseased animals). This also made us question ourselves: what causes the switch from a non-pathogenic Mycoplasma community to a pathogenic one? We know that the disease is a result of multiple factors that can be related to the ability of the bacteria to cause disease (virulence factors, evasion mechanisms, cytotoxicity), to the susceptibility of the host (individual factors such as age, weight, nutrition, immune system, genetic factors), or to the environment (overpopulation, temperature, ventilation). But, other than this, how can an unbalance in this microbiome cause disease? We proposed here that carbohydrate availability is a key factor for this switch. This is based on our hypothesis that competition with fast-growing species results in a higher virulence of M. hyopneumoniae due to less carbohydrate availability and hence, in a higher production of hydrogen peroxide.

The study of differential gene expression is one of the ongoing works of this PhD thesis. Up to this point, we were already able to retrieve some interesting information on promoter sequences from several Mycoplasma species. The detection of a Pribnow box was already expected and is in agreement with many other studies on mycoplasmas. However, the study of more specialized promoter sequences, specific to a particular protein function, was never performed in these species. Our aim here was to check if these specific promoters exist and, if that was the case, which proteins regulate their transcription.

In this way, this PhD thesis will serve as a basis for the study of the differential metabolisms and pathologies caused by the mycoplasmas in the swine respiratory tract and may help to propose ways to prevent disease development in the future. Overall, the main differences among the species we reported here might indeed be related to virulence and pathogenicity at different levels.

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Appendix A
Appendix: Metabolic Network Reconstructions








Table S2b: Metabolites present in reconstructed models of M. hyopneumoniae, M. hyorhinis and M. flocculare.




| cnd |  | соннззоз | $\bigcirc$ |  |
| :---: | :---: | :---: | :---: | :---: |
| gambp |  | С6н $\mathbf{H z N o s p}^{\text {P }}$ | . 1 | Cobl 4 Nosp |
| s7p | Seathepuluse 7 P.phosphato | C7H13010P | - 2 | C7H15010P |
| daap |  | C10H122NSOPP | 3 | CTOH15N509P2 |
| damp |  | C10H1255S68 | , | C10HH4N5S6P |
| datp |  | C10H12N5012P3 | 4 | C107H1655012P3 |
| dacap |  | Cathinsoiop | 3 | C9H15N3000P2 |
| domp |  | С9Н12N307P | - | C9H14N3O7P |
| dap |  | C9H12N3013P3 | 4 |  |
| drad | Deamno-NAD+ | C2IH2ancoisp | - 2 | C21H27N6015P2 |
| dogntep $^{\text {a }}$ |  | C6Heosp | 3 |  |
| dascoce |  | C6tBo7 | 0 |  |
| ${ }^{20419}$ | 2.Doexy-0.ritose 1-phosphate | CSHeorp | - 2 |  |
| abagn |  | C6HOO6 | ${ }^{-1}$ | cetriog |
| ${ }^{2}$ atisp | 2.Doexy-P.ribose 5.phosphate | CSHeorp | - 2 |  |
| dad.2 | Deoryadenosine | $\mathrm{ClOH}_{13 \mathrm{SN} 53}$ | $\bigcirc$ |  |
| don | Dooxcenadine | соннззоо4 | 0 |  |
| dasn | Dooxyguansse | C10HI3S504 | $\bigcirc$ |  |
| din | Dooxminosine | C10712N004 | - |  |
| duri | Doexyundine | conirveos | - |  |
| dpooa | Dephoseso.CoA |  | - 2 |  |
| ${ }^{\text {di2dg }}$ |  | C4636+186720150 | 0 |  |
| dadp |  | Cliotilinsoiop | 3 |  |
| dgmp |  | Ciohlinsoorp | - |  |
| dgp |  | C10H12256013P3 | 4 | с10НH6N5013P3 |
| ${ }^{12969}$ | 12.Onamalyereal | C5H605R2 | 0 |  |
| dit |  | C19HISNob | - 2 | C19H21 1NTO |
| drap |  | Сзн5обр | - 2 | сзнrob |
| dna |  | Сз4603 | 0 |  |
| dna |  | с3н603 | 0 |  |
| ana |  | с3н603 | - |  |
| a2 |  | С558004 | 0 |  |
| ${ }^{\text {al }}$ |  | Сك5604 | $\bigcirc$ |  |
| a2 |  | CSthou | $\bigcirc$ |  |
| ${ }^{13809}$ | 3.Phospho-D.agrecory p phosphato | C34400002 | 4 |  |
| duap |  | Csthineorip ${ }^{\text {a }}$ | 3 | C9H14N2OITP2 |
| dump |  | Coth11208P | - | С9+13N2O8P |
| dup |  | C9H11N201493 | 4 | C9H15N2O1493 |
| asp |  | C44707P | - | CAHBOTP |
| eot |  | с2нво | - |  |
| eloh |  | с2нво | 0 |  |
| etoh |  | с2460 | 0 |  |
| tad | FFaxn adenne dinucteotico oxdized | Cz7431300152 | - |  |
| ${ }^{102}$ |  | Fo | 2 |  |
| ${ }^{102}$ |  | Fo | 2 |  |
| ${ }^{102}$ |  | Fo | 2 |  |
| $1{ }^{103}$ |  | Fo | 3 |  |
| ${ }_{\text {tm }}$ |  | Cithiomaosp | - | C17H2INGO9P |
| for |  | Cighiento | ${ }^{-1}$ | Cighignob |
| tol |  | ciehieño | ${ }^{-1}$ | cishignoo |
| tol |  |  | ${ }^{-1}$ | C19H199Nob |
| tald |  | Cr2o | $\bigcirc$ |  |
| tald |  | с +2 O | - |  |
| tald |  | crro | - |  |
| for |  | CHIO2 | - -1 |  |
| tor |  | CH102 | ${ }^{-1}$ |  |
| for |  | CH102 | ${ }^{-1}$ |  |
| tu | D.Frucose | C6H1206 | - |  |
| HP | D-Frucose 1.phosshale | C6FH1109P | - | Cehti3o9P |
| ${ }_{\text {top }}$ |  | Ceth10012P2 | 4 | Ceth1012P2 |
| ${ }_{180}$ | D.F.ruciose 6.phosphato | C6thliosp | - 2 | Centi39P |
| tu | D.fucase | C6FH206 | - |  |
| tu | D.fucuse | C6H1206 | - |  |
| km |  | Catroa | - 2 |  |
| tum |  | Catr204 | - |  |
| tum |  | C41204 | - 2 |  |
| 3 mg | 3.Phospho.-.agyearate | СЗН407p | 3 | C3F707P |
| gal | DGGatasose | C6th206 | - |  |
| gal | D.Gatacose | C6H1206 | 0 |  |
| gal | D.Gatacose | C6H1206 | 0 |  |
| ${ }^{33}$ |  | C3H5608 | - | C3Frosp |
| sodp |  | C10H1255011P2 | 3 |  |
| gcod | D.Cbucse | C6H1208 | 0 |  |
| 910 | D.Gucose T.phosphato | Cobliog | - 2 | Centi3osp |
| ${ }^{66}$ | D.Gluosese ephosshate | Cothlosp | - | Coht3osp |
| 9cob | D.Gucose | C6tr1206 | 0 |  |
| gco. | D.GGuose | C6H1206 | 0 |  |
| gm .2 |  | C5HHON2O3 | $\bigcirc$ | C5H10N2O3 |
| gme. |  | C5H10N2O3 | $\bigcirc$ | C5H10N2O3 |
| gme. |  | C5HHON2O3 | 0 | C5H10N2O3 |
| Gin-GIn |  | C10hismos | 0 |  |
| Gin-an |  |  | - |  |
| tragn |  |  |  |  |
| gut |  | CStheno4 | $\rightarrow$ | CSHeso4 |
| gut |  | CSteno 4 | $\stackrel{1}{4}$ | CSHeso4 |
| guw- |  | CStreno4 | - | CSHswo4 |
| 6il.al |  |  | $\bigcirc$ |  |
| 6ing |  |  | $\bigcirc$ |  |
| tragu |  |  |  |  |
| gam |  | cehtanos | 1 | сенізоо |
| gam |  | C6H14Nos | 1 | C6H13305 |
| av |  | C2rssoz | $\bigcirc$ | C2hbsoz |
| $9 y$ |  | C2H5NO2 | 0 | C2H5NO2 |
| av |  | C2HESO2 | $\bigcirc$ | C2H5NO2 |
| Glyay |  | Cahencoi | $\bigcirc$ |  |
| aryay |  | Cahanzos | $\bigcirc$ |  |
| tmagy |  |  |  |  |
| gyad |  | Сзноз | - |  |
| gyad |  | с3н603 | 0 |  |
| gyad |  | сзноо | $\bigcirc$ |  |
| 9 go |  | Catratiob | 1 | Cabrow ${ }^{\text {a }}$ |
| 9 ge |  | Cshrinoor | 1 | с8нгомов |
| ${ }_{\text {ax }}$ |  | Cartivob | 1 |  |
| ave |  | Сзнвоз | 0 |  |
| avesp |  | C3F7rosp | - | СЗНеов |
| ${ }_{\text {ancesp }}^{\substack{\text { avesp }}}$ |  |  | 2 |  |

 $\qquad$ Extrachluar






C6HON3O2
C6HON3O2
CEHON3O2

 $\square$
74051
74051 8
$6027-13 \cdot 0$
$\quad 7722-84 \cdot 1$
$7722-8 \cdot 1$
$772-841$

| C00155 | 6027-13.0 |
| :---: | :---: |
| c00027 | 7722-84, |
| c00027 | 7722-84, |








$\qquad$
$\qquad$
$\qquad$

|  |  |
| :---: | :---: |
|  |  | 8







| $\begin{array}{r}\text { C6H1206 } \\ \hline \text { C6H1206 }\end{array}$ |  |
| :---: | :---: |
|  |  |
|  | C14427 |
|  | C14427 |
|  | C14427 |
| C3H14NO9P |  |
|  | C8H15NO6 |
|  | C8H15NO6 |
|  | C8H15NO6 |
|  | C8H14NO9P |
|  | C1H18809 |
|  | C1H18NO9 |




$>\square \square>\square \square>\square \square \square \square>\square \square \square \square \square$

| SPERMIDINE.b | spermidine | spmd |  | C7H22N3 | 3 | C7\% $\mathrm{CHN}^{\text {N }}$ | coosis | $12420 \cdot 9$ |  | Extracolluar | $\checkmark$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| spermidine. | spermidine | spmd |  | С7Н22N3 | 3 | C7H19N3 | coos15 | $12420 \cdot 9$ |  | Extracelluar | $\square$ |
| spm. 6 | Sphingomyelin | spm |  | C33H79N206P |  |  | coos50 |  |  | Extraclluar | $\checkmark$ |
| spm. | Sphingomyelin | spm |  | C39H79N206P |  |  | coos50 |  |  | Extraclluar | $\square$ |
| Starch b | starch | starch |  | C12H20010 | 0 |  | coo369 |  |  | Extracalluar | $\checkmark$ |
| STARCH. | starch | starch |  | C12H20010 | 0 |  | coo369 |  |  | Extracelluar | $\square$ |
| Steanic aclo | Stearic Acid | steac |  | C17735 | 0 |  | co1530 |  |  | Cyusol | $\square$ |
| Stearic acid b | Stearic Acid | staac |  | С17735 | $\bigcirc$ |  | C01530 |  |  | Extracolluar | $\checkmark$ |
| Stearlc acid. | Stearic Acid | steac |  | C17735 | 0 |  | co1530 |  |  | Extraclulur | $\square$ |
| suc | sucatinate | succ |  | C4H404 | - 2 |  | c00042 | ${ }^{110-15.6}$ |  | Crosol | $\square$ |
| suc. b | sucinate | suce |  | $C^{4} 4404$ | $\cdots$ |  | cooo42 | ${ }^{110-15-6}$ |  | Extracolluar | $\checkmark$ |
| suc COA | succiny-CoA | succoa |  | C25H33N7019P3S | . 5 | C25H40N70199P3S | cooos | 604-98.8 |  | Cytosol | $\square$ |
| suc. | succinate | succ |  | C44404 | - 2 |  | c00042 | ${ }^{110-15-6}$ |  | Extraclluar | ] |
| sucrose | sucrose | sucr |  | C12H22011 | - |  | cooos9 | 57.50.1 |  | Cytosol | $\square$ |
| SUCRose b | sucrose | sucr |  | C12H2O11 | 0 |  | cooos9 | 57-50.1 |  | Extracelluar | $\checkmark$ |
| SUCFose. ${ }^{\text {e }}$ | sucrose | sucr |  | C12H2OO11 | 0 |  | cooos9 | 57.50.-1 |  | Extracolluar | $\square$ |
| SULFate | sultate | so4 |  | 045 | - | H204s | cooos9 | 766493.9 |  | Cyrosol | ] |
| sulfate b | sulate | so4 |  | 045 | - | H204s | cooos9 | 766493.9 |  | Extracolluar | $\checkmark$ |
| Sulfate. | sulate | so4 |  | 045 | - 2 | H204s | cooos9 | 766493.9 |  | Extracalluar | $\square$ |
| Sulturated Sultur Accepplors | a suturated [sulur camier] | scaariers |  |  |  |  |  |  |  | Cyrosol | $\square$ |
| SUPER OXIDE | superoxide | ${ }^{02 s}$ | Superoxide anion | 02 | $\cdot 1$ |  | c00704 |  |  | Cytosol | $\square$ |
| SUPER OXIDE - b | superixide | ${ }^{025}$ | Superoxide anion | 02 | ${ }^{-1}$ |  | cooro4 |  |  | Extracelluar | $\checkmark$ |
| SUPER OXIDE.0 | superoxide | ${ }^{225}$ | Superoxide anion | 02 | - 1 |  | c00704 |  |  | Extraclulur | $\square$ |
| TDP | dTDP | ditop |  | $\mathrm{ClOH}^{13 N 20111 P 2}$ | $\cdots$ | $\mathrm{ClOH}^{16 \mathrm{~N} 2 \mathrm{O}} 1 \mathrm{P}$ 2 | coo3s3 |  |  | Cyosol | $\square$ |
| THCHDO | 3D-(3,5/4) <br> trihydroxycyclohexane-1,2-dione | thenso |  | с6нво5 | 0 |  | C04287 |  |  | cytosol | $\square$ |
| THF | terahydrotolate | ${ }^{\text {tit }}$ | 5,6,7.8.Terahydrotolate | C19H21N7O6 | -2 | C19H23N706 | c0010 | ${ }^{135-16.0}$ |  | Cyrosol | $\square$ |
| Thamine Prgophosphate | thiamin diphosphate | thmpp |  | C12H16N407P2S | - | $\mathrm{C}^{12 H 19 N 407 P 2 S ~}$ | cooosb | 15487.0 |  | Cytosol | $\square$ |
| THAMME PY\%OPHOSPHATE . ${ }^{\text {b }}$ | thiamin diphosshate | thmpe |  | C12H16N407P2S | - | C12H19N407P2S | cooose | 15487.0 |  | Extractluar | $\checkmark$ |
| THAMME PYYOOPHOSPhate. | thiamin diphosphate | thmpp |  | C12H16N407P2S | - 2 | C12H19N407P2S | cooose | 15487.0 |  | Extracallar | $\square$ |
| тия | L-trroonine | thr-L |  | C4HONO3 | 0 | C4H9NO3 | c00188 | 72-19.5 |  | Cyrosol | $\square$ |
| THR. ${ }^{\text {b }}$ | L-trroonine | thr-L |  | C4HONO3 | 0 | CAHONO3 | c00188 | 72-19.5 |  | Extraciluar | $\checkmark$ |
| THR. | L-trreonine | throL |  | Cahtonos | 0 | C4HONO3 | coors | 72:19.5 |  | Extractluar | $\square$ |
| The. Thr | dipepiliee $\mathrm{Th} \cdot \mathrm{Thr}$ | Thr-Thr |  | C8H16n205 | 0 |  |  |  | 73665 | Cyrosol | $\square$ |
| The.Thr. ${ }^{\text {c }}$ | dipepilie Thr-Thr | ThreThr |  | C8H16N2O5 | 0 |  |  |  | 73665 | Extractluar | $\square$ |
| thr rinas | trathr | tmathr |  |  |  |  | C01651 |  |  | Cyrosol | $\square$ |
| thymidine | thymidine | thymd |  | C10H14N2O5 | 0 |  | c00214 | 50-89.5 |  | cytosol | $\square$ |
| THYMINE | thymine | thym |  | C5H6N2O2 | 0 |  | C00178 | $65.71-4$ |  | cyiosol | $\square$ |
| THYMNE b | thymine | thym |  | C5H6N2O2 | 0 |  | c00178 | $65.71-4$ |  | Extracolluar | $\checkmark$ |
| THYMINE. | thymine | thym |  | C5H6N2O2 | 0 |  | c00178 | ${ }^{65-71.4}$ |  | Extracelluar | $\square$ |
| TMP | वTMP | dtmp |  | C10H13N2O8P | - 2 | C10H15N2OBP | cooss | 365-07.1 |  | Cyosol | $\square$ |
| trehalose | trealose | tre |  | C12H22011 | 0 |  | C01083 | 99-20.7 |  | Cytosol | $\square$ |
| trehalose gr | alpha,aphat trehalose 6 -phosphate | tre6p |  | C12H21014P | - 2 |  | coobe9 | 4484-88-2 |  | Cyrosol | $\square$ |
| trehalose b | appha, alpha.trenalose | tre | Trenalose | C12H2OO11 | 0 |  | C01083 | 99-20-7 |  | Extracalluar | $\checkmark$ |
| trehalose. | appha, alpha.trehalose | tre | Trenalose | C12H22011 | - |  | C01083 | 99-20.7 |  | Extracelluar | $\square$ |
| TRP | L-tryelophan | tp-L |  | C11H12N2O2 | 0 | C11H12N2O2 | cooors | 73-22-3 |  | Cytosol | $\square$ |
| TRP. b | L-trypopophan | up-L |  | C11H12N2O2 | 0 | C11H12N2O2 | c00078 | 73-22-3 |  | Extracaluar | $\checkmark$ |
| TRP.0 | L-tryplophan | tp-L |  | C11H12N2O2 | 0 | C11H2N2O2 | c00078 | ${ }^{73} 322 \cdot 3$ |  | Extraciluar | $\square$ |
| TRP.tanas | tratte | tratrp |  |  |  |  | C01652 |  |  | Cyrosol | $\square$ |
| Trp Tip | dipepilide Tp.TTp | Trp.Tip |  | C22H22N4O3 | $\bigcirc$ |  |  |  | 74876 | Cyrosol | $\square$ |
| TTp. TTP. ${ }^{\text {a }}$ | dipepide $T$ Tp.Tp | Trp-Tp |  | C22H22N403 | 0 |  |  |  | 74876 | Extracelluar | $\square$ |
| TTP | ditp | ditp |  | C10H13N2O14P3 | 4 | $\mathrm{COHPH}^{17 \mathrm{~N} 21493}$ | C00459 |  |  | Cyrosol | $\square$ |
| TYR | L-tyrosine | ty-L |  | C9H11NO3 | 0 | С9H11NO3 | C00082 | ${ }^{60-18.4}$ |  | Cytosol | $\square$ |
| TYR. ${ }^{\text {b }}$ | L-trosine | ty-L |  | C9H11NO3 | $\bigcirc$ | С9Н11NO3 | c00082 | 60-18-4 |  | Extracelluar | $\checkmark$ |
| TVR. | L-tyrosine | tyr-L |  | С9H11NO3 | $\bigcirc$ | C9H11NO3 | c00082 | 60.18 .4 |  | Extracelluar | $\square$ |
| trr lrmas | tratyr | trayr |  |  |  |  | c00787 |  |  | Cyrosol | $\square$ |
| Tyr Tyr | dipepidio Ty-Tyr | Tym-Tyr |  | C18H20N205 | 0 |  |  |  | 60987 | Cylosol | $\square$ |
| Tyy- Tyy 0 | dipepidid Ty-Tyr | Tyr-Tyr |  | C18H2ON2O5 | 0 |  |  |  | 60987 | Extracaluar | $\square$ |
| UDP | UDP | udp |  | C9H11N2012P2 | 3 | C9H14N2OOPP2 | c00015 | 58.98.0 |  | Cytosol | $\square$ |
| UDP gat | UDP-0.galactose | udpal |  | C15H22N2017P2 | - | C15H24N2017P2 | cooos2 |  |  | Cytosol | $\square$ |
| UDP. CLC | UDP.--glucose | uppg |  | C15H22N2017P2 | -2 | C15H24N2017P2 | coooze |  |  | Cytusol | $\square$ |
| UMP | UMP | ump |  | C9HH1 1 N299P | -2 | C9H13N209P | c00105 | 58-97-9 |  | Cyrosol | $\square$ |
| Unsulurated. Sulutur Acceplors | an unsulurated [sulfur carrier) | s-carrier |  |  |  |  |  |  |  | Crosol | $\square$ |
| UFACIL | uracil | ura |  | CAHAN2O2 | $\bigcirc$ |  | c0006 | ${ }^{66-22 \cdot 8}$ |  | Cyosol | $\square$ |
| URACLL_b | uracil | ura |  | C4H4N2O2 | 0 |  | C00106 | 66-22-8 |  | Extracaluar | $\checkmark$ |
| URACLI. ${ }^{\text {a }}$ | uracil | ura |  | C4H4N2O2 | $\bigcirc$ |  | c0006 | ${ }^{66-22-8}$ |  | Extracolluar | $\square$ |
| URIINE | uridine | uri |  | C9H12N2O6 | $\bigcirc$ |  | cooze9 | 56.96-8 |  | Cytosol | $\square$ |
| UTP | UTP | up |  | CSH11N2O15P3 | ${ }^{4}$ | CSH15N2O15P3 | couors | 63-39.8 |  | Cyrosol | $\square$ |
| vat | L.valine | val- |  |  | 0 | $\mathrm{CSHH}^{\text {H NO2 }}$ | c00183 | 72-18.4 |  | Cytosol | $\square$ |
| VaL. ${ }^{\text {b }}$ | L-valine | vall |  | C5H11NO2 | $\bigcirc$ | C5H11NO2 | C00183 | 72-18.4 |  | Extracolluar | $\checkmark$ |
| vaL. | L-valine | val- |  | CSH11NO2 | 0 | C5H11NO2 | c00183 | 72-18-4 |  | Extracalluar | $\square$ |
| val manas | tanaval | tmaval |  |  |  |  | C01653 |  |  | Cyosol | $\square$ |
| Val val | dipepilid Valval | valval |  | C10H2ON2O3 | 0 |  |  |  | 73704 | Cyrosol | $\square$ |
| Val Val.e | dipepidio Valval | valval |  | C10H2ON2O3 | 0 |  |  |  | 73704 | Extracaluar | $\square$ |
| water | H2O | n20 |  | H2O | 0 |  | cooool | ${ }^{733}$-18.5 |  | Crosol | $\square$ |
| Water.b | H2O | n20 |  | H2O | 0 |  | c0000 | 7732-18.5 |  | Extracelluar | $\checkmark$ |
| water. | H2O | n20 |  | H2O | 0 |  | cooool | 7732-18-5 |  | Extracaluar | $\square$ |
| XANTHINE | xanthine | xan |  | CSHAN4O2 | 0 |  | cooss5 | ${ }^{69.89 .6}$ |  | Cyrosol | $\square$ |
| Xanthosine | xanthosine | xss |  | C10H12N406 | $\bigcirc$ |  | c01762 | 146-80.5 |  | Criosol | $\square$ |
| XANTHOSINE 5. P. P | xMP | xmp | Xantrosine 5-pposphate | C10H11N4OSP | - |  | coobs5 | 523-98.8 |  | cyrosol | $\square$ |
| XYLULOSE S. ${ }^{\text {P }}$. | D-xylubse 5.phosphate | xu50.D |  | C5HOOBP | - | ${ }^{\text {C5HH108P }}$ | c00231 |  |  | Cyosol | $\square$ |
| 2N2 | zn2+ | $2 \mathrm{z2}$ | Zne | zn | 2 |  | cooosb | 7440.66.6 |  | Cyosol | $\square$ |
| $\mathrm{ZNS}_{2} \mathrm{~b}$ | $2 \mathrm{n} 2+$ | ${ }^{2 n 2}$ | Zne | 2 n | 2 |  | cooose | $7440.66 \cdot 6$ |  | Extracelluar | $\checkmark$ |
| ZN2. | Zn2+ | 2 2 2 | Zino | Zn | 2 |  | cooos8 | 7440.66.6 |  | Extracelluar | $\square$ |

Appendix B
Appendix: NMR Data

Table S7a: Metabolites detected in growth of M. hyopneumoniae, M. hyorhinis and M. flocculare in complex Friis medium.

| Species | Species Abbreviation | Time | Replicate | Niacinamide | Niacinamide | Formate | Inosine | Inosine |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Friss medium | Friss medium | 0 h (Negative Control) | 1 | 1538466.875 | 1375438.625 | 7321876.25 | 10584600.13 | 10400696.63 |
| Friss medium | Friss medium | 0 h (Negative Control) | 2 | 1825666.5 | 1719124.5 | 7806012.25 | 7928716.5 | 8587448.5 |
| Friss medium | Friss medium | 0 h (Negative Control) |  | 1497956 | 1747770.375 | 7401734.375 | 10301270.25 | 10406477.88 |
| M. flocculare | MFL27716 | 10 H | 1 | 1830285.75 | 1345424.75 | 7926530.25 | 8809888.25 | 8972710 |
| M. flocculare | MFL27716 | 10H | 2 | 1753688.625 | 1392266.25 | 7785085.125 | 8224043.125 | 8666983.25 |
| M. flocculare | MFL27716 | 10 H | 3 | 1935485.5 | 1548079.5 | 8223789.75 | 7010514 | 7477658.5 |
| M. flocculare | MFL27716 | 24 H | 1 | 2007431 | 1676576.5 | 8846697.5 | 5831498.75 | 6038570 |
| M. flocculare | MFL27716 | 24 H | 2 | 1962292.875 | 1330830.375 | 8665696.625 | 6170909.875 | 6594410 |
| M. flocculare | MFL27716 | 24 H | 3 | 1990687.75 | 1548406.375 | 8352892.375 | 5538413.5 | 6138190.25 |
| M. flocculare | MFL27716 | 32 H | 1 | 1763714.25 | 1430784.25 | 9009263.25 | 4057185.25 | 4364456.5 |
| M. flocculare | MFL27716 | 32 H | 2 | 1881216.75 | 1616500.25 | 9128054 | 3148748.5 | 3565537.75 |
| M. flocculare | MFL27716 | 32 H | 3 | 1758449.75 | 1564236.75 | 9222190.75 | 3890201.75 | 4309946.75 |
| M. flocculare | MFL27716 | 48H | 1 | 1780442 | 1380573 | 10023346 | 752023 | 1312310.5 |
| M. flocculare | MFL27716 | 48H | 2 | 2357063.5 | 1597716.5 | 13252359.5 | 686712.5 | 1223772 |
| M. flocculare | MFL27716 | 48 H | 3 | 2435343.5 | 1809785.75 | 13273498.75 | 574327.25 | 1128308.25 |
| M. flocculare | MFL27716 | 8 H | 1 | 2333522.75 | 1666526.75 | 11053286.5 | 10615251.75 | 10669206.75 |
| M. flocculare | MFL27716 | 8 H | 2 | 2510244.5 | 2164042.25 | 11998426 | 9764898.5 | 9981630 |
| M. flocculare | MFL27716 | 8 H | 3 | 2369345.5 | 2076127.75 | 11913141.25 | 11260735.5 | 11421679 |
| M. hyopneumoniae | MHP7448 | 10 H | 1 | 1857219 | 1560128.5 | 8953725.25 | 8025799 | 8287509.25 |
| M. hyopneumoniae | MHP7448 | 10 H | 2 | 1735687.75 | 1359464.5 | 8490025 | 7367461.75 | 7629389.25 |
| M. hyopneumoniae | MHP7448 | 10 H | 3 | 1791955 | 1445965.75 | 8837925.5 | 7796927.75 | 8037138.75 |
| M. hyopneumoniae | MHP7448 | 24 H | 1 | 1857566 | 1368582.75 | 8769729.25 | 2105221.75 | 2282135.5 |
| M. hyopneumoniae | MHP7448 | 24 H | 2 | 1789697.25 | 1483766.5 | 9338168.25 | 2953316.25 | 3514565 |
| M. hyopneumoniae | MHP7448 | 24 H | 3 | 1521421 | 1377352.25 | 8823924.25 | 2865437.5 | 3237807.5 |
| M. hyopneumoniae | MHP7448 | 32 H | 1 | 2013931 | 1681954.25 | 9836627.25 | 357666.25 | 1069907 |
| M. hyopneumoniae | MHP7448 | 32 H | 2 | 1493757.25 | 1397592.5 | 9915332.75 | 877859 | 1098036.5 |
| M. hyopneumoniae | MHP7448 | 32 H | 3 | 1728597 | 1535098 | 10152946 | 501130 | 837934 |
| M. hyopneumoniae | MHP7448 | 48H | 1 | 1739001 | 1405562 | 10353763 | 476210 | 791961 |
| M. hyopneumoniae | MHP7448 | 48H | 2 | 1771500 | 1322480 | 11241657 | 233161.5 | 1101951 |
| M. hyopneumoniae | MHP7448 | 8 H | 1 | 1788273 | 1651316.75 | 8198043.25 | 8014969.875 | 8366031.125 |
| M. hyopneumoniae | MHP7448 | 8 H | 2 | 2238654.25 | 1710574.375 | 8380627.125 | 8126161.625 | 8323838.25 |
| M. hyopneumoniae | MHP7448 | 8 H | 3 | 1701074 | 1742926.5 | 8357442.375 | 7911250.375 | 8107849 |
| M. hyopneumoniae J | MHPJ | 10 H | 1 | 1553714 | 1004889.5 | 10293612.75 | 5813296.5 | 6581973.75 |
| M. hyopneumoniae J | MHPJ | 10 H | 2 | 1852135.5 | 1593469.5 | 12352373 | 7063272 | 7342156.5 |
| M. hyopneumoniae J | MHPJ | 10 H | 3 | 1768972 | 1522763.75 | 10904519 | 6189367 | 6594603 |
| M. hyopneumoniae J | MHPJ | 10 H | 4 | 1984099.75 | 1465027.25 | 10630940.5 | 6479819.25 | 6960769.5 |
| M. hyopneumoniae J | MHPJ | 24 H | 1 | 2193640.416 | 1945332.503 | 13040443.27 | 980732.4163 | 1944779.353 |
| M. hyopneumoniae $J$ | MHPJ | 24 H | 2 | 0 | 0 | 16555714.11 | 0 | 0 |
| M. hyopneumoniae J | MHPJ | 24 H | 4 | 2061624.5 | 1601549.5 | 11909939 | 396904.5 | 1255226.75 |
| M. hyopneumoniae $J$ | MHPJ | 32 H | 1 | 2431487.909 | 1856025.411 | 12206812.18 | 1020939.513 | 1975690.574 |
| M. hyopneumoniae $J$ | MHPJ | 32 H | 2 | 1772063.5 | 1453116.5 | 12390589.5 | 585528 | 1918960.5 |
| M. hyopneumoniae J | MHPJ | 32 H | 3 | 1671150.276 | 1722367.478 | 11750660.67 | 896101.4392 | 2049454.377 |
| M. hyopneumoniae J | MHPJ | 48 H | 1 | 2182700.5 | 1617337.5 | 12873625 | 964659 | 1661844.5 |
| M. hyopneumoniae J | MHPJ | 48 H | 2 | 1829989 | 1699049 | 12304002 | 803122 | 1833847 |
| M. hyopneumoniae J | MHPJ | 48 H | 3 | 1731442.661 | 1520965.537 | 11478198.02 | 1488313.641 | 2496140.639 |
| M. hyopneumoniae J | MHPJ | 48 H | 4 | 1396925 | 1138376.5 | 11199128 | 300209.5 | 1716642 |
| M. hyopneumoniae J | MHPJ | 8 H | 1 | 1996764.75 | 1776575.25 | 10365510.5 | 7451636.5 | 7901080.5 |
| M. hyopneumoniae J | MHPJ | 8 H | 2 | 1135553.25 | 530514.75 | 10861303.25 | 6537073 | 6996819.5 |
| M. hyopneumoniae J | MHPJ | 8 H | 3 | 1885493.5 | 1483914.75 | 10558174.75 | 7109314.5 | 7555943.5 |
| M. hyopneumoniae J | MHPJ | 8 H | 4 | 1348843.7 | 955783.0614 | 10495856.84 | 5435172.624 | 6292985.518 |
| M. hyorhinis | MHR17981 | 10 H | 1 | 1740867 | 1413968 | 12539932 | 8386168.5 | 8432391.25 |
| M. hyorhinis | MHR17981 | 10 H | 2 | 1704753.375 | 1506288.75 | 10446301.25 | 7824756.875 | 8450593.125 |
| M. hyorhinis | MHR17981 | 10 H | 3 | 1322304.5 | 1496625.5 | 10841005 | 7403356.5 | 8011506 |
| M. hyorhinis | MHR17981 | 10 H | 4 | 1839658.75 | 1541235.5 | 10471067.5 | 7721578.75 | 8445149.5 |
| M. hyorhinis | MHR17981 | 24 H | 1 | 1549404.5 | 1147954.25 | 10737991.5 | 2318375.75 | 3370226.25 |
| M. hyorhinis | MHR17981 | 24 H | 2 | 1826606.5 | 1642952.75 | 13039235.75 | 3388647.25 | 4066928 |
| M. hyorhinis | MHR17981 | 24 H | 3 | 2044898.84 | 1889289.643 | 11191482.24 | 2930046.528 | 3630409.435 |
| M. hyorhinis | MHR17981 | 24 H | 4 | 1808216.833 | 1343090.407 | 9957543.848 | 2730693.161 | 3465979.75 |
| M. hyorhinis | MHR17981 | 32 H | 1 | 2102276.5 | 1575500.75 | 12058819.25 | 1460701.75 | 2303866.25 |
| M. hyorhinis | MHR17981 | 32 H | 2 | 1814384.75 | 1549691.75 | 11850792 | 1225264 | 1952573 |
| M. hyorhinis | MHR17981 | 32 H | 3 | 1927942.25 | 1751333.5 | 12450602.25 | 1447131 | 2301488.5 |
| M. hyorhinis | MHR17981 | 32 H | 4 | 1990805.25 | 1532645.25 | 10953002.5 | 1270067.5 | 2447268.25 |
| M. hyorhinis | MHR17981 | 48 H | 1 | 1950296.25 | 1529214 | 11258037.5 | 582326.25 | 1633890.75 |
| M. hyorhinis | MHR17981 | 48 H | 2 | 2010841.75 | 1831973.75 | 11267709 | 718159 | 1957345.25 |
| M. hyorhinis | MHR17981 | 48 H | 3 | 1872160.5 | 1610136.25 | 10396358.5 | 581024.5 | 1473520.5 |
| M. hyorhinis | MHR17981 | 48 H | 4 | 1968635.002 | 1614201.976 | 11199144.8 | 518602.6644 | 1839892.767 |
| M. hyorhinis | MHR17981 | 8 H | 1 | 1921385.125 | 1740568.875 | 12081180.13 | 8931988.875 | 8971582.625 |
| M. hyorhinis | MHR17981 | 8 H | 2 | 1869925.75 | 1690599.25 | 10979417.5 | 8480354 | 8693794 |
| M. hyorhinis | MHR17981 | 8 H | 3 | 1966003.5 | 1604806.25 | 10702679.25 | 6807956.5 | 7623392 |
| M. hyorhinis | MHR17981 | 8 H | 4 | 1982854.25 | 1494598.25 | 10943183.25 | 8483394 | 8979934.5 |


| Species | Hypoxanthine | Hypoxanthine | x 1 | Uridine +Methylhistidine +Cytidine | Cytidine | Tryptophan | Tryptophan | Phenylalanine |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Friss medium | 8067217.75 | 9063830.25 | 11404955 | 86366756 | 47966533.5 | 13609330.75 | 11699300.25 | 188548653.1 |
| Friss medium | 10363255.75 | 11103026.75 | 12512470 | 92774914 | 21874082.75 | 13913704.5 | 12136466 | 200212276.3 |
| Friss medium | 8840368.5 | 9652772.625 | 12725801.38 | 91937935.25 | 47411464.25 | 13910959.5 | 12008378.5 | 199926717.5 |
| M. flocculare | 10788904.5 | 11427943.75 | 11655189 | 92899288.75 | 15719276.75 | 14128757.75 | 15129173.25 | 209076272.3 |
| M. flocculare | 10608225.5 | 11209051.63 | 11458778.63 | 90554073.75 | 15490439.88 | 14057751.63 | 15075693.38 | 205694269.3 |
| M. flocculare | 11984167 | 12543952.25 | 9462637 | 94866713.25 | 13723559.75 | 14345667.5 | 15276041.25 | 212324973.3 |
| M. flocculare | 12965284.75 | 13653487 | 8974077.5 | 89029016 | 13106277.25 | 14640874.75 | 17777425.5 | 210461287.8 |
| M. flocculare | 12995750.5 | 13792894.63 | 10592235.88 | 91840207.88 | 21220197.88 | 14849495.13 | 17424458.88 | 217201957.3 |
| M. flocculare | 13041431.13 | 13487281.25 | 9374727.625 | 90625758.25 | 13252169 | 14642235.88 | 17595320.13 | 212787103.3 |
| M. flocculare | 14067896.25 | 14473006.5 | 8167628.5 | 86929359.75 | 12456501 | 14550693.5 | 19075028.75 | 209773554.8 |
| M. flocculare | 14817207.25 | 15255145.75 | 7624764.75 | 88269114.75 | 12317344.5 | 14887198.25 | 20055941 | 213557194.8 |
| M. flocculare | 14159053.75 | 14538002.5 | 8112266.25 | 87507650.5 | 12876155 | 14647896.25 | 18969326.5 | 211523190.5 |
| M. flocculare | 17286626 | 17571513.5 | 5397300 | 77363852 | 5569777 | 16394225.5 | 33230218.5 | 229688991.5 |
| M. flocculare | 18890542 | 19191438 | 5131397 | 84048875 | 8013183.5 | 16796857.5 | 32896152 | 235793306 |
| M. flocculare | 19112209.25 | 19527317.75 | 4900674.75 | 84481304.75 | 7481953.5 | 17008652.25 | 32996540.75 | 237012963.5 |
| M. flocculare | 11386928.75 | 11491139.25 | 10372239.25 | 95519675 | 14478968.75 | 14710838.25 | 16465353.25 | 220629953.5 |
| M. flocculare | 13130535.5 | 13310965.25 | 10235824.25 | 102333418.5 | 15514178 | 15238976 | 17023589.25 | 236042263 |
| M. flocculare | 11734184.25 | 12079793.75 | 10369859 | 102766592.3 | 14564670.5 | 15155032.75 | 17176406.25 | 234956816 |
| M. hyopneumoniae | 11135912.75 | 11873736 | 11211193 | 87747003.25 | 49751179.75 | 14035228.5 | 15780413.75 | 204797572.3 |
| M. hyopneumoniae | 10382146.25 | 11178527.75 | 10752397.5 | 82664501.5 | 43275982.5 | 13385562 | 15185315.5 | 191407662.5 |
| M. hyopneumoniae | 10826695.5 | 11599202.25 | 12598824.25 | 89067711 | 17777252 | 13947666 | 15716070.25 | 205343973 |
| M. hyopneumoniae | 14295031.75 | 15334449.25 | 8784798 | 80107800.75 | 14032735 | 13488535.5 | 20203600.25 | 198490728.5 |
| M. hyopneumoniae | 14540117.25 | 15245055 | 9139087.75 | 87000217.5 | 13559546 | 14268649 | 20412232 | 211300560.3 |
| M. hyopneumoniae | 14251258.75 | 15011179.25 | 8891984 | 83060286.25 | 12649553 | 13666916.5 | 19789129.5 | 202935240.5 |
| M. hyopneumoniae | 16243064.5 | 17180721.5 | 6363482.75 | 78971794.5 | 9944013.5 | 14344111.75 | 25800843.75 | 210706044.8 |
| M. hyopneumoniae | 15823228.5 | 16307766.75 | 5059293.75 | 76053214 | 9018997 | 13822038 | 25753868.25 | 207511304.3 |
| M. hyopneumoniae | 16408914 | 17132488 | 5530747 | 79627886.5 | 9074747 | 14431344 | 25898644 | 211041438.5 |
| M. hyopneumoniae | 16205484 | 16369552 | 5639316 | 70173660 | 5513077 | 14251343 | 29950538 | 206102233 |
| M. hyopneumoniae | 16531401 | 16765840.5 | 5936340 | 73494391 | 5992304.5 | 14583033 | 30486018.5 | 216088335 |
| M. hyopneumoniae | 10693021.63 | 11519637.63 | 12361879.38 | 88941474.75 | 27446252.5 | 13767942 | 14993508.75 | 201261363.5 |
| M. hyopneumoniae | 11056352.88 | 11511876.13 | 10928047.75 | 84220557.88 | 40789387.88 | 13724699.75 | 15332279.75 | 192432349.1 |
| M. hyopneumoniae | 10758696.25 | 11761605.25 | 12231209.38 | 89511745.13 | 16288547.88 | 13808569.13 | 15183183.75 | 202386776.9 |
| M. hyopneumoniae J | 10325498 | 10695907.75 | 9865990.25 | 81235504.25 | 11680698.25 | 11612538 | 14493142.25 | 185287831.5 |
| M. hyopneumoniae J | 10666327.5 | 11302518 | 10265059 | 80619103 | 12404262.5 | 13082015 | 15247592.5 | 183357586 |
| M. hyopneumoniae J | 10416687 | 10986167 | 10262894.25 | 81653321 | 12166238.25 | 12841324.5 | 14369788 | 178078491.8 |
| M. hyopneumoniae J | 10498277.5 | 11280811.5 | 10389211 | 82200699.5 | 12686562.75 | 13002221.25 | 14557326.5 | 179262114 |
| M. hyopneumoniae J | 15306169.78 | 15491009.02 | 7026851.926 | 76802635.6 | 9599150.937 | 13702281.83 | 23955802.07 | 188037741.1 |
| M. hyopneumoniae J | 30994693.58 | 30539904.32 | 8813345.744 | 123776626.9 | 10711855.39 | 9883232.562 | 36820400.68 | 448211143.8 |
| M. hyopneumoniae J | 14711436.25 | 15807742.5 | 7127136.75 | 75239604 | 9290090.75 | 13347544.5 | 23019202.75 | 185278000 |
| M. hyopneumoniae J | 15557773.92 | 15925797.68 | 6855058.139 | 73399870.45 | 6082577.97 | 14588288.36 | 27603210.55 | 190227653.1 |
| M. hyopneumoniae $J$ | 15255271.5 | 15090723.5 | 7262245.5 | 69077177 | 5491054 | 13686989.5 | 27126180 | 187519969.5 |
| M. hyopneumoniae J | 14964168.93 | 15093157.04 | 6761051.005 | 71724828.29 | 6127904.457 | 13453949.04 | 27094589.45 | 190919361.7 |
| M. hyopneumoniae J | 15831624 | 16189423 | 6754143.5 | 69404349.5 | 4361955 | 14614064.5 | 29048394.5 | 192369347.5 |
| M. hyopneumoniae J | 15084133 | 14763825 | 6219446 | 66494940 | 4268977 | 13883950 | 27676945 | 185154230 |
| M. hyopneumoniae J | 15222285.57 | 14716658.78 | 6204047.087 | 63418205.56 | 4087223.03 | 13668676.95 | 28299420.27 | 190687719.4 |
| M. hyopneumoniae J | 15728917.5 | 15210305.5 | 6108528.5 | 66972199.5 | 3770465.5 | 13845365.5 | 29473841.5 | 200597846.5 |
| M. hyopneumoniae J | 10611787 | 11362145.75 | 11258778.5 | 83668743 | 11931212.5 | 13572916.5 | 15017622.5 | 183323031.3 |
| M. hyopneumoniae J | 9045436.5 | 9453981.25 | 9994509.75 | 73412132.5 | 11251120.5 | 11153357.75 | 13008221.75 | 177031235 |
| M. hyopneumoniae J | 9768958.5 | 10570641.5 | 10831214.5 | 80996343.5 | 12923529.5 | 13037310 | 14135236.75 | 175970760 |
| M. hyopneumoniae J | 9336518.427 | 9905433.483 | 10096031.85 | 76248826.85 | 12339516.19 | 11222706.4 | 13407568.2 | 173605445.1 |
| M. hyorhinis | 9767250 | 10707807.5 | 13110516.25 | 80163676.75 | 15805124.75 | 13312809.5 | 14640114.25 | 183864373.5 |
| M. hyorhinis | 9785961.25 | 10560913 | 11105405.88 | 83617080.5 | 13431008.5 | 13262318.63 | 14657915.88 | 181192655.5 |
| M. hyorhinis | 9552928 | 10135821.5 | 11426921.5 | 80299085.5 | 12324847.5 | 12447576 | 14656846.5 | 179703963 |
| M. hyorhinis | 9473018.25 | 10086424.75 | 11167922.25 | 80836842.75 | 12617278.25 | 12755966 | 13663763.25 | 173770180 |
| M. hyorhinis | 13218897 | 13657814.5 | 7615750.75 | 76434195.25 | 9924186.25 | 12032801.25 | 18735741.75 | 184466701.5 |
| M. hyorhinis | 13626193.25 | 14049862.5 | 7852216.75 | 77977981 | 10922450 | 12762968 | 19435536.5 | 186010998.3 |
| M. hyorhinis | 13723242.02 | 14084100.96 | 8108521.118 | 80877503.58 | 10648529.02 | 12699159.59 | 19738460.38 | 187341276.4 |
| M. hyorhinis | 13144412.45 | 13808040.48 | 7914624.321 | 79166638.76 | 10421089.06 | 12182496.42 | 18817339.38 | 180738131.6 |
| M. hyorhinis | 14083321.25 | 15052743.25 | 7021446 | 78043021 | 10049579.75 | 12960208 | 21413573.5 | 184564148.3 |
| M. hyorhinis | 14640591.25 | 15496671.75 | 6628273.25 | 78235081.25 | 10705460.25 | 13188613 | 21991418.75 | 188399332.3 |
| M. hyorhinis | 14505573.25 | 15254184.25 | 7246976.25 | 77897662.75 | 9784098.25 | 12744371.75 | 21490085.75 | 183515918 |
| M. hyorhinis | 15639725.5 | 15418186.5 | 7584121.25 | 80979093 | 9825901.5 | 13372861.25 | 23712084.5 | 200694480.5 |
| M. hyorhinis | 15053178.75 | 16344618.5 | 6737816.75 | 69232946 | 5427676 | 13064573.75 | 27238928.25 | 190386651 |
| M. hyorhinis | 15291566.75 | 16214596.75 | 6901462.75 | 71811770.75 | 5707276.5 | 12862621.5 | 27550229.25 | 190707963 |
| M. hyorhinis | 15142988.5 | 15981509.75 | 6560024.75 | 70144885 | 5900084.25 | 12797977.25 | 26898554 | 189487936.3 |
| M. hyorhinis | 15653267.98 | 15997005.73 | 6600596.946 | 75284079.11 | 6126529.926 | 12855048.98 | 27799131.36 | 197874096.4 |
| M. hyorhinis | 9654723.5 | 10889312.13 | 10940410.13 | 80548189.25 | 13970902.13 | 13408023.25 | 14635683.5 | 184437432.6 |
| M. hyorhinis | 9192738.25 | 10413174.5 | 11201393.5 | 81701230.5 | 13170316.75 | 13175310.5 | 14335366.5 | 178615083.3 |
| M. hyorhinis | 10122454.25 | 11329776.25 | 12253798 | 84138012.75 | 14103074.25 | 13056112.75 | 14278251.5 | 184115796 |
| M. hyorhinis | 9274263.75 | 10390676 | 11501686.75 | 82622648.25 | 13507210.5 | 13197722.5 | 14278785.5 | 178483415.8 |


| Species | Phenylalanine | Phenylalanine | Tyrosine | Methylhistidine | Tyrosine | Fumarate | Inosine | Cytidine |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Friss medium | 129578523.3 | 221123350 | 102019657.8 | 51807738.88 | 88871381.63 | 2089883.875 | 10014595.5 | 7905032.125 |
| Friss medium | 134743863.5 | 233305229.8 | 107457372.3 | 55691466.5 | 93924038.75 | 2369075.25 | 7778588.25 | 8257010.25 |
| Friss medium | 136580154 | 233998715.9 | 108104904.6 | 55778881.88 | 94293896.5 | 2312435.375 | 10136537.38 | 8263046.25 |
| M. flocculare | 138374179.3 | 242015750.8 | 114211788 | 59606520.25 | 100316231.3 | 2990219.75 | 8282154.5 | 7834791.25 |
| M. flocculare | 135314574.3 | 237497841.5 | 112033062 | 58486649.13 | 98472037.25 | 2892792.25 | 8122275.75 | 7550158.75 |
| M. flocculare | 139097694.5 | 245169114.3 | 115333315.3 | 60816569 | 101623409.3 | 3108232.25 | 6946673.5 | 7871630.25 |
| M. flocculare | 136934620.8 | 242915652.5 | 116269382.5 | 59694821.25 | 101958252.5 | 3512661.25 | 5399837 | 7196438 |
| M. flocculare | 141028002.1 | 249739691.5 | 119485623 | 61258520 | 105137410.8 | 3031105.375 | 5619003.5 | 7517558.5 |
| M. flocculare | 138439686 | 245383167.6 | 117384333.6 | 60584663.5 | 103562256.8 | 3235426.875 | 5599325.75 | 7284840.625 |
| M. flocculare | 136204394.5 | 242167492 | 116680590.8 | 60242301 | 102630791.8 | 3413438.25 | 3410943 | 6224617.25 |
| M. flocculare | 137932789.5 | 245317070.3 | 119425655.8 | 61480129.5 | 104782933.3 | 3523342.25 | 2941790.75 | 6761976 |
| M. flocculare | 137011706.8 | 243810966.8 | 117392751.5 | 61099793.25 | 103313238.8 | 3407974.75 | 3704035.75 | 6543899 |
| M. flocculare | 146633722 | 263278153.5 | 131714149 | 71607893.5 | 116890045.5 | 3650649 | -75838.5 | 209748 |
| M. flocculare | 146784664 | 268411501 | 132983307 | 72821150.5 | 117457912.5 | 4668857.5 | -183184.5 | 2436470.5 |
| M. flocculare | 147368242.8 | 269698688.3 | 134043690.8 | 73019918.5 | 118384726.5 | 4880610.5 | 270168.25 | 2794399.5 |
| M. flocculare | 139065248.5 | 251430090.8 | 119461595.3 | 64200187 | 104944129.5 | 3686329 | 10394886.75 | 8656960.25 |
| M. flocculare | 147706673 | 267728168 | 127356790 | 68960817.25 | 111849780 | 3982662.25 | 9155784.5 | 9232630.75 |
| M. flocculare | 147784022.8 | 267929989.8 | 126941801.5 | 68361159 | 111524682.5 | 3973183.75 | 10895965.25 | 9190014 |
| M. hyopneumoniae | 136202611 | 237166588 | 110256045.8 | 56947672.25 | 96432451.5 | 2540819.25 | 7896175 | 7380506.25 |
| M. hyopneumoniae | 127950181 | 222693924 | 103458441.3 | 53379555.75 | 90651791 | 2349645.75 | 7435734.75 | 6881908.25 |
| M. hyopneumoniae | 136517173.3 | 238178863 | 110563070.8 | 57266980.25 | 96983553.5 | 2718964 | 8034096.5 | 7565052.5 |
| M. hyopneumoniae | 130106244.8 | 228614616 | 107607756 | 56230586.5 | 94449890 | 2707370.5 | 1660078.75 | 5524192.5 |
| M. hyopneumoniae | 139438214 | 244858748.5 | 114698616.3 | 59318640.5 | 100620331.8 | 2964141.5 | 2714874.5 | 6258995 |
| M. hyopneumoniae | 133410530.8 | 234578178.5 | 109836388.3 | 57341811 | 96710595.75 | 2917561.75 | 2692971.75 | 6156025.25 |
| M. hyopneumoniae | 137695799.3 | 242324191 | 114691266.8 | 60032241 | 100713236 | 2923604.25 | -115665.75 | 2922038.75 |
| M. hyopneumoniae | 135757306.8 | 237205692.8 | 112256430.5 | 59147765 | 99174878.75 | 3935623.75 | 3334816.5 | 6331200.5 |
| M. hyopneumoniae | 137846430.5 | 243052786.5 | 114892960.5 | 60161910.5 | 100834942.5 | 3011052 | 183492 | 3406502 |
| M. hyopneumoniae | 133278197 | 236956239 | 112986515 | 61373893 | 99455623 | 3052995 | 402633 | 268636 |
| M. hyopneumoniae | 139813001.5 | 248385898 | 118936604 | 65610800 | 105328293 | 3178324 | 253199.5 | 368953.5 |
| M. hyopneumoniae | 134536218.5 | 233932442 | 108609222.9 | 56158687 | 94981667 | 2551678.125 | 7674727 | 7440426.875 |
| M. hyopneumoniae | 128092168 | 221086912.9 | 102421944.4 | 52784275.88 | 90241480.13 | 2645345.75 | 7859267.625 | 7265706.875 |
| M. hyopneumoniae | 135121044.9 | 234923783 | 109380247.4 | 56860172.13 | 95829412.63 | 2439798.125 | 7649740 | 7407798.5 |
| M. hyopneumoniae J | 127609774.8 | 212788990.8 | 97341586.75 | 50412920.75 | 84938538.5 | 1802239 | 4721984.5 | 4585380 |
| M. hyopneumoniae J | 126920607.5 | 212557355.5 | 98974082.5 | 52764844 | 86544165 | 2904570 | 6076677 | 6161197 |
| M. hyopneumoniae J | 123876957.3 | 210108185.5 | 98315412.25 | 52391481.25 | 85606058.5 | 2836184.5 | 5828161.25 | 6280035 |
| M. hyopneumoniae J | 124536366.3 | 209470340.3 | 98042973.75 | 51887716.5 | 85449222.5 | 2673436 | 5983270.5 | 5742113.5 |
| M. hyopneumoniae J | 126623856.4 | 212771005.2 | 101409941.1 | 55598343.95 | 88630748.57 | 3694876.732 | 922210.929 | 4445366.284 |
| M. hyopneumoniae J | 302000115.4 | 528234422.5 | 248394851.2 | 139004692 | 227263567.4 | 4316597.289 | -18256362.98 | -5794497.736 |
| M. hyopneumoniae J | 125648880 | 216654489.8 | 102984068.3 | 57695418.5 | 89528529 | 3253512.75 | -210742.5 | 3471599.75 |
| M. hyopneumoniae J | 128463292.1 | 218432207 | 105498347.6 | 61826550.81 | 92194948.15 | 3840043.141 | 202009.2416 | 559129.6247 |
| M. hyopneumoniae J | 125376993.5 | 213586159 | 101862062.5 | 59035093 | 88515402.5 | 3446240 | 493781.5 | 745812.5 |
| M. hyopneumoniae J | 128593095.9 | 216208543.8 | 104623538.5 | 60876268.39 | 91072671.4 | 3124721.576 | -150296.8176 | -101670.6359 |
| M. hyopneumoniae J | 127728873 | 223542183.5 | 108004667.5 | 63050995.5 | 94209846.5 | 3510191 | -592352.5 | -345747.5 |
| M. hyopneumoniae J | 122925754 | 210854081 | 102293797 | 58803440 | 88515933 | 2930918 | -804507 | -591430 |
| M. hyopneumoniae J | 127732993.6 | 211810883.2 | 102775099.4 | 59119174.31 | 89383001.15 | 3022353.285 | -618308.7841 | -317533.1793 |
| M. hyopneumoniae J | 131433324 | 222269171.5 | 106912809.5 | 60162912 | 94227937 | 3819036.5 | 1190659 | 1487373 |
| M. hyopneumoniae J | 127619552 | 215102860 | 99905804.25 | 54142909.25 | 87134867.75 | 2818709.75 | 6182808 | 5595021.5 |
| M. hyopneumoniae J | 123158779.3 | 203906921 | 92805425.75 | 47327697.25 | 81557756.25 | 1660117.75 | 5554894.5 | 4847555.25 |
| M. hyopneumoniae J | 123396815 | 208320700.8 | 96609927 | 50901889.25 | 84195234.25 | 2791516.25 | 6757606 | 6129161.25 |
| M. hyopneumoniae J | 120467199.8 | 194328675.5 | 90618727.13 | 48288422.66 | 81790285.47 | 2919243.263 | 6500391.197 | 6748253.316 |
| M. hyorhinis | 128660570.5 | 217300830 | 100321793.3 | 53394670.75 | 87693320.75 | 3214463.5 | 7604832.5 | 6106659 |
| M. hyorhinis | 126885044.4 | 214641252.8 | 99595463.63 | 52998053.25 | 86793541.38 | 3365658.25 | 7507916.875 | 6100251.5 |
| M. hyorhinis | 124219809.5 | 205437919.5 | 94201805.5 | 49496461.5 | 81673783.5 | 2487404 | 6861921 | 5832081 |
| M. hyorhinis | 121812678.3 | 204416020.8 | 95073531.25 | 50336834.5 | 82470904.25 | 2586217.5 | 6681967.25 | 5332785 |
| M. hyorhinis | 123842484.3 | 212117061.8 | 97465216 | 51333809.5 | 85126512.75 | 2342012.75 | 853404.5 | 3400563.5 |
| M. hyorhinis | 126095584 | 214973263.8 | 100489561.5 | 54318640.25 | 88040650.5 | 3554721.75 | 2324370 | 4630008.5 |
| M. hyorhinis | 126565557.5 | 215667195.6 | 101155813.9 | 55467632.48 | 88094663.29 | 3181495.777 | 2014881.726 | 4440792.488 |
| M. hyorhinis | 123253635.5 | 211664234.1 | 99779450.01 | 54039612.95 | 87032025.03 | 3169681.29 | 1944110.347 | 4409578.404 |
| M. hyorhinis | 124040486.8 | 214892233 | 100804129.8 | 54821776 | 87803999.75 | 3308669.25 | 752542.75 | 3602636.5 |
| M. hyorhinis | 126631279.5 | 219684963.8 | 103139947.5 | 55537077 | 90086949.25 | 3541622 | 515900.5 | 3532494 |
| M. hyorhinis | 123767693 | 214603688 | 100675587.3 | 54684089 | 87693926.5 | 3414600.5 | 520743.25 | 3864299 |
| M. hyorhinis | 133892282.3 | 227961561 | 105432652 | 60407086 | 92316221.5 | 3345362.25 | 162330.75 | 3356296.5 |
| M. hyorhinis | 125549437 | 221039522 | 104759708 | 60492238.25 | 91537467.75 | 3813255 | -28460.5 | 292495.25 |
| M. hyorhinis | 125575444.5 | 218672407.8 | 103584213.5 | 59303466.25 | 90398066 | 3750386 | -133202.25 | -85710.25 |
| M. hyorhinis | 125506614 | 220265851.8 | 104602890 | 61216030 | 91475238.75 | 3714308 | 85264 | 173742.5 |
| M. hyorhinis | 130310153.8 | 225681135.5 | 107159950.7 | 62869029.79 | 93408488 | 3896704.949 | 114277.2255 | 215861.9138 |
| M. hyorhinis | 129539002.1 | 217864383.1 | 101018675.9 | 53226802.75 | 87880924.25 | 3291739.75 | 7839280.125 | 6016441.125 |
| M. hyorhinis | 125545845.5 | 211358075.8 | 97851990.5 | 51441633.25 | 85028390.25 | 2882643.5 | 7714327.75 | 5784407.25 |
| M. hyorhinis | 127059420.8 | 215884049.5 | 100508765.5 | 53465831.5 | 87158609 | 3073549.75 | 6398895.5 | 6303547 |
| M. hyorhinis | 125389114.5 | 210414004.3 | 97565276 | 51545236 | 84931446.5 | 3059139 | 7605317.5 | 5828770 |


| Species | Uridine+Cytidine | x2 | x3 | aGlucose | Trehalose | bGlucose | Threonine | Pyroglutamate |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Friss medium | 35297021.25 | 3732556.125 | 5225640.625 | 269025019.4 | 50523361.5 | 623360636.3 | 163107652.6 | 246009243.3 |
| Friss medium | 36111170 | 4244581.75 | 9811590.75 | 292725049 | 38804412.5 | 659518529.5 | 170494399.8 | 259294268.8 |
| Friss medium | 37063172.75 | 4153465.625 | 6923219.125 | 293421050.1 | 49636811.88 | 671597262.1 | 174294869.4 | 262715425.3 |
| M. flocculare | 33151467.5 | 6467996.25 | 4065121.25 | 268544331.5 | 24727280.5 | 619812619 | 177472477.5 | 257126660.8 |
| M. flocculare | 32626289.75 | 6356394.875 | 4195108.125 | 264180656.4 | 25825624.75 | 604977671.1 | 171084735 | 250303663.3 |
| M. flocculare | 32742857.25 | 6555012.75 | 6749716.25 | 272898781.5 | 21161883 | 620342700 | 176631746.3 | 259929134 |
| M. flocculare | 27564662 | 8217091.75 | 2716335.75 | 253544878.5 | 18237472.5 | 582662195.3 | 174166109.5 | 249926016.3 |
| M. flocculare | 28694826.63 | 7550956.625 | 2001244.875 | 252298765.9 | 20960556.25 | 595265536.4 | 182481239 | 258342701 |
| M. flocculare | 27984031.25 | 8075941.75 | 2500319.625 | 258952645.9 | 17804850 | 596066572.6 | 176481278.1 | 252748133.9 |
| M. flocculare | 23702583.75 | 8945969.5 | 1781969.5 | 240799105 | 15304898.25 | 566450120.5 | 175578404.5 | 248026002 |
| M. flocculare | 22832775.25 | 10025624 | 2244705.75 | 244213426.8 | 15525233 | 556857193.3 | 174481707 | 242722004.8 |
| M. flocculare | 24005637.75 | 8683126 | 1934725.5 | 241893524.3 | 15600631.5 | 578691396.5 | 175703784.8 | 250003010.8 |
| M. flocculare | 214917.5 | 19908655 | 469303 | 153679010 | 10573632.5 | 427605475 | 201054892 | 272575790 |
| M. flocculare | 4530477.5 | 19091385.5 | 219461 | 247462351 | 10071351.5 | 551465400 | 186655220 | 240710994.5 |
| M. flocculare | 4986726.75 | 19161932 | 826225 | 250858435.3 | 11632867.25 | 552827437.5 | 186409965 | 241022902.8 |
| M. flocculare | 37177225 | 6463059.25 | 2994994.25 | 326972248.5 | 12831894.75 | 679471326.3 | 179505666.3 | 227667585.8 |
| M. flocculare | 38598596 | 6752801.5 | 5237614.75 | 351386046 | 11737052.25 | 726674843 | 194443738.8 | 249282835 |
| M. flocculare | 39401297.25 | 6728646.5 | 2901278 | 348329568 | 12563904 | 723640154.5 | 192904037.8 | 244106976.3 |
| M. hyopneumoniae | 30401566 | 7425783.5 | 2964951 | 295276687.3 | 28979211 | 661242025 | 173762553 | 254859546.5 |
| M. hyopneumoniae | 28604582.25 | 7004372.5 | 3023464 | 275379406 | 27276014 | 613411163.3 | 159366046 | 236717991 |
| M. hyopneumoniae | 30942644.5 | 7499584.75 | 3637664.25 | 298848185.3 | 30095656.5 | 659676892.8 | 171929909.8 | 255023013.5 |
| M. hyopneumoniae | 16494068 | 11096218.25 | 1409689 | 277857459.8 | 14262808.25 | 621244348.5 | 163779270.8 | 242128371 |
| M. hyopneumoniae | 19972161 | 11277996.25 | 1975452.5 | 295331393.5 | 26525224.75 | 655013534.8 | 176390171.3 | 256398811.3 |
| M. hyopneumoniae | 19444230 | 10981282.25 | 1750536.25 | 283612928 | 25931639.5 | 624562057.3 | 167764063.5 | 245106101.5 |
| M. hyopneumoniae | 5636125.75 | 15878359.75 | 100173.5 | 278351805.8 | 18134813.5 | 635708772.3 | 174038364.8 | 253120870.5 |
| M. hyopneumoniae | 13582961.5 | 21949148.75 | 13489103.5 | 321486424.8 | 66598912 | 965215220.8 | 220981054.8 | 280058436.8 |
| M. hyopneumoniae | 6496154.5 | 16291570.5 | 925289 | 277975438.5 | 21284735 | 617087490 | 172427785 | 247572474 |
| M. hyopneumoniae | 604390 | 18718563 | 775636 | 207342801 | 8586666 | 493811893 | 168633794 | 238387042 |
| M. hyopneumoniae | 376114 | 19300322 | 485562.5 | 208889414 | 10665621.5 | 509598731 | 177664139 | 253785055 |
| M. hyopneumoniae | 30734423.63 | 6709160.125 | 3352620.25 | 297650859.1 | 19456048.25 | 672325524.4 | 171835178.6 | 259386117.3 |
| M. hyopneumoniae | 29488270.13 | 7158475.875 | 3851774.625 | 277745896.8 | 19945777.13 | 624648184 | 161254145.3 | 243463501.3 |
| M. hyopneumoniae | 30924166.25 | 7090346 | 3945830.375 | 300578598.3 | 20520893.25 | 672217473.6 | 170086847.4 | 259827452.9 |
| M. hyopneumoniae J | 22875250.5 | 5272827.5 | 1754476.5 | 255058960 | 93782600.5 | 592471724 | 156003968.5 | 252828484.3 |
| M. hyopneumoniae J | 24364279.5 | 5850084.5 | 2086891 | 245298411 | 83674872 | 612538474.5 | 175157087.5 | 261440329.5 |
| M. hyopneumoniae J | 23954831 | 6146961.5 | 2634421.25 | 244719752.5 | 83859418.75 | 584915034.8 | 155980461.3 | 247942334.8 |
| M. hyopneumoniae J | 23564695 | 5648534.5 | 1237432 | 240080338.5 | 86397023.75 | 604779295.8 | 162176286.8 | 254436542.3 |
| M. hyopneumoniae J | 6648902.32 | 13727509.17 | 1764180.189 | 227977117.2 | 44574396.52 | 554972106 | 159007158.5 | 247063656 |
| M. hyopneumoniae $J$ | $-3762393.437$ | 17943290.89 | -5230621.16 | 550834255.1 | 73046473.52 | 1247463435 | 387081943.9 | 590343077.2 |
| M. hyopneumoniae J | 4620720 | 12636678 | -271186.5 | 220299347.3 | 37033953.5 | 557381989 | 160634576.8 | 246835530.3 |
| M. hyopneumoniae J | 505100.3939 | 15732749.34 | 50976.04529 | 152219352 | 20585871.41 | 468233254.1 | 168281903.3 | 258585597.3 |
| M. hyopneumoniae $J$ | 1546316.5 | 16330072 | 1523495.5 | 191702554 | 24045984 | 502101670 | 159746722 | 244774854.5 |
| M. hyopneumoniae J | 257258.4243 | 14922472.07 | -354525.8995 | 149864508.8 | 15226560.91 | 469425503.2 | 167750572.5 | 256749450.9 |
| M. hyopneumoniae J | -695367 | 15235428 | -1128424 | 66950712 | 2592696 | 317969116.5 | 166474518.5 | 250442291.5 |
| M. hyopneumoniae J | -1005906 | 13673642 | -1796849 | 57929458 | 2213841 | 309766925 | 160502802 | 243853748 |
| M. hyopneumoniae J | -428633.4926 | 14110428 | 430900.8716 | 152022850.3 | 135222851.1 | 309545424.6 | 173468350.8 | 256967380.6 |
| M. hyopneumoniae J | 3506017 | 18798556.5 | 4963206.5 | 91824476 | 27383603 | 275005063.5 | 152499093 | 243104764 |
| M. hyopneumoniae J | 23999839.5 | 4970175.25 | 1175502.25 | 246083917.5 | 85438179.5 | 623681239 | 167404124 | 263583999.3 |
| M. hyopneumoniae J | 22963396.5 | 5117828.5 | 3053229.75 | 245318315.5 | 111466618 | 553564637.8 | 157174858.5 | 245552328.5 |
| M. hyopneumoniae J | 24117419.5 | 5938004.5 | 2733060.25 | 237248484 | 106569688.5 | 575722401.3 | 155924798.3 | 251162185 |
| M. hyopneumoniae J | 27628087.18 | 12736477.53 | 36171833.94 | 1180648771 | 1189359690 | 597517958.6 | 161818507.6 | 251287506.1 |
| M. hyorhinis | 25973211.5 | 6538384.75 | 2792956 | 254139033.3 | 108609669.8 | 602498123.8 | 172491412.3 | 260299311.5 |
| M. hyorhinis | 25735018.13 | 6069927.25 | 2676760.125 | 252374332.1 | 106409907.3 | 597718983 | 159772921.4 | 256363470.3 |
| M. hyorhinis | 24798218 | 5999860 | 3787840.5 | 248956133.5 | 104224353 | 589179928.5 | 155178301.5 | 249393974 |
| M. hyorhinis | 23470000.5 | 4632062.25 | 947139.25 | 237723440.3 | 104753498.3 | 607497381 | 160652706.5 | 253421887 |
| M. hyorhinis | 14318683.5 | 8748172.5 | -315585.75 | 245096622.3 | 62257080 | 577728440.8 | 154075172.5 | 240448388.5 |
| M. hyorhinis | 15946850.5 | 9691401.75 | 514141.25 | 243085263.3 | 57007855.25 | 599801721 | 175218345.3 | 254250717.5 |
| M. hyorhinis | 15214445.13 | 9747397.605 | 244538.1824 | 242761260.5 | 51779602.9 | 609538092.6 | 167188679.3 | 255024426.6 |
| M. hyorhinis | 14977917.86 | 9496667.892 | 267623.9229 | 238877845.2 | 54882811.61 | 586915916.3 | 159773946.3 | 244827244.2 |
| M. hyorhinis | 10207208 | 11322216 | -310375.75 | 233966627.5 | 44395022.5 | 578106480.3 | 158595008.5 | 241976612 |
| M. hyorhinis | 9563302 | 12574011.5 | -386495.25 | 238168067.5 | 42024686 | 583374939.5 | 163097896 | 246980225 |
| M. hyorhinis | 10680225 | 11450456 | -327317.75 | 234030296.5 | 44065540.25 | 574617060 | 159132321 | 242256860.8 |
| M. hyorhinis | 9980715 | 12640042.75 | 751321.75 | 257099124.8 | 57429279 | 608037489.8 | 169714039 | 264300578.3 |
| M. hyorhinis | 333847 | 17261453.5 | 398119.5 | 203021028.5 | 18603939.75 | 495171613.5 | 157250072.5 | 235497096.3 |
| M. hyorhinis | -130370.5 | 16103109.5 | -958638 | 188817474 | 15862015 | 510414998 | 166070774.5 | 243203769.3 |
| M. hyorhinis | -362777.5 | 16452540 | -202367 | 193737383.5 | 17995025 | 500785394 | 161537945.5 | 239827864.3 |
| M. hyorhinis | 567075.2109 | 17159206.54 | 520695.3148 | 211670615.5 | 20857305.84 | 543339411.7 | 171110050.6 | 255821895.2 |
| M. hyorhinis | 26291133.38 | 6239973.375 | 3495650.125 | 256022071.3 | 109704356.6 | 600203512.5 | 172999869.6 | 261729805.6 |
| M. hyorhinis | 24965526.5 | 5439770.5 | 2569272.5 | 244658215.8 | 111909440.8 | 596793031.5 | 159238134 | 255400550.5 |
| M. hyorhinis | 24670297.75 | 5523629.75 | 5332447.75 | 261855704.5 | 83508257.5 | 632651034.8 | 164319439.5 | 260253544.5 |
| M. hyorhinis | 24560678 | 5279944 | 1693817.75 | 242086116.8 | 113670273.3 | 606962985.3 | 163550634.8 | 259931128.5 |


| Species | Proline+Lactate | Valine | Threonine | Glycine | Glucose | Glucose | Carnithine? | Phenylalanine |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Friss medium | 507121496.1 | 251920086.1 | 170626794.6 | 624332511.4 | 569484684.5 | 372720050.9 | 159810502.3 | 189515958.6 |
| Friss medium | 550788303 | 268881187.5 | 183225449.8 | 703267787.8 | 620074870 | 402399125.8 | 169432729.8 | 199983488.3 |
| Friss medium | 544767193.8 | 271772943.1 | 185191890.4 | 684485764.3 | 621405828.8 | 406424890.8 | 171481937.4 | 203002396.9 |
| M. flocculare | 577826267.3 | 282349322 | 193478368.3 | 765811696 | 602380225.3 | 368315981 | 168872943.8 | 203689118.3 |
| M. flocculare | 559499692 | 277401312 | 190621381.3 | 762440551.6 | 588415268.5 | 363906275.8 | 166604706.8 | 201458823.6 |
| M. flocculare | 586938647 | 286091808 | 197386895.5 | 800558601.3 | 615582872.8 | 373158990 | 170807559 | 202500015.5 |
| M. flocculare | 576639408.8 | 283024965 | 194845453 | 807529277.5 | 587462521.3 | 347925315.5 | 169308550 | 199398424.5 |
| M. flocculare | 595355437.4 | 292794553.9 | 201894042 | 832063044.3 | 581244695.9 | 356460689.6 | 175705193.3 | 209884138 |
| M. flocculare | 580782660.4 | 288089962.4 | 198462861.8 | 820816402 | 594858114.9 | 357507757.5 | 171704547.4 | 201702449.8 |
| M. flocculare | 576891650.5 | 284248686 | 195802730 | 831025430.3 | 573611881.5 | 334142946.8 | 169443108.3 | 196792342.3 |
| M. flocculare | 575647191.3 | 287170328 | 199432047.5 | 867268438.8 | 584525371.5 | 334914499.5 | 171892465.5 | 200177405.8 |
| M. flocculare | 581030685.5 | 285160422 | 197046546.5 | 827808619.3 | 572731796.3 | 337840076.5 | 171203041.3 | 196594753.8 |
| M. flocculare | 667969449.5 | 308838175.5 | 213191078.5 | 918270523.5 | 418961313 | 257038464 | 188546194 | 200935689.5 |
| M. flocculare | 618817558 | 313648865.5 | 225604736 | 1101754808 | 644615231.5 | 344481861.5 | 192703249.5 | 208563737 |
| M. flocculare | 621539510 | 314925782.5 | 229273626.3 | 1115010300 | 648815591.5 | 345574455.5 | 194394999 | 209329427 |
| M. flocculare | 583573216.3 | 294027432.3 | 209110666.8 | 1027478433 | 772303779 | 413390349.8 | 177677241.8 | 204662838.8 |
| M. flocculare | 637043212.3 | 318974848.3 | 226018739.5 | 1139331174 | 840710944.8 | 447215891.5 | 190936742.8 | 221352753.8 |
| M. flocculare | 627737541.8 | 314963433.8 | 223279367.8 | 1098655114 | 826246696.5 | 443819941.5 | 190407866.8 | 215747783.8 |
| M. hyopneumoniae | 531520281.3 | 273067201.3 | 187825843.5 | 746651497.8 | 651651513.5 | 385305954.8 | 166020857.8 | 201760426 |
| M. hyopneumoniae | 491762462.3 | 254032311.8 | 174567566.3 | 690198997.8 | 598514907.8 | 364053946 | 156417484.8 | 188691601.8 |
| M. hyopneumoniae | 533876318.3 | 274721786.3 | 189254160.8 | 745705885.5 | 650255962.8 | 397351067.8 | 168892935.8 | 202211082 |
| M. hyopneumoniae | 472960474.5 | 263164420.3 | 182458629.3 | 737250456.5 | 623788799.5 | 360927672 | 160445213.5 | 194991381.8 |
| M. hyopneumoniae | 501815851.5 | 283874954.3 | 196644696.3 | 797151168 | 671445265 | 378917869.8 | 179366371 | 205033172 |
| M. hyopneumoniae | 479201881.5 | 271413017.8 | 188211042.5 | 768218740.3 | 640146283.8 | 365064133.3 | 171727714.8 | 196864301.5 |
| M. hyopneumoniae | 468613962.8 | 282146371 | 196580797.5 | 802732758.5 | 634919370 | 363947257 | 183738883.5 | 202534618 |
| M. hyopneumoniae | 517884498.8 | 279910541.3 | 198635675.3 | 799896433.3 | 635645453.8 | 355175804 | 182286611.5 | 197290641.5 |
| M. hyopneumoniae | 468700650 | 281840470.5 | 197541207 | 828480271 | 648028032 | 353560462 | 184808589 | 195827495 |
| M. hyopneumoniae | 463905990 | 268315719 | 191044103 | 798337418 | 513004783 | 266050199 | 192858204 | 188202946 |
| M. hyopneumoniae | 459899095.5 | 284041890 | 202276080.5 | 829334330 | 522484685.5 | 271789087.5 | 205591637 | 197514011.5 |
| M. hyopneumoniae | 533206219.6 | 268212768.1 | 183669276.3 | 715647605.1 | 645688397.5 | 399060913.8 | 160317233.4 | 201988392.4 |
| M. hyopneumoniae | 497418843.3 | 249238329 | 170799249.1 | 660910391.6 | 598242538.8 | 365056075.8 | 147784176.3 | 187391798.8 |
| M. hyopneumoniae | 530075707 | 269667514.4 | 184993044.4 | 712715173.9 | 645656236.5 | 401293208.1 | 161308927.5 | 202435644.8 |
| M. hyopneumoniae J | 488851251 | 240446541.8 | 166244570.8 | 515793232.5 | 523459342.8 | 335249218.3 | 134839279 | 176522670.3 |
| M. hyopneumoniae J | 532204557.5 | 241143713 | 166376234 | 509610364.5 | 522698968 | 337699736.5 | 136194965.5 | 183550887 |
| M. hyopneumoniae J | 481883464 | 237703670 | 162435745.3 | 510371213.3 | 517926437.5 | 335032387.5 | 136310613 | 181190980.5 |
| M. hyopneumoniae J | 489306246.3 | 238847319.5 | 164646889.5 | 504907434.3 | 514599887.5 | 335393391.8 | 136453098.5 | 184939280.3 |
| M. hyopneumoniae J | 489622031.5 | 240753691.9 | 170981602.7 | 558219974.1 | 495490216.5 | 292352196.7 | 145729304.1 | 174683266.8 |
| M. hyopneumoniae J | 1262134006 | 590888143.5 | 406196989.3 | 1397187030 | 1233956438 | 730719166.5 | 359424458.3 | 417849272.5 |
| M. hyopneumoniae J | 493449484.8 | 245132042.5 | 170007895.8 | 565263691.5 | 494836888.5 | 299929460.5 | 151783321 | 180142539.5 |
| M. hyopneumoniae J | 530378519 | 242871001.8 | 177518872.7 | 568568696.2 | 372182399.4 | 216018574.2 | 175918059.6 | 181056352 |
| M. hyopneumoniae J | 522388623 | 238389990.5 | 172900916 | 561194217 | 442020818 | 254075492 | 155204493.5 | 172538915 |
| M. hyopneumoniae J | 517457044.2 | 242582055.7 | 182106568.8 | 557258589.9 | 377811442.7 | 211553567.5 | 172317503.3 | 180715571.1 |
| M. hyopneumoniae J | 674076976.5 | 242384908 | 177408230 | 597843025 | 217645284 | 134405321 | 202555822 | 175211437.5 |
| M. hyopneumoniae J | 621839986 | 230706792 | 173839376 | 555646484 | 197768026 | 122612877 | 192485513 | 167355425 |
| M. hyopneumoniae $J$ | 682188516.8 | 236990779 | 180757118.7 | 556363780.5 | 198871588.9 | 123310368.5 | 193585799.4 | 169375350.8 |
| M. hyopneumoniae J | 656209345 | 240826173.5 | 179348053.5 | 610292039.5 | 211556561 | 125278489 | 197676746.5 | 164823723.5 |
| M. hyopneumoniae J | 507547756.3 | 243135155.3 | 166919334.3 | 507091532 | 520660078 | 348633911 | 140878859.3 | 181630881.5 |
| M. hyopneumoniae J | 497979775.8 | 228564361.3 | 156459456.5 | 481158194 | 489026453.8 | 321536462.5 | 127856877.5 | 169077821.3 |
| M. hyopneumoniae J | 483194441.5 | 234362523.8 | 159157193.5 | 488158924.3 | 490885777.5 | 335745742.5 | 137043322.5 | 182881458 |
| M. hyopneumoniae J | 471589548.1 | 233345358.7 | 170734280.2 | 484814881.6 | 520016551.8 | 328596100 | 125697234.8 | 180838445.9 |
| M. hyorhinis | 538950558.3 | 247062540.5 | 166509704.8 | 525093606 | 528077595.5 | 357392531.5 | 145203918.8 | 189433767 |
| M. hyorhinis | 498894342.3 | 243642910.4 | 164495007 | 521143706.9 | 524639050.1 | 356151597.8 | 144001566.8 | 187722438.8 |
| M. hyorhinis | 483501347 | 231794108.5 | 163254875.5 | 497499083 | 511920288 | 336423076.5 | 133050875.5 | 173852379 |
| M. hyorhinis | 482594662.8 | 235650708.3 | 161380202.3 | 495369049.5 | 505042764.8 | 342685302.5 | 138632515.5 | 183113736.3 |
| M. hyorhinis | 501996174.8 | 243464188 | 168218191.3 | 576944216.3 | 512564605.8 | 335262228.3 | 170207157.8 | 174270430.8 |
| M. hyorhinis | 551858231.3 | 248960669.5 | 172185733.8 | 583756885.8 | 527563523.3 | 339219350.3 | 173823851 | 181450188.8 |
| M. hyorhinis | 527937460.4 | 251459967.3 | 175443272.9 | 590453568.7 | 530798412 | 345669625 | 175875467.7 | 181525332.1 |
| M. hyorhinis | 507890485.7 | 247283540.5 | 169824439.1 | 582423519.9 | 525542490.8 | 337528418.3 | 174642120.3 | 179691084.6 |
| M. hyorhinis | 507477877.5 | 248331149.8 | 171440181.8 | 607561679 | 514485532.8 | 334352121.3 | 186495541.8 | 181374262.5 |
| M. hyorhinis | 527461485.3 | 254008528.5 | 175750473.5 | 624736566 | 521425664.8 | 338787479 | 191423569.8 | 192425452.8 |
| M. hyorhinis | 511055087.8 | 247921331.3 | 170443217.8 | 608783543 | 513856829 | 335515997.3 | 186806667 | 179634530.5 |
| M. hyorhinis | 559556597 | 266051238 | 187303262 | 652917500 | 561315612.5 | 349769029 | 197901843.8 | 183903538.5 |
| M. hyorhinis | 541915039.5 | 255387924 | 176116903.5 | 666986113.5 | 447978550.8 | 306884724.8 | 204992354.3 | 181024645.8 |
| M. hyorhinis | 564677354.8 | 255260631.5 | 180115193 | 663102788.5 | 443637984 | 288372535 | 199922085 | 180529516.5 |
| M. hyorhinis | 558532271.3 | 257776948.5 | 178561783.3 | 674234485.3 | 451949857.5 | 292795874.3 | 205242362.3 | 182812385.3 |
| M. hyorhinis | 577537545.4 | 268134931.3 | 189367301.4 | 701498316.7 | 494361260.7 | 309367374.6 | 209621720.2 | 186708886.1 |
| M. hyorhinis | 543746189.6 | 246887960.1 | 165701272.8 | 518888113 | 526728280.1 | 360575988.9 | 145178576 | 189739579.8 |
| M. hyorhinis | 490023551.8 | 239340406 | 162095587.8 | 502872213.3 | 509133796.3 | 350756217.8 | 141081364.5 | 185922603.8 |
| M. hyorhinis | 513647367.3 | 247918357 | 169371911.8 | 550323461.5 | 550665648.3 | 368394153.8 | 142860629 | 191620940.5 |
| M. hyorhinis | 496579395.8 | 239658136.3 | 162997027 | 499492057.8 | 507242574 | 349187021.3 | 141465858 | 186323890.5 |


| Species | Asparagine | Asparagine | Aspartate | Sarcosine? | Aspartate | Methionine | Succinate | Pyruvate |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Friss medium | 108224903.4 | 97081987.38 | 132846610.8 | 48180441.5 | 171039638.4 | 149574978.5 | 206571105.1 | 193140267.5 |
| Friss medium | 114131140.8 | 101781192.8 | 140688252.3 | 50978636.75 | 181343835.8 | 154311136.5 | 222338692.3 | 199615096.8 |
| Friss medium | 116341963.6 | 104180865.9 | 143788694.5 | 51472521 | 185711418.4 | 158935487.9 | 222063115.8 | 203847617.3 |
| M. flocculare | 119019417.3 | 108359105.3 | 138145613.8 | 50823685.5 | 180266441.5 | 157620007.3 | 235766259 | 234144994.3 |
| M. flocculare | 116706119.8 | 105918425.5 | 136777022.4 | 49664735.5 | 176756344.8 | 156104334.8 | 232355430.9 | 226761514.1 |
| M. flocculare | 119601233 | 108737721.3 | 139482906 | 51628526.25 | 180720239.3 | 160685057.8 | 243251634.8 | 231647440.8 |
| M. flocculare | 118013909 | 106989905.5 | 138162311.5 | 52273463 | 179663793.3 | 154841182.8 | 245632802.5 | 231982259.3 |
| M. flocculare | 121573189.8 | 110971605.4 | 142422163.4 | 53349010.38 | 186789538.6 | 161027004.5 | 251529451.8 | 240120798.5 |
| M. flocculare | 119981738.5 | 108970146.8 | 140850052.3 | 53145224.75 | 183506130.9 | 157711298 | 249340371.3 | 233514760.8 |
| M. flocculare | 118082944 | 107371380.3 | 139655077.3 | 53190575.25 | 183149621.3 | 153459048.5 | 252147368.8 | 237914892 |
| M. flocculare | 114405979.3 | 103481684.5 | 146370882 | 53747880 | 193835674.8 | 153333217 | 259614206.8 | 243967163.5 |
| M. flocculare | 118667222.5 | 108191448.8 | 140409999 | 53488009.75 | 183191506.8 | 154210684 | 250679374.5 | 236000714 |
| M. flocculare | 126948272.5 | 116421986 | 143000564 | 56103134 | 198245173.5 | 164678022.5 | 285312962.5 | 346216861.5 |
| M. flocculare | 102731408 | 88507001.5 | 197782713 | 60288648.5 | 247662593.5 | 158662121.5 | 305291306 | 326767132 |
| M. flocculare | 103037220.8 | 88728064.75 | 198209381.8 | 60628326.75 | 248561756.8 | 159662613.5 | 307163651.8 | 327291018.8 |
| M. flocculare | 96252610 | 83035848.25 | 184574394.5 | 55092124.5 | 227139666.8 | 155511820.3 | 271718809.5 | 241325264.5 |
| M. flocculare | 103161790.3 | 89645876.25 | 202865045.5 | 59883709 | 247481073 | 170293578.5 | 302429943.8 | 260501519.8 |
| M. flocculare | 103228215.5 | 89004517.5 | 197881426 | 59356736.5 | 245144968 | 165938807.5 | 288073353.8 | 255411899.5 |
| M. hyopneumoniae | 113065108.5 | 102386630 | 143068909.5 | 52895433.75 | 185268171 | 153914035.5 | 235859271.5 | 225499244.3 |
| M. hyopneumoniae | 105967075.3 | 94983850.5 | 132638395.3 | 49164658.75 | 169346762.5 | 144377183.5 | 218362408 | 206212679 |
| M. hyopneumoniae | 114229021.8 | 102192014.8 | 142889520.3 | 52959815 | 184057781 | 154296939.3 | 233842304 | 219775062.5 |
| M. hyopneumoniae | 110258190.3 | 99118757 | 136276638.5 | 53559627 | 174173654.3 | 146221392 | 239008632.5 | 210978306.5 |
| M. hyopneumoniae | 117498738 | 104621512.3 | 145075756.5 | 56681360.75 | 188466106.3 | 153410750.3 | 258059357.5 | 235221998.5 |
| M. hyopneumoniae | 112556643.5 | 100475898.8 | 139593016 | 53984032.75 | 179593624.8 | 147816358 | 246526368.5 | 222767052.5 |
| M. hyopneumoniae | 116976859 | 105112907 | 147592673 | 58231457.25 | 188738581.8 | 154095262.8 | 261279272.3 | 222904633.3 |
| M. hyopneumoniae | 117371974 | 106161356.3 | 150288846.3 | 57986526.75 | 195215751.5 | 152055171.8 | 260744383.8 | 227672829.3 |
| M. hyopneumoniae | 114985764.5 | 101854826 | 147422381.5 | 57936476 | 192333474 | 149765556.5 | 267822826.5 | 232345728.5 |
| M. hyopneumoniae | 111688628 | 99124096 | 145559111 | 61173154 | 189489708 | 141195731 | 270416104 | 225546802 |
| M. hyopneumoniae | 120161895.5 | 107109996 | 152112452.5 | 66552874.5 | 204191776 | 146124529 | 294239914.5 | 242993758 |
| M. hyopneumoniae | 114064996.4 | 103032787.9 | 141145394 | 52072242.13 | 180723561.6 | 154906673.4 | 229263574.3 | 213449614.4 |
| M. hyopneumoniae | 104415659.8 | 93913955.75 | 130263448 | 48052601 | 167527229.1 | 145365950.3 | 216227533 | 199435442.6 |
| M. hyopneumoniae | 114175134 | 103226215.1 | 140837866.9 | 51983744.75 | 180911110.5 | 155233977.1 | 230343580.9 | 213929551 |
| M. hyopneumoniae J | 109242713 | 97661028 | 107075012.3 | 48426757.75 | 151013328 | 134040147 | 211372000 | 211949282.5 |
| M. hyopneumoniae J | 117704859.5 | 108168346 | 115918546.5 | 51233840.5 | 161479109 | 140931137 | 209744883 | 210332332.5 |
| M. hyopneumoniae J | 116554152 | 104755934.3 | 112647778.5 | 50314014.75 | 153816771.5 | 137133707.8 | 207886060.5 | 205849858.5 |
| M. hyopneumoniae J | 117851248 | 107424756.8 | 114109941.3 | 50547333.25 | 156616207.3 | 139693856.8 | 208479353.8 | 206995233.3 |
| M. hyopneumoniae J | 115500632 | 106479931.3 | 112485320.1 | 53830365.07 | 156779143.8 | 133000332.6 | 224310987.9 | 204855122.5 |
| M. hyopneumoniae J | 277036881.8 | 253314251.4 | 272192079 | 131812708 | 388760867.2 | 323899793.2 | 550372928.8 | 501329844 |
| M. hyopneumoniae J | 120671780.3 | 110687356.8 | 115921559.5 | 55492629.75 | 160446497.3 | 137363713.3 | 226957596.5 | 202824434 |
| M. hyopneumoniae J | 122713266.1 | 116582441.3 | 119636277.8 | 60509388.39 | 168473251.9 | 136091116.9 | 246439146.4 | 215572371.5 |
| M. hyopneumoniae J | 114826234.5 | 108268950.5 | 112679344 | 55066670.5 | 159937044 | 131509625 | 223426520.5 | 211016855 |
| M. hyopneumoniae J | 120586890.5 | 115527479.5 | 117826408.5 | 60175000.36 | 170199408 | 133119674.2 | 243852448.5 | 226645893.3 |
| M. hyopneumoniae J | 122725010.5 | 115027357.5 | 118194437.5 | 61149753 | 168994436.5 | 130397457.5 | 250266934 | 221734562.5 |
| M. hyopneumoniae J | 118210469 | 110546684 | 112944707 | 58589774 | 163091648 | 125205376 | 238497123 | 219011028 |
| M. hyopneumoniae J | 118708835.1 | 112808691.1 | 115499992.6 | 59743542.25 | 167627209.7 | 125783206.3 | 245790174.2 | 225096483.9 |
| M. hyopneumoniae J | 109837133.5 | 102361826.5 | 109613147.5 | 58662993.5 | 161643226.5 | 121393165.5 | 254444610 | 225759196.5 |
| M. hyopneumoniae J | 120359960 | 110947734.5 | 115590141.3 | 51158435.5 | 159095513.5 | 143599142.5 | 212119546.5 | 206729364.8 |
| M. hyopneumoniae J | 103739451 | 92947999 | 102885615.3 | 45879397 | 146733393.3 | 130184710 | 202349789.3 | 199753374.3 |
| M. hyopneumoniae J | 116406042.3 | 104669482 | 111792173.5 | 48803092 | 152290610 | 139542929 | 204169517.5 | 198247631 |
| M. hyopneumoniae J | 112149229.5 | 106418933.7 | 111902979.9 | 48666157.78 | 151286923.7 | 136264701.4 | 202786788.9 | 204249179.1 |
| M. hyorhinis | 120931042 | 109503353.8 | 107765374.8 | 49419802.25 | 152660292 | 147004007.3 | 211562410 | 307512507.3 |
| M. hyorhinis | 119363691.4 | 107165531.1 | 104466163.9 | 48482836.75 | 146562599.1 | 142578854.1 | 209286718.6 | 304484914.8 |
| M. hyorhinis | 108728552.5 | 98024738 | 95921085 | 45000403 | 137971645 | 132913423 | 199276443.5 | 293419826.5 |
| M. hyorhinis | 116728216.8 | 106269400 | 103440649.3 | 47303391.75 | 144255812.3 | 139674836 | 201010147 | 288563363.5 |
| M. hyorhinis | 111507379.3 | 101021945 | 87521815.25 | 46924687.5 | 129975943 | 135109749.3 | 211457495.8 | 471708216.8 |
| M. hyorhinis | 119290705.8 | 111023189.5 | 96037377.25 | 50410337.25 | 142362360.3 | 142683199.5 | 216867352.3 | 478198019.3 |
| M. hyorhinis | 120788416.5 | 112156702.2 | 96343602.29 | 50323585.4 | 139872075.6 | 144453785 | 217009174.5 | 462891261.9 |
| M. hyorhinis | 120576250.5 | 110243674.1 | 94320998.66 | 49856911.92 | 137655705.5 | 140807685.7 | 212734181.4 | 459244801.2 |
| M. hyorhinis | 120586912.3 | 110486887.5 | 92091468.5 | 50132201 | 133650708 | 140814603.8 | 213442828 | 549803547 |
| M. hyorhinis | 123319207.8 | 112895529.3 | 93176429.5 | 51339144 | 137309199.3 | 144240780.8 | 218480501.3 | 572304613.3 |
| M. hyorhinis | 120583005.8 | 110311353.3 | 91727469.5 | 50103043 | 133040882.3 | 140697052.3 | 213168862 | 546608895.8 |
| M. hyorhinis | 122295597.3 | 112894448 | 92185615 | 52169458.75 | 138543102.3 | 144467128 | 233630916.5 | 595087775 |
| M. hyorhinis | 122422191.8 | 112347519 | 81599013.75 | 51452991.25 | 124060867.5 | 141916032.8 | 218409071.8 | 828397556.8 |
| M. hyorhinis | 123192264.3 | 114942239.8 | 83204917.25 | 52143290.25 | 128379533.8 | 141059315 | 220500168 | 819118957.5 |
| M. hyorhinis | 124518660.3 | 114495588 | 82960739.75 | 52542478.75 | 127983220 | 142053629 | 221459271.5 | 815873832 |
| M. hyorhinis | 127072421.3 | 118360547.8 | 86213028.65 | 54333300.09 | 132997217.7 | 146861578.1 | 236783862.6 | 835486601 |
| M. hyorhinis | 121025491.4 | 109482382.3 | 108718771.9 | 49365980.63 | 154148368.8 | 149066625.6 | 212786105.5 | 293872060.1 |
| M. hyorhinis | 117771625.8 | 106318689.8 | 104855789.5 | 47673032.75 | 146311296.8 | 143074944.8 | 205256699.8 | 288463786.3 |
| M. hyorhinis | 121364394.5 | 109543907.5 | 106090709.8 | 49851735 | 147135714 | 147835332.5 | 211861255 | 283556729.5 |
| M. hyorhinis | 118973020.5 | 107627804.3 | 105430128.5 | 47963557 | 147297170 | 143763010.5 | 206318834.8 | 289472998.5 |


| Species | Methionine | Acetate | Alanine | Lactate | Ethanol | Valine | Isoleucine | Valine |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Friss medium | 305592574.9 | 614326247.6 | 988665559.1 | 1144761869 | 344438041.3 | 549408995 | 374597876.4 | 569649727.6 |
| Friss medium | 320507887.5 | 651384008.8 | 1071585556 | 1223305509 | 320549366.8 | 596199023.8 | 400306216.3 | 611646197.8 |
| Friss medium | 327032877.3 | 654495990.3 | 1069481238 | 1239088115 | 334010345.6 | 596155964.3 | 402103813.9 | 616362948.5 |
| M. flocculare | 342590211.8 | 1029203068 | 1174715754 | 1336941597 | 247434475.3 | 641199784.5 | 420438630.8 | 653049402.5 |
| M. flocculare | 335948507.9 | 1019076928 | 1164729555 | 1320080421 | 226918899.8 | 632161272.9 | 413902861.3 | 643568539.9 |
| M. flocculare | 343967735.5 | 1047780998 | 1202660832 | 1348542850 | 230984597.5 | 653279364.5 | 426326404.8 | 668825874.3 |
| M. flocculare | 341671654.8 | 1248450539 | 1209610134 | 1353098493 | 237660594.5 | 653122318.3 | 421031033.3 | 663580977.3 |
| M. flocculare | 351562633.6 | 1308180990 | 1256460713 | 1390855764 | 301258230.3 | 675808377 | 436653412.4 | 688406394.1 |
| M. flocculare | 346860020.1 | 1251068207 | 1228560975 | 1370377420 | 229342496.6 | 664687461.4 | 428343579.6 | 675658089 |
| M. flocculare | 341013154 | 1384120130 | 1219636429 | 1352674563 | 236387836.8 | 659175095.8 | 423065368.5 | 670551249.5 |
| M. flocculare | 343668661.3 | 1459611156 | 1261078077 | 1366726238 | 219654481 | 670886627.8 | 429390423.3 | 684333741 |
| M. flocculare | 343620204 | 1400826156 | 1227896993 | 1361159088 | 241016404 | 661330421.8 | 424018486 | 671960520.3 |
| M. flocculare | 368853947.5 | 2357040566 | 1381072294 | 1642407493 | 293153051.5 | 729282272.5 | 455884373 | 732693001.5 |
| M. flocculare | 377198756 | 1942358172 | 1481048467 | 1441535763 | 192556824.5 | 749280172.5 | 483524428.5 | 761107590.5 |
| M. flocculare | 380404397.3 | 1958513172 | 1495978617 | 1455212259 | 189404937.8 | 756490264.3 | 486467292.3 | 769853211.3 |
| M. flocculare | 354674550 | 922956084.8 | 1337281226 | 1281241829 | 187991095 | 691172338.8 | 452800436.5 | 712766220.3 |
| M. flocculare | 379451496.3 | 994841114 | 1468715055 | 1403935288 | 173222694 | 754403440.5 | 489382765 | 776813572.5 |
| M. flocculare | 381960264.8 | 986095694.8 | 1426846326 | 1369130283 | 204776937.3 | 738216858 | 481455270.3 | 761490471.5 |
| M. hyopneumoniae | 328526608 | 886968185.5 | 1130334684 | 1114565028 | 306468611.5 | 615922940 | 412313890.8 | 632372694.5 |
| M. hyopneumoniae | 306812620.3 | 827853271.8 | 1057916277 | 1038623738 | 279851442.8 | 574724458 | 383858252.3 | 589876359 |
| M. hyopneumoniae | 331136313.3 | 891409738.3 | 1130367961 | 1120227487 | 303146539.3 | 619390395.3 | 411498386.5 | 634165766.8 |
| M. hyopneumoniae | 316694167 | 1251906558 | 1132718085 | 883464835.3 | 216867320.5 | 612889529.5 | 399389581.3 | 621445544 |
| M. hyopneumoniae | 339113200.3 | 1339397711 | 1210644061 | 918897437.3 | 264428234 | 655790534.5 | 424933388.3 | 669691076 |
| M. hyopneumoniae | 324857636.5 | 1283324019 | 1165798572 | 885112706.3 | 240456457.5 | 630933294 | 409001278.8 | 642314036.5 |
| M. hyopneumoniae | 338392627.8 | 1714454174 | 1227139665 | 798110698.8 | 238221287.8 | 664925644.5 | 425507337.5 | 674688402.5 |
| M. hyopneumoniae | 329802372.3 | 1652619387 | 1149199146 | 806442012.5 | 345338107.8 | 644033275.3 | 416977128 | 661281780.8 |
| M. hyopneumoniae | 336950871 | 1716502916 | 1225578429 | 801819526 | 356881206.5 | 664110556 | 424583088.5 | 678834236 |
| M. hyopneumoniae | 322778989 | 2479031797 | 1210328912 | 856406069 | 326784290 | 657990985 | 415156357 | 667393200 |
| M. hyopneumoniae | 344879490.5 | 2908457635 | 1289040388 | 734177358.5 | 250652853 | 697776647.5 | 438084091.5 | 704246089 |
| M. hyopneumoniae | 325966735.8 | 856223769.4 | 1108047938 | 1124579012 | 231696757.4 | 604005259.8 | 405069152.6 | 614586143.5 |
| M. hyopneumoniae | 302306159.4 | 800382672 | 1026715818 | 1043628577 | 229652879.8 | 558806292.4 | 376438689.6 | 573143990.8 |
| M. hyopneumoniae | 327074292.6 | 858222485.6 | 1108012429 | 1117964518 | 234994443.1 | 607109370.9 | 406724611 | 617663968.9 |
| M. hyopneumoniae J | 290589141 | 985767313.3 | 947886106.5 | 1172979000 | 267749987.3 | 528504298.5 | 364980487.8 | 544805219 |
| M. hyopneumoniae J | 288548302 | 961482599.5 | 896537291.5 | 1194021129 | 279500940 | 523174465.5 | 360941677.5 | 535667224 |
| M. hyopneumoniae J | 284674752.5 | 964737227.3 | 931808200.5 | 1156429118 | 264374915 | 522921424.5 | 353427642 | 529526660.8 |
| M. hyopneumoniae J | 285844065 | 950146180 | 927825665 | 1153568067 | 276891996.5 | 521108529.3 | 355149518.5 | 531025153.8 |
| M. hyopneumoniae J | 292664264.8 | 1565518002 | 986418230.9 | 1181034832 | 254957575.8 | 555258218.2 | 367934817.4 | 563751578.3 |
| M. hyopneumoniae J | 728823802.6 | 3876063720 | 2395521057 | 3027663243 | 650517066.4 | 1379694483 | 910190987.9 | 1393796422 |
| M. hyopneumoniae J | 297181972.3 | 1600414587 | 1002761651 | 1206044651 | 269809978.5 | 564117070.3 | 363970573.8 | 565091440 |
| M. hyopneumoniae J | 299379137.4 | 2406571404 | 1031677553 | 1340388334 | 263460857.3 | 580789502.1 | 372660997.7 | 578783656.2 |
| M. hyopneumoniae J | 293485130 | 1782673075 | 984980836 | 1372756415 | 255360983.5 | 558698710.5 | 370229899.5 | 565845381.5 |
| M. hyopneumoniae J | 298359979.8 | 2344026251 | 1028125470 | 1330268658 | 252903679.5 | 581337734.6 | 384460844.3 | 590635088.7 |
| M. hyopneumoniae J | 305295007 | 2711388635 | 1051361394 | 2158439769 | 288589433 | 591371809 | 369584887 | 582780155.5 |
| M. hyopneumoniae J | 287605655 | 2579781806 | 996975995 | 2011289568 | 250536705 | 562120542 | 363046870 | 563531485 |
| M. hyopneumoniae J | 298296746.9 | 2602789594 | 971080274.4 | 2060972526 | 256654107 | 575677044.4 | 375328034.2 | 580611758.4 |
| M. hyopneumoniae J | 305447573.5 | 2688178136 | 1078243858 | 2183570148 | 248974740 | 604460447.5 | 384152193 | 605610589.5 |
| M. hyopneumoniae J | 290000445 | 942431111 | 931830311.8 | 1186002223 | 302881257.8 | 527981511 | 356626274.3 | 533319689.8 |
| M. hyopneumoniae J | 277024008.3 | 896799426 | 853108350 | 1161506572 | 268972829.8 | 496752338 | 346000344.5 | 516647396.8 |
| M. hyopneumoniae J | 279551186.8 | 903267153.3 | 897396787.5 | 1155129303 | 280034808.8 | 503705545.3 | 345197121 | 514052989.8 |
| M. hyopneumoniae J | 273505121.3 | 854582351.7 | 881658596.8 | 1076741290 | 234620075.3 | 506018559.6 | 362268959.2 | 525246212.8 |
| M. hyorhinis | 293237138 | 733495085.8 | 966218254.5 | 1252009120 | 285136405.3 | 526212493 | 361312723.8 | 539411220 |
| M. hyorhinis | 288393702.4 | 727568724.4 | 1002568193 | 1206822861 | 275949129.1 | 523853452 | 357712715.9 | 534340867 |
| M. hyorhinis | 276588877.5 | 689731541.5 | 955658970.5 | 1149718433 | 255528627 | 498555629.5 | 356081119 | 522295216.5 |
| M. hyorhinis | 277605839 | 686795158.8 | 958548942.8 | 1153944614 | 260571582 | 503190883.3 | 348448267.5 | 516368329.8 |
| M. hyorhinis | 288288239.8 | 764446495.5 | 1117219417 | 1247661920 | 259106847.3 | 542932039 | 367288813.5 | 555577634.5 |
| M. hyorhinis | 293490561.3 | 760196551.8 | 1087717419 | 1289285267 | 268517166.5 | 550827303.5 | 371391132.8 | 559784753 |
| M. hyorhinis | 294559679 | 782008920.1 | 1137608870 | 1296459773 | 257800004.6 | 555802886 | 376925870.3 | 566310830 |
| M. hyorhinis | 290819723 | 766949491.2 | 1134499010 | 1289493733 | 254954440.1 | 552021291.5 | 368763852.9 | 556847596.5 |
| M. hyorhinis | 291398366 | 778032901.5 | 1171329639 | 1250487840 | 261553037 | 556355156.3 | 369550698.3 | 561937958 |
| M. hyorhinis | 299315304 | 807715287.3 | 1217630099 | 1325635965 | 268933328.8 | 571412982.3 | 381295924.5 | 577700580.8 |
| M. hyorhinis | 291212037 | 777248776.5 | 1178633399 | 1256944618 | 266772071.5 | 556746553.5 | 367942684.8 | 560484985.5 |
| M. hyorhinis | 314567282.5 | 842718478.3 | 1271209080 | 1395034967 | 285907517.8 | 602724467.8 | 404304767 | 612072751.8 |
| M. hyorhinis | 298867418.3 | 846825040.5 | 1327885409 | 1466425185 | 284853395 | 586403348.5 | 379298782.3 | 587463312 |
| M. hyorhinis | 298058892 | 849781821.8 | 1334309589 | 1540136786 | 259288498.5 | 583748731.5 | 386513255.5 | 588442356.3 |
| M. hyorhinis | 301956422.3 | 854714144.5 | 1349568877 | 1565815413 | 253877723 | 593890543.8 | 386833504.3 | 594053189.3 |
| M. hyorhinis | 314412934.5 | 870261046.9 | 1408507251 | 1545463750 | 268935369.4 | 618578968.4 | 407075695.4 | 623558224.3 |
| M. hyorhinis | 293757310.5 | 720877999.9 | 947769791.9 | 1256546293 | 309191943.8 | 524467759.4 | 360325634.3 | 536604671.3 |
| M. hyorhinis | 283948146.3 | 705804785.3 | 965588146.5 | 1180637913 | 272950428 | 509591053.8 | 351869246.3 | 520013407.8 |
| M. hyorhinis | 291818841.8 | 722728790.5 | 1025954043 | 1214906421 | 262276356.5 | 534036783.8 | 367419805.3 | 545411323.8 |
| M. hyorhinis | 284181342.3 | 700805454.8 | 970413443 | 1186057316 | 281366711.8 | 509934377.8 | 351796474.3 | 521613758.5 |


| Species | Leucine | Isoleucine |
| :---: | :---: | :---: |
| Friss medium | 1435177837 | 763874857.8 |
| Friss medium | 1516746221 | 809793625.3 |
| Friss medium | 1527578382 | 843310043.9 |
| M. flocculare | 1610462386 | 793893390 |
| M. flocculare | 1582255756 | 782415317 |
| M. flocculare | 1623441718 | 796785396 |
| M. flocculare | 1622623831 | 763824691.3 |
| M. flocculare | 1673879082 | 799435162.9 |
| M. flocculare | 1654746374 | 781831042.8 |
| M. flocculare | 1658213523 | 745435070 |
| M. flocculare | 1699554793 | 738781335.3 |
| M. flocculare | 1641609376 | 761381679.8 |
| M. flocculare | 1844925710 | 736249687 |
| M. flocculare | 1871152670 | 682371833 |
| M. flocculare | 1879149004 | 697805492 |
| M. flocculare | 1699289543 | 722888354.8 |
| M. flocculare | 1839095970 | 773372595 |
| M. flocculare | 1788272090 | 790666694.3 |
| M. hyopneumoniae | 1566841931 | 780238971.5 |
| M. hyopneumoniae | 1461274324 | 729918591 |
| M. hyopneumoniae | 1563803094 | 789468422.8 |
| M. hyopneumoniae | 1535373745 | 737175151.5 |
| M. hyopneumoniae | 1647930779 | 781102100.3 |
| M. hyopneumoniae | 1574867275 | 748266273.3 |
| M. hyopneumoniae | 1638952889 | 781364073.8 |
| M. hyopneumoniae | 1608722131 | 733571510.8 |
| M. hyopneumoniae | 1678013335 | 738392321 |
| M. hyopneumoniae | 1664601971 | 683011710 |
| M. hyopneumoniae | 1769240098 | 698488457.5 |
| M. hyopneumoniae | 1539011207 | 804008734.5 |
| M. hyopneumoniae | 1437475253 | 760387414.6 |
| M. hyopneumoniae | 1548778569 | 799972203.1 |
| M. hyopneumoniae J | 1426102499 | 774470765.5 |
| M. hyopneumoniae J | 1407228182 | 746457454.5 |
| M. hyopneumoniae J | 1407638278 | 742732155 |
| M. hyopneumoniae J | 1396781144 | 754109315.8 |
| M. hyopneumoniae J | 1441114962 | 720333578.2 |
| M. hyopneumoniae J | 3648555446 | 1812884649 |
| M. hyopneumoniae J | 1470812572 | 728855843.5 |
| M. hyopneumoniae J | 1515894232 | 693665610.5 |
| M. hyopneumoniae J | 1452008914 | 683076758.5 |
| M. hyopneumoniae J | 1533165086 | 686674277.2 |
| M. hyopneumoniae J | 1564555817 | 660392072 |
| M. hyopneumoniae J | 1502784152 | 635021659 |
| M. hyopneumoniae J | 1498831947 | 639377418.7 |
| M. hyopneumoniae J | 1588372908 | 675621672 |
| M. hyopneumoniae J | 1405530914 | 790376211.5 |
| M. hyopneumoniae J | 1352774425 | 751296957.5 |
| M. hyopneumoniae J | 1367097896 | 778769425 |
| M. hyopneumoniae J | 1313554134 | 705141866 |
| M. hyorhinis | 1427011653 | 786693175.5 |
| M. hyorhinis | 1419772765 | 778937163 |
| M. hyorhinis | 1363853085 | 749531706.5 |
| M. hyorhinis | 1357744502 | 759942009.3 |
| M. hyorhinis | 1401764996 | 748693004.3 |
| M. hyorhinis | 1416904022 | 740830446.5 |
| M. hyorhinis | 1422689819 | 754556417.8 |
| M. hyorhinis | 1431315976 | 735081460.9 |
| M. hyorhinis | 1416628698 | 737198933.3 |
| M. hyorhinis | 1462795309 | 750543522.3 |
| M. hyorhinis | 1418599166 | 736345219.3 |
| M. hyorhinis | 1526550453 | 778544480.8 |
| M. hyorhinis | 1452602644 | 751367035 |
| M. hyorhinis | 1459525666 | 713906622.5 |
| M. hyorhinis | 1486566758 | 729566397.5 |
| M. hyorhinis | 1533543743 | 752966447.1 |
| M. hyorhinis | 1415550506 | 807276125.6 |
| M. hyorhinis | 1384491216 | 782697455.5 |
| M. hyorhinis | 1424872554 | 792847417.5 |
| M. hyorhinis | 1378045896 | 791235521.5 |

Table S7b: Metabolites detected in growth of M. hyopneumoniae, M. hyorhinis and M. flocculare in defined Yus medium.

| Espèce | Temp | Replicate | Acetate | Adenine | Alanine | Arginine | Asparagine | Aspartate | Cystine | Cytidine | Ethanol | Formate | Glucose | Glutamate | Glutamine |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| CTRL- | to | 1 | 0.3 | 0.2 | 3.3 | 1.7 | 4.4 | 3.1 | 1.0 | 0.0 | 33.5 | 0.0 | 42.6 | 4.3 | 1.9 |
| CTRL- | t0 | 2 | 2.3 | 0.2 | 3.3 | 4.0 | 4.8 | 2.8 | 0.9 | 0.1 | 33.6 | 0.2 | 53.6 | 4.3 | 2.1 |
| CTRL- | t1 | 1 | 0.0 | 0.2 | 3.3 | 3.5 | 3.9 | 3.4 | 1.0 | 0.1 | 43.2 | 0.0 | 43.4 | 3.7 | 2.2 |
| CTRL- | t1 | 2 | 1.1 | 0.1 | 3.3 | 3.9 | 3.4 | 2.8 | 1.0 | 0.1 | 36.4 | 0.1 | 40.7 | 3.2 | 1.9 |
| CTRL- | t2 | 1 | 0.0 | 0.2 | 3.0 | 3.2 | 3.2 | 2.7 | 1.0 | 0.1 | 37.5 | 0.0 | 44.5 | 1.5 | 1.4 |
| CTRL- | t2 | 2 | 0.0 | 0.1 | 3.8 | 2.8 | 2.5 | 2.8 | 1.0 | 0.1 | 38.0 | 0.0 | 39.4 | 2.0 | 5.3 |
| CTRL- | t3 | 1 | 0.5 | 0.1 | 3.5 | 3.7 | 3.4 | 2.1 | 0.9 | 0.1 | 30.1 | 0.1 | 43.4 | 2.7 | 2.0 |
| CTRL- | t3 | 2 | 0.0 | 0.2 | 2.9 | 3.7 | 3.0 | 2.2 | 0.9 | 0.1 | 30.7 | 0.0 | 45.7 | 4.0 | 1.7 |
| CTRL- | t4 | 1 | 0.0 | 0.2 | 3.2 | 4.5 | 4.3 | 3.2 | 1.0 | 0.1 | 40.9 | 0.0 | 49.7 | 4.0 | 2.2 |
| CTRL- | t4 | 2 | 2.1 | 0.2 | 3.4 | 3.7 | 3.4 | 2.5 | 0.8 | 0.1 | 36.9 | 0.2 | 43.4 | 3.7 | 1.9 |
| CTRL- | t5 | 1 | 0.3 | 0.2 | 3.3 | 3.6 | 3.2 | 2.5 | 0.9 | 0.1 | 40.5 | 0.0 | 45.5 | 4.0 | 2.2 |
| CTRL- | t5 | 2 | 0.0 | 0.0 | 3.5 | 3.7 | 3.4 | 2.8 | 0.9 | 0.1 | 18.1 | 0.0 | 47.2 | 3.9 | 2.2 |
| CTRL- | t6 | 1 | 0.0 | 0.1 | 2.6 | 3.0 | 3.0 | 1.8 | 0.7 | 0.1 | 31.0 | 0.0 | 33.7 | 2.7 | 1.3 |
| CTRL- | t6 | 2 | 0.2 | 0.2 | 4.0 | 3.5 | 1.9 | 2.1 | 0.8 | 0.0 | 35.3 | 0.1 | 40.4 | 2.9 | 1.5 |
| MFL27716 | t0 | 1 | 1.1 | 0.2 | 3.3 | 3.3 | 3.3 | 2.0 | 0.7 | 0.1 | 30.2 | 0.2 | 36.4 | 3.2 | 1.7 |
| MFL27716 | to | 2 | 2.3 | 0.1 | 3.3 | 3.4 | 2.8 | 1.6 | 0.7 | 0.0 | 21.7 | 0.2 | 41.9 | 3.0 | 1.5 |
| MFL27716 | t1 | 1 | 1.8 | 0.1 | 2.9 | 4.0 | 2.5 | 2.0 | 0.7 | 0.0 | 26.0 | 0.2 | 38.1 | 3.1 | 1.9 |
| MFL27716 | t1 | 2 | 0.0 | 0.2 | 2.8 | 3.0 | 2.3 | 2.0 | 0.9 | 0.0 | 32.1 | 0.1 | 40.6 | 4.2 | 1.2 |
| MFL27716 | t2 | 1 | 0.0 | 0.1 | 2.9 | 2.9 | 2.5 | 1.3 | 1.1 | 0.0 | 32.1 | 0.0 | 40.4 | 3.2 | 1.2 |
| MFL27716 | t2 | 2 | 0.0 | 0.1 | 2.2 | 2.9 | 2.9 | 1.7 | 0.9 | 0.0 | 35.8 | 0.0 | 39.0 | 3.7 | 1.3 |
| MFL27716 | t3 | 2 | 0.0 | 0.2 | 3.2 | 3.4 | 2.4 | 2.6 | 0.9 | 0.1 | 32.8 | 0.0 | 43.5 | 3.0 | 1.4 |
| MFL27716 | t4 | 2 | 0.0 | 0.1 | 3.7 | 3.1 | 2.9 | 1.7 | 1.0 | 0.1 | 39.8 | 0.0 | 40.5 | 5.7 | 2.0 |
| MFL27716 | t4 | 2 | 1.9 | 0.2 | 3.5 | 3.7 | 3.1 | 2.6 | 0.8 | 0.1 | 37.7 | 0.2 | 44.5 | 4.1 | 2.2 |
| MFL27716 | t5 | 2 | 1.1 | 0.2 | 3.4 | 3.7 | 2.2 | 2.5 | 0.8 | 0.1 | 45.2 | 0.1 | 42.6 | 3.4 | 2.2 |
| MFL27716 | t5 | 2 | 2.5 | 0.2 | 3.4 | 3.6 | 3.3 | 2.5 | 0.8 | 0.0 | 32.6 | 0.2 | 44.9 | 4.2 | 1.6 |
| MFL27716 | t6 | 1 | 0.0 | 0.2 | 3.2 | 3.5 | 3.2 | 2.6 | 0.9 | 0.1 | 37.6 | 0.0 | 48.7 | 3.9 | 2.4 |
| MFL27716 | t6 | 2 | 1.7 | 0.1 | 3.5 | 3.6 | 3.1 | 2.5 | 1.0 | 0.1 | 33.2 | 0.2 | 44.4 | 3.5 | 1.8 |
| MHPJ | to | 1 | 3.6 | 0.2 | 3.4 | 1.4 | 1.3 | 1.2 | 0.5 | 0.1 | 15.4 | 0.3 | 7.9 | 4.4 | 0.3 |
| MHPJ | to | 2 | 0.0 | 0.1 | 3.5 | 3.2 | 2.9 | 1.8 | 0.9 | 0.1 | 30.7 | 0.0 | 36.9 | 3.0 | 1.8 |
| MHPJ | t1 | 1 | 0.0 | 0.1 | 3.1 | 3.2 | 3.1 | 1.6 | 0.7 | 0.0 | 31.6 | 0.0 | 43.8 | 2.2 | 1.4 |
| MHPJ | t1 | 2 | 0.0 | 0.1 | 2.7 | 3.1 | 2.8 | 1.6 | 0.9 | 0.1 | 34.0 | 0.0 | 40.6 | 1.9 | 1.3 |
| MHPJ | t2 | 1 | 0.0 | 0.2 | 3.2 | 2.9 | 3.2 | 2.6 | 0.8 | 0.1 | 42.0 | 0.0 | 47.0 | 3.6 | 2.3 |
| MHPJ | t2 | 2 | 0.0 | 0.2 | 3.4 | 3.6 | 2.6 | 2.7 | 0.9 | 0.1 | 14.8 | 0.0 | 47.0 | 3.1 | 2.2 |
| MHPJ | t3 | 1 | 1.7 | 0.2 | 3.9 | 4.4 | 3.9 | 3.0 | 1.0 | 0.1 | 41.1 | 0.3 | 55.2 | 4.8 | 2.0 |
| MHPJ | t3 | 2 | 0.8 | 0.2 | 3.7 | 4.2 | 3.6 | 3.0 | 0.9 | 0.1 | 41.9 | 0.1 | 49.2 | 3.9 | 2.4 |
| MHPJ | t4 | 1 | 0.0 | 0.2 | 3.6 | 4.2 | 3.8 | 2.8 | 1.0 | 0.1 | 37.3 | 0.0 | 50.8 | 4.8 | 2.5 |
| MHPJ | t4 | 2 | 0.0 | 0.2 | 3.6 | 4.2 | 3.6 | 3.3 | 1.0 | 0.1 | 38.2 | 0.1 | 64.6 | 3.4 | 1.7 |
| MHPJ | t5 | 1 | 0.8 | 0.2 | 3.8 | 3.9 | 3.8 | 3.7 | 1.1 | 0.1 | 32.9 | 0.1 | 49.1 | 3.8 | 2.2 |
| MHPJ | t5 | 2 | 0.9 | 0.2 | 3.4 | 4.0 | 3.2 | 2.6 | 0.9 | 0.0 | 31.7 | 0.2 | 48.5 | 2.9 | 2.1 |
| MHPJ | t6 | 1 | 1.6 | 0.2 | 3.5 | 3.5 | 3.0 | 2.7 | 0.9 | 0.0 | 35.8 | 0.2 | 45.7 | 5.4 | 1.8 |
| MHPJ | t6 | 2 | 2.0 | 0.2 | 3.5 | 3.8 | 3.1 | 2.7 | 1.0 | 0.0 | 21.4 | 0.2 | 45.6 | 4.6 | 2.0 |
| MHR17981 | t0 | 1 | 0.0 | 0.2 | 3.1 | 3.6 | 2.9 | 2.8 | 0.9 | 0.1 | 33.8 | 0.0 | 45.3 | 4.7 | 2.0 |
| MHR17981 | t0 | 1 | 2.3 | 0.2 | 3.6 | 4.0 | 3.8 | 2.7 | 0.8 | 0.1 | 38.2 | 0.2 | 45.9 | 4.4 | 1.8 |
| MHR17981 | t1 | 1 | 1.3 | 0.2 | 3.2 | 3.7 | 3.3 | 2.4 | 0.8 | 0.1 | 20.4 | 0.1 | 43.5 | 4.1 | 1.9 |
| MHR17981 | t1 | 2 | 0.8 | 0.1 | 3.0 | 3.7 | 2.9 | 2.8 | 0.9 | 0.1 | 28.2 | 0.1 | 43.6 | 3.3 | 1.3 |
| MHR17981 | t2 | 1 | 1.0 | 0.2 | 3.4 | 4.0 | 3.3 | 2.8 | 0.9 | 0.1 | 30.3 | 0.2 | 45.6 | 4.2 | 2.0 |
| MHR17981 | t2 | 2 | 0.0 | 0.1 | 2.1 | 3.4 | 2.6 | 2.1 | 0.7 | 0.1 | 21.2 | 0.0 | 36.3 | 2.9 | 1.4 |
| MHR17981 | t3 | 1 | 0.0 | 0.1 | 2.6 | 3.5 | 2.6 | 2.5 | 0.8 | 0.0 | 23.8 | 0.0 | 38.7 | 3.5 | 3.8 |
| MHR17981 | t3 | 2 | 0.0 | 0.1 | 2.7 | 3.7 | 2.7 | 2.1 | 0.8 | 0.1 | 25.9 | 0.0 | 41.2 | 3.5 | 1.7 |
| MHR17981 | t4 | 1 | 1.5 | 0.1 | 2.0 | 3.2 | 2.3 | 1.5 | 0.6 | 0.0 | 20.6 | 0.1 | 38.6 | 3.0 | 1.2 |
| MHR17981 | t4 | 2 | 0.0 | 0.2 | 3.2 | 3.6 | 2.8 | 2.4 | 0.8 | 0.0 | 30.0 | 0.0 | 45.5 | 3.1 | 1.5 |
| MHR17981 | t5 | 1 | 1.5 | 0.2 | 3.3 | 3.5 | 2.4 | 2.4 | 0.8 | 0.0 | 35.1 | 0.1 | 39.4 | 4.0 | 1.6 |
| MHR17981 | t5 | 2 | 1.2 | 0.1 | 3.8 | 3.7 | 3.2 | 2.5 | 0.9 | 0.0 | 29.6 | 0.1 | 50.2 | 4.2 | 1.8 |
| MHR17981 | t6 | 1 | 0.6 | 0.1 | 2.1 | 3.6 | 2.8 | 2.3 | 0.9 | 0.1 | 21.2 | 0.1 | 43.9 | 3.0 | 1.6 |
| MHR17981 | t6 | 2 | 0.6 | 0.1 | 2.1 | 3.6 | 2.5 | 1.8 | 0.6 | 0.1 | 15.5 | 0.1 | 40.1 | 3.1 | 2.1 |


| Espèce | Temp | Replicate | Glycerol | Histidine | Hypoxanthine | Isoleucine | Leucine | Lysine | Methionine | Phenylalanine | Proline | Pyroglutamate | Serine |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| CTRL- | t0 | 1 | 486.5 | 3.2 | 0.0 | 3.1 | 3.9 | 3.2 | 7.4 | 1.1 | 4.4 | 1.1 | 0.8 |
| CTRL- | to | 2 | 726.2 | 4.3 | 0.1 | 3.5 | 4.8 | 4.0 | 6.9 | 1.5 | 4.0 | 2.1 | 1.4 |
| CTRL- | t1 | 1 | 433.6 | 4.0 | 0.0 | 2.8 | 3.8 | 3.5 | 6.7 | 1.2 | 3.2 | 1.4 | 0.9 |
| CTRL- | t1 | 2 | 536.9 | 3.7 | 0.1 | 2.9 | 4.1 | 3.7 | 6.0 | 1.6 | 3.3 | 1.7 | 1.0 |
| CTRL- | t2 | 1 | 383.2 | 1.9 | 0.0 | 2.2 | 2.9 | 2.2 | 4.8 | 0.9 | 3.0 | 0.7 | 1.0 |
| CTRL- | t2 | 2 | 524.8 | 3.5 | 0.0 | 3.2 | 3.7 | 3.4 | 4.5 | 1.4 | 3.4 | 0.9 | 1.2 |
| CTRL- | t3 | 1 | 492.4 | 2.3 | 0.1 | 3.2 | 4.4 | 4.2 | 5.2 | 1.0 | 2.7 | 1.5 | 1.0 |
| CTRL- | t3 | 2 | 378.5 | 3.1 | 0.0 | 2.8 | 4.4 | 2.7 | 5.3 | 0.9 | 3.3 | 1.3 | 1.0 |
| CTRL- | t4 | 1 | 410.8 | 2.9 | 0.0 | 2.7 | 0.5 | 4.6 | 6.2 | 1.0 | 2.5 | 1.0 | 1.1 |
| CTRL- | t4 | 2 | 558.0 | 2.8 | 0.1 | 2.8 | 3.8 | 3.7 | 4.5 | 1.3 | 3.2 | 1.2 | 1.4 |
| CTRL- | t5 | 1 | 415.6 | 2.9 | 0.0 | 2.7 | 3.2 | 3.7 | 6.5 | 1.0 | 1.9 | 0.8 | 0.9 |
| CTRL- | t5 | 2 | 445.0 | 3.0 | 0.0 | 2.9 | 3.4 | 3.2 | 6.8 | 1.0 | 2.4 | 1.1 | 1.1 |
| CTRL- | t6 | 1 | 289.4 | 2.4 | 0.0 | 1.8 | 2.9 | 2.9 | 4.5 | 0.8 | 2.4 | 0.9 | 0.7 |
| CTRL- | t6 | 2 | 359.2 | 3.3 | 0.0 | 1.9 | 2.3 | 3.4 | 3.5 | 1.0 | 2.6 | 1.6 | 0.9 |
| MFL27716 | t0 | 1 | 471.9 | 2.6 | 0.1 | 2.0 | 4.2 | 3.6 | 3.8 | 1.1 | 2.7 | 1.0 | 1.0 |
| MFL27716 | to | 2 | 535.5 | 2.3 | 0.1 | 3.5 | 4.2 | 4.1 | 5.1 | 1.1 | 2.5 | 1.6 | 1.1 |
| MFL27716 | t1 | 1 | 521.7 | 2.5 | 0.1 | 2.4 | 2.9 | 2.8 | 4.9 | 1.1 | 3.1 | 2.0 | 1.0 |
| MFL27716 | t1 | 2 | 327.4 | 2.1 | 0.0 | 3.1 | 3.7 | 3.3 | 5.6 | 0.9 | 2.6 | 1.8 | 1.0 |
| MFL27716 | t2 | 1 | 389.9 | 2.3 | 0.0 | 3.0 | 4.0 | 3.2 | 5.6 | 1.0 | 2.7 | 1.4 | 0.9 |
| MFL27716 | t2 | 2 | 290.2 | 2.4 | 0.0 | 1.9 | 3.2 | 2.8 | 4.3 | 0.9 | 2.4 | 0.7 | 0.9 |
| MFL27716 | t3 | 2 | 371.7 | 2.3 | 0.0 | 2.3 | 3.7 | 2.0 | 6.0 | 0.8 | 2.9 | 1.4 | 0.9 |
| MFL27716 | t4 | 2 | 286.0 | 2.1 | 0.0 | 3.4 | 2.6 | 3.1 | 4.3 | 1.2 | 3.2 | 0.6 | 0.7 |
| MFL27716 | t4 | 2 | 589.0 | 2.9 | 0.1 | 2.8 | 3.8 | 3.3 | 4.4 | 1.3 | 3.0 | 1.2 | 1.3 |
| MFL27716 | t5 | 2 | 493.1 | 2.9 | 0.1 | 2.8 | 3.7 | 3.9 | 3.5 | 1.3 | 3.0 | 1.1 | 1.3 |
| MFL27716 | t5 | 2 | 480.6 | 3.1 | 0.1 | 2.8 | 2.0 | 6.1 | 3.3 | 1.3 | 3.1 | 1.3 | 1.3 |
| MFL27716 | t6 | 1 | 408.1 | 2.8 | 0.0 | 2.7 | 3.4 | 3.9 | 6.2 | 1.0 | 2.2 | 0.8 | 1.1 |
| MFL27716 | t6 | 2 | 673.9 | 2.4 | 0.1 | 3.4 | 3.7 | 4.2 | 4.6 | 1.2 | 3.0 | 2.5 | 1.2 |
| MHPJ | to | 1 | 106.7 | 0.7 | 0.3 | 3.2 | 5.7 | 3.4 | 0.9 | 2.1 | 2.6 | 2.0 | 2.2 |
| MHPJ | to | 2 | 359.6 | 2.2 | 0.0 | 2.8 | 3.0 | 2.7 | 4.3 | 0.9 | 2.4 | 1.0 | 0.7 |
| MHPJ | t1 | 1 | 331.0 | 2.3 | 0.0 | 3.4 | 3.0 | 3.0 | 3.6 | 1.0 | 2.3 | 0.9 | 0.8 |
| MHPJ | t1 | 2 | 422.2 | 2.5 | 0.0 | 2.4 | 3.1 | 3.6 | 4.0 | 1.0 | 2.4 | 1.2 | 1.0 |
| MHPJ | t2 | 1 | 423.1 | 2.7 | 0.0 | 2.7 | 3.1 | 3.2 | 6.1 | 1.0 | 2.9 | 0.8 | 0.8 |
| MHPJ | t2 | 2 | 447.2 | 3.0 | 0.0 | 2.9 | 3.3 | 3.3 | 6.6 | 1.1 | 2.9 | 1.0 | 1.1 |
| MHPJ | t3 | 1 | 777.5 | 2.7 | 0.1 | 2.9 | 4.5 | 4.8 | 7.0 | 1.6 | 5.1 | 1.7 | 1.2 |
| MHPJ | t3 | 2 | 544.7 | 3.9 | 0.1 | 3.2 | 4.0 | 4.2 | 6.9 | 1.6 | 2.9 | 1.7 | 1.1 |
| MHPJ | t4 | 1 | 517.5 | 4.3 | 0.0 | 3.6 | 3.3 | 3.8 | 7.8 | 1.4 | 4.4 | 1.3 | 1.1 |
| MHPJ | t4 | 2 | 541.0 | 3.1 | 0.0 | 3.0 | 4.1 | 4.1 | 6.9 | 1.0 | 3.5 | 1.7 | 1.0 |
| MHPJ | t5 | 1 | 664.7 | 3.7 | 0.1 | 3.3 | 4.2 | 4.1 | 6.8 | 1.3 | 3.3 | 2.0 | 1.1 |
| MHPJ | t5 | 2 | 568.8 | 2.9 | 0.1 | 2.8 | 4.3 | 3.6 | 6.6 | 1.2 | 3.3 | 1.0 | 1.1 |
| MHPJ | t6 | 1 | 563.1 | 2.4 | 0.1 | 2.8 | 4.3 | 3.8 | 6.3 | 1.2 | 3.5 | 1.3 | 1.3 |
| MHPJ | t6 | 2 | 585.8 | 3.8 | 0.1 | 3.4 | 4.2 | 3.6 | 4.8 | 1.2 | 3.6 | 1.4 | 1.2 |
| MHR17981 | to | 1 | 425.5 | 3.3 | 0.0 | 3.0 | 3.7 | 3.5 | 6.2 | 1.1 | 3.3 | 1.1 | 0.9 |
| MHR17981 | to | 1 | 614.6 | 3.2 | 0.1 | 3.0 | 4.2 | 3.7 | 5.7 | 1.3 | 3.4 | 1.4 | 1.5 |
| MHR17981 | t1 | 1 | 546.1 | 2.7 | 0.1 | 2.6 | 3.3 | 3.6 | 5.6 | 1.1 | 3.1 | 1.1 | 1.2 |
| MHR17981 | t1 | 2 | 515.7 | 2.5 | 0.1 | 2.7 | 3.6 | 3.6 | 6.0 | 1.1 | 3.0 | 1.7 | 1.0 |
| MHR17981 | t2 | 1 | 603.1 | 2.8 | 0.1 | 3.2 | 4.1 | 4.0 | 5.2 | 1.2 | 3.4 | 2.1 | 1.1 |
| MHR17981 | t2 | 2 | 300.5 | 3.0 | 0.0 | 2.1 | 2.8 | 2.9 | 4.6 | 0.9 | 2.2 | 1.1 | 0.6 |
| MHR17981 | t3 | 1 | 471.8 | 2.4 | 0.0 | 3.1 | 3.1 | 3.3 | 5.6 | 0.9 | 2.7 | 1.5 | 0.8 |
| MHR17981 | t3 | 2 | 376.7 | 2.9 | 0.0 | 2.3 | 3.8 | 3.3 | 6.1 | 1.1 | 2.6 | 1.3 | 0.8 |
| MHR17981 | t4 | 1 | 368.8 | 1.9 | 0.1 | 2.1 | 3.1 | 1.6 | 5.0 | 1.1 | 2.4 | 1.7 | 0.9 |
| MHR17981 | t4 | 2 | 388.0 | 2.5 | 0.0 | 3.4 | 3.2 | 3.2 | 5.8 | 1.1 | 2.8 | 1.5 | 0.7 |
| MHR17981 | t5 | 1 | 465.3 | 2.8 | 0.1 | 2.7 | 3.9 | 3.8 | 5.3 | 1.2 | 2.3 | 1.0 | 1.2 |
| MHR17981 | t5 | 2 | 511.1 | 3.3 | 0.1 | 3.0 | 4.0 | 4.1 | 5.1 | 1.2 | 3.2 | 2.1 | 1.2 |
| MHR17981 | $t 6$ | 1 | 464.4 | 2.2 | 0.1 | 2.2 | 2.9 | 2.3 | 5.2 | 1.1 | 2.3 | 0.6 | 0.8 |
| MHR17981 | t6 | 2 | 438.6 | 3.4 | 0.1 | 2.1 | 2.6 | 2.3 | 5.9 | 1.0 | 3.0 | 1.8 | 0.8 |


| Espèce | Temp | Replicate | Threonine | Trehalose | Tryptophan | Tyrosine | Uracil | Valine |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| CTRL- | to | 1 | 3.6 | 0.0 | 0.4 | 0.5 | 0.2 | 6.7 |
| CTRL- | t0 | 2 | 3.9 | 0.3 | 0.6 | 0.6 | 0.2 | 7.4 |
| CTRL- | t1 | 1 | 3.3 | 0.0 | 0.5 | 0.5 | 0.1 | 6.1 |
| CTRL- | t1 | 2 | 3.2 | 0.3 | 0.5 | 0.6 | 0.2 | 5.1 |
| CTRL- | t2 | 1 | 2.7 | 0.0 | 0.4 | 0.4 | 0.1 | 5.3 |
| CTRL- | t2 | 2 | 3.4 | 0.0 | 0.4 | 0.4 | 0.1 | 5.9 |
| CTRL- | t3 | 1 | 3.6 | 0.2 | 0.3 | 0.6 | 0.1 | 5.5 |
| CTRL- | t3 | 2 | 3.1 | 0.0 | 0.3 | 0.5 | 0.1 | 5.4 |
| CTRL- | t4 | 1 | 3.0 | 0.0 | 0.4 | 0.5 | 0.1 | 5.6 |
| CTRL- | t4 | 2 | 2.9 | 0.3 | 0.4 | 0.6 | 0.2 | 5.6 |
| CTRL- | t5 | 1 | 2.9 | 0.0 | 0.4 | 0.5 | 0.1 | 5.8 |
| CTRL- | t5 | 2 | 3.1 | 0.0 | 0.4 | 0.5 | 0.1 | 6.1 |
| CTRL- | t6 | 1 | 2.0 | 0.0 | 0.3 | 0.4 | 0.1 | 4.3 |
| CTRL- | t6 | 2 | 3.4 | 0.0 | 0.4 | 0.5 | 0.2 | 5.1 |
| MFL27716 | t0 | 1 | 3.9 | 0.3 | 0.4 | 0.6 | 0.1 | 4.6 |
| MFL27716 | to | 2 | 3.5 | 0.2 | 0.3 | 0.6 | 0.2 | 5.9 |
| MFL27716 | t1 | 1 | 3.7 | 0.2 | 0.3 | 0.6 | 0.1 | 5.5 |
| MFL27716 | t1 | 2 | 3.0 | 0.0 | 0.3 | 0.4 | 0.1 | 5.5 |
| MFL27716 | t2 | 1 | 2.8 | 0.0 | 0.3 | 0.5 | 0.1 | 5.4 |
| MFL27716 | t2 | 2 | 2.0 | 0.0 | 0.3 | 0.5 | 0.1 | 3.7 |
| MFL27716 | t3 | 2 | 2.9 | 0.0 | 0.3 | 0.4 | 0.1 | 5.8 |
| MFL27716 | t4 | 2 | 3.2 | 0.0 | 0.3 | 0.4 | 0.1 | 4.6 |
| MFL27716 | t4 | 2 | 3.0 | 0.3 | 0.4 | 0.6 | 0.2 | 5.8 |
| MFL27716 | t5 | 2 | 2.9 | 0.3 | 0.4 | 0.6 | 0.2 | 5.8 |
| MFL27716 | t5 | 2 | 2.9 | 0.3 | 0.3 | 0.6 | 0.2 | 5.4 |
| MFL27716 | t6 | 1 | 2.9 | 0.0 | 0.4 | 0.5 | 0.1 | 5.5 |
| MFL27716 | $t 6$ | 2 | 2.9 | 0.3 | 0.4 | 0.8 | 0.1 | 5.9 |
| MHPJ | t0 | 1 | 1.3 | 2.6 | 0.2 | 0.9 | 0.1 | 2.6 |
| MHPJ | to | 2 | 2.7 | 0.0 | 0.5 | 0.4 | 0.1 | 5.2 |
| MHPJ | t1 | 1 | 3.3 | 0.0 | 0.3 | 0.5 | 0.1 | 5.1 |
| MHPJ | t1 | 2 | 2.8 | 0.0 | 0.3 | 0.5 | 0.1 | 5.0 |
| MHPJ | t2 | 1 | 2.9 | 0.0 | 0.4 | 0.5 | 0.1 | 5.5 |
| MHPJ | t2 | 2 | 3.0 | 0.0 | 0.3 | 0.5 | 0.1 | 6.0 |
| MHPJ | t3 | 1 | 3.8 | 0.3 | 0.6 | 0.7 | 0.2 | 6.9 |
| MHPJ | t3 | 2 | 3.4 | 0.3 | 0.5 | 0.6 | 0.2 | 5.9 |
| MHPJ | t4 | 1 | 3.8 | 0.0 | 0.5 | 0.6 | 0.2 | 7.5 |
| MHPJ | t4 | 2 | 3.5 | 0.0 | 0.6 | 0.5 | 0.2 | 7.0 |
| MHPJ | t5 | 1 | 3.6 | 0.3 | 0.5 | 0.5 | 0.1 | 5.6 |
| MHPJ | t5 | 2 | 3.4 | 0.3 | 0.5 | 0.7 | 0.1 | 6.0 |
| MHPJ | t6 | 1 | 3.8 | 0.3 | 0.4 | 0.6 | 0.2 | 5.9 |
| MHPJ | t6 | 2 | 3.8 | 0.3 | 0.4 | 0.6 | 0.2 | 5.9 |
| MHR17981 | to | 1 | 3.2 | 0.0 | 0.4 | 0.5 | 0.1 | 5.9 |
| MHR17981 | to | 1 | 3.3 | 0.3 | 0.4 | 0.7 | 0.2 | 6.3 |
| MHR17981 | t1 | 1 | 2.9 | 0.3 | 0.4 | 0.6 | 0.1 | 5.4 |
| MHR17981 | t1 | 2 | 2.8 | 0.3 | 0.4 | 0.7 | 0.1 | 4.6 |
| MHR17981 | t2 | 1 | 3.3 | 0.3 | 0.5 | 0.5 | 0.1 | 5.8 |
| MHR17981 | t2 | 2 | 2.0 | 0.0 | 0.5 | 0.5 | 0.1 | 3.7 |
| MHR17981 | t3 | 1 | 2.7 | 0.0 | 0.4 | 0.4 | 0.2 | 4.9 |
| MHR17981 | t3 | 2 | 2.8 | 0.0 | 0.4 | 0.4 | 0.1 | 0.0 |
| MHR17981 | t4 | 1 | 2.7 | 0.2 | 0.4 | 0.5 | 0.2 | 3.8 |
| MHR17981 | t4 | 2 | 2.5 | 0.0 | 0.4 | 0.4 | 0.1 | 5.0 |
| MHR17981 | t5 | 1 | 2.9 | 0.3 | 0.3 | 0.6 | 0.2 | 5.6 |
| MHR17981 | t5 | 2 | 2.9 | 0.3 | 0.4 | 0.5 | 0.2 | 5.9 |
| MHR17981 | t6 | 1 | 2.4 | 0.2 | 0.4 | 0.4 | 0.1 | 4.4 |
| MHR17981 | t6 | 2 | 2.0 | 0.2 | 0.4 | 0.5 | 0.1 | 3.1 |

## Appendix C

## Appendix: Essential Reactions

Table S8: Prediction of essential reactions in the pan-network.

| Reaction ID | Reaction Name | Reaction ID | Reaction Name |
| :---: | :---: | :---: | :---: |
| R002 | 1TRANSKETO-RXN | R200 | RXN-9386 |
| R003 | 2.4.1.46-RXN | R208 | S-ADENMETSYN-RXN |
| R006 | 2.7.7.15-RXN | R209 | SERINE--TRNA-LIGASE-RXN |
| R008 | 2PGADEHYDRAT-RXN | R213 | THREONINE--TRNA-LIGASE-RXN |
| R009 | 2TRANSKETO-RXN | R217 | TRANSALDOL-RXN |
| R018 | 3PGAREARR-RXN | R219 | TRIOSEPISOMERIZATION-RXN |
| R022 | 6.3.5.7-RXN | R220 | TRYPTOPHAN--TRNA-LIGASE-RXN |
| R023 | 6PFRUCTPHOS-RXN | R221 | TYROSINE--TRNA-LIGASE-RXN |
| R032 | ALANINE--TRNA-LIGASE-RXN | R222 | UDPGLUCEPIM-RXN |
| R036 | ARGININE--TRNA-LIGASE-RXN | R223 | UDPKIN-RXN |
| R040 | ASPARTATE--TRNA-LIGASE-RXN | R226 | URACIL-PRIBOSYLTRANS-RXN |
| R042 | BETA-PHOSPHOGLUCOMUTASE-RXN | R227 | URPHOS-RXN |
| R044 | BIOMASS-RXN | R228 | VALINE--TRNA-LIGASE-RXN |
| R048 | CDPDIGLYSYN-RXN | T001 | DIFFUSION-Ammonia |
| R050 | CDPREDUCT-RXN | T004 | DIFFUSION-Oxygen |
| R051 | CHOLINE-KINASE-RXN | T011 | TRANS-Arg-ABC |
| R052 | CMPKI-RXN | T017 | TRANS-Ca-ATPase |
| R053 | CTPSYN-RXN | T019 | TRANS-CI |
| R054 | CYSTEINE--TRNA-LIGASE-RXN | T020 | TRANS-Cobalt |
| R056 | CYTIDEAM2-RXN | T021 | TRANS-Copper-ATPase |
| R058 | DADPKIN-RXN | T022 | TRANS-Cys-ABC |
| R059 | DCDPKIN-RXN | T024 | TRANS-Cytidine-ABC |
| R071 | DGDPKIN-RXN | T027 | TRANS-Fe-ATPase |
| R072 | DIHYDROFOLATEREDUCT-RXN | T036 | TRANS-GIn-ABC |
| R078 | F16ALDOLASE-RXN | T040 | TRANS-Glucose-ABC |
| R079 | FADSYN-RXN | T042 | TRANS-Gly-ABC |
| R081 | FORMATETHFLIG-RXN | T051 | TRANS-His-ABC |
| R084 | GAPOXNPHOSPHN-RXN | T057 | TRANS-K |
| R087 | GLUC1PURIDYLTRANS-RXN | T060 | TRANS-Lauric-Acid |
| R090 | GLURS-RXN | T061 | TRANS-Leu-ABC |
| R092 | GLYCEROL-3-PHOSPHATE-OXIDASE-RXN | T063 | TRANS-Linoleic-Acid |
| R095 | GLYCINE--TRNA-LIGASE-RXN | T070 | TRANS-Met-ABC |
| R096 | GLYOHMETRANS-RXN | T072 | TRANS-Mg-mgtE |
| R100 | HISTIDINE--TRNA-LIGASE-RXN | T073 | TRANS-Mn |
| R107 | ISOLEUCINE--TRNA-LIGASE-RXN | T074 | TRANS-Molybdate-ABC |
| R109 | LEUCINE--TRNA-LIGASE-RXN | T076 | TRANS-Myristic-Acid |
| R111 | LYSINE--TRNA-LIGASE-RXN | T081 | TRANS-Oleic-Acid |
| R116 | METHIONINE--TRNA-LIGASE-RXN | T082 | TRANS-Palmitic-Acid |
| R153 | PEPDEPHOS-RXN | T083 | TRANS-Palmotoleic-Acid |
| R155 | PGPPHOSPHA-RXN | T090 | TRANS-Putrescine-ABC |
| R156 | PHENYLALANINE--TRNA-LIGASE-RXN | T091 | TRANS-Pyridoxal |
| R159 | PHOSPHAGLYPSYN-RXN | T093 | TRANS-Riboflavin |
| R160 | PHOSPHATIDATE-PHOSPHATASE-RXN | T097 | TRANS-Spermidine-ABC |
| R161 | PPENTOMUT-RXN | T099 | TRANS-Stearic-Acid |
| R162 | PROLINE--TRNA-LIGASE-RXN | T102 | TRANS-Sulfate-ABC |
| R165 | PYRIDOXKIN-RXN | T104 | TRANS-Thiamine |
| R170 | RIB5PISOM-RXN | T110 | TRANS-Trp-ABC |
| R171 | RIBOFLAVINKIN-RXN | T114 | TRANS-Uracil-ABC |
| R174 | RIBULP3EPIM-RXN | T115 | TRANS-Val-ABC |
| R177 | RXN-12002 | T117 | TRANS-Zn |
| R178 | RXN-1225 | S001 | SPONTANEOUS-FATTY-ACID-GENERIC-RXN |
| R180 | RXN-12588 | S002 | SPONTANEOUS-H2O-IONIZATION |
| R181 | RXN-1381 | S003 | SPONTANEOUS-RXN-11811 |
| R190 | RXN-1623 | S005 | SPONTANEOUS-RXN-12540 |
| R192 | RXN-5781 | S009 | SYNTHETIC-DRAIN-RXN-12588 |
| R196 | RXN-7904 |  |  |

Appendix D
Appendix: Submitted Paper

# Insights on the virulence of swine respiratory tract mycoplasmas through genome-scale metabolic modeling 

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#### Abstract

The respiratory tract of swines is colonized by several bacteria, among which are three mycoplasma species: Mycoplasma flocculare, Mycoplasma hyopneumoniae, and Mycoplasma hyorhinis. In this work, we created whole-genome metabolic network reconstructions for these three Mycoplasma species and were able to show that they all have similar metabolic capabilities. Differential metabolism includes the myo-inositol catabolism, a more complete system for glycerol uptake in $M$. hyopneumoniae and a wider range of carbohydrate uptake in $M$. hyorhinis; which, in turn may also explain why this species is a widely known contaminant in cell cultures. Glycerol conversion to dihydroxyacetone-phosphate, an activity missing only in M. flocculare, produces toxic hydrogen peroxide and may explain the lack of pathogenicity of this species. Growth experiments showed that defined media described for other Mycoplasma species are not suitable for the growth of these mycoplasmas. Metabolomic data suggest that even though these species are extremely similar from a genomic and metabolic point of views, the different products and reaction rates may be the result of differential expression in each species.


## 1. Introduction

The respiratory tract of swines is colonized by several bacteria, among which are three mycoplasma species: Mycoplasma flocculare, Mycoplasma hyopneumoniae,-and Mycoplasma hyorhinis (Mare and Switzer 1965; Meyling and Friis 1972; Rose, Tully, and Wittler 1979). The genome sizes of mycoplasmas range from 580 kb (Mycoplasma genitalium) to more than $1,358 \mathrm{~kb}$ (Mycoplasma penetrans), representing an important example of genome reduction during the evolutionary process. It is possible that in an initial symbiotic phase, the host provided a broad range of metabolites for these bacteria. This, together with the ability of the bacteria to uptake such compounds, made several activities dispensable for the bacterial life. Over the course of evolution, these bacteria would have lost some of the genes that became unnecessary for life in an environment conditioned by another genome (Andersson and Kurland 1998).
While M. flocculare is usually described as a commensal bacterium (Kobisch and Friis 1996), M. hyopneumoniae and M. hyorhinis are considered pathogenic. Enzootic pneumonia, caused by M. hyopneumoniae, is widespread in pig populations and is considered as a major cause of economic loss in the pig industry (Maes et al. 1996); M. hyorhinis is frequently present in cases of polyserositis and arthritis and $M$. flocculare has high prevalence in swine herds worldwide, but up to date, no disease has been per se associated with this species (Kobisch and Friis 1996). While mycoplasma diseases in swines have been extensively studied, they have not been explored from a mathematical and computational point of view, mostly because the genome sequences of $M$. hyopneumoniae and $M$. flocculare were not available until recently (Vasconcelos et al. 2005; Siqueira et al. 2013). Moreover, although these three species are related genetically, it is not yet clear what causes the specific pathogenicity or lack thereof in each of them.
There is little metabolic experimental data available for the three species, which makes the reconstruction of a reliable metabolic model an extremely time-consuming work. Together with the fact that these mycoplasmas are generally grown in complex media, with high serum concentrations, we needed additional experimental growth data in order to compare the in silico metabolic networks from the three species. With the aid of NMR analyses in both complex and defined media, we were able to identify similarities and differences in the metabolism of $M$. hyopneumoniae, M. hyorhinis and $M$. flocculare, correlate them with the reconstructed networks and discuss some aspects related to the possible consequences for the virulence of these species.
Virulence factors such as antigen surface proteins and lipopolysaccharide capsule have already been well-described in the literature for both M. hyopneumoniae and M. hyorhinis (Tajima et al. 1982; Citti et al. 1997; Djordjevic et al., 2004; Seymour et al. 2012; Tully et al. 2012). What has yet to be better understood is the direct participation of metabolism in the development of the diseases caused by these two species. For instance, although adhesion factors are related to pathogenicity, M. hyopneumoniae and M. flocculare harbor similar sets of adhesion proteins (Siqueira et al. 2014), and have been shown to adhere to cilia in a similar way (Young et al. 2000). Thus, the inability of $M$. flocculare to cause disease may arise from some kind of cytotoxicity absent in this species.
Indeed, in the present work, we show that one of the mechanisms that may explain why $M$. hyorhinis and $M$. hyopneumoniae are pathogenic while M. flocculare is not, is their ability to use glycerol as a carbon source, thus enabling the production of the highly toxic hydrogen peroxide. This trait may be directly involved in cytotoxicity, as already described for two lung pathogenic mycoplasmas, namely Mycoplasma pneumoniae in human (Hames et al. 2009) and Mycoplasma mycoides subsp. mycoides in ruminants (Vilei et al. 2001).
Additionally, the reconstructed networks show that $M$. hyorhinis and $M$. hyopneumoniae have extra sets of enzymes that may help them overgrow the non-pathogenic species $M$. flocculare. M. hyorhinis seems to have a wider carbohydrate uptake range, and probably for this reason is seen as a well-known contaminant of cell cultures (Nikfarjam and Farzaneh 2012). M. hyopneumoniae, on the other hand, might use myo-inositol as a carbon source producing acetyl-coenzyme-A as a byproduct. This feature is of particular interest for the production of cofactor Coenzyme-A in this species, since most enzymes from the biosynthetic pathway are missing in all species. Growth rates and fitness have been correlated in the past to virulence in several other organisms (Gulig et al 1993; Paisley et al. 2005; Amarsaikhan et al. 2014) and may be a key factor for the difference in pathogenicity among these three mycoplasmas. Metabolomics experiments, in turn, corroborated with the reconstructed models and suggested two new features in particular: (i) the uptake of myo-inositol in $M$. hyopneumoniae might be related to a higher acetate production, and (ii) $M$. hyorhinis showed a surprisingly reduced ability to convert pyruvate to acetate in the growth conditions used in this study. All these in silico and in vivo metabolic differences might influence the different levels of pathogenicity in each of the species studied here.
We present in this work the metabolic networks reconstructed from the annotated genomes of the three species and the comparison done with growth rates and metabolomic experiments performed in vitro in order to better understand the basis of the pathologies caused by these bacteria which might help prevent their development in the future.

## 2. Materials and Methods

## Modeling Methods

## Network Reconstruction and Refinement

We reconstructed the metabolic networks of 6 strains of $M$. hyopneumoniae (pathogenics 232, 7422, 7448, 168 and nonpathogenics $J$ and 168L), 4 strains of $M$. hyorhinis (HUB-1, GDL-1, SK-76 and ATCC 17981 strain BTS7) and 2 strains of $M$. flocculare (ATCC 27716 and ATCC 27399). The semi-automated reconstructions were generated by the Pathologic tool from the Pathway Tools software (Karp, Paley, and Romero 2002) using the complete genomes available online. From now on, the species strains will be abbreviated according to Table 1.

Table 1. Selected species and strains, abbreviation, NCBI accession numbers and experimental availability of strains.

| Species | Strain | Accession Number | Abbreviation | Pathogenicity Level | Experimental Availability ${ }^{* 1}$ | Reference |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Mycoplasma hyopneumoniae | 168 | NC_017509 | MHP168 | Pathogenic | Not available | Liu et al., 2011 |
|  | 168L | CP003131 | MHP168L | Attenuated | Not available | Liu et al., 2013 |
|  | 232 | AE017332 | MHP232 | Pathogenic | Not available | Minion et al., 2004 |
|  | 7422 | NC_021831 | MHP7422 | Pathogenic | Available in Brazil | Siqueira et al., 2013 |
|  | 7448 | AE017244 | MHP7448 | Pathogenic | Available in Brazil | Vasconcelos et al., 2005 |
|  | J, ATCC 25934 | AE017243 | MHPJ | Attenuated | Available in Brazil and France | Vasconcelos et al., 2005 |
|  | All strains | NA | MHP | NA | NA | NA |
| Mycoplasma hyorhinis | HUB-1 | NC_014448 | MHRHUB1 | Pathogenic | Not available | Liu et al., 2010 |
|  | GDL-1 | NC_016829 | MHRGDL1 | $N A^{* 2}$ | Not available | Calcutt et al., 2012 |
|  | SK76 | NC_019552 | MHRSK76 | Pathogenic | Not available | Goodison et al., 2013 |
|  | ATCC 17981 | ARTL00000000 | MHR17981 | Attenuated | Available in Brazil and France | Kyrpides et al., 2013 ${ }^{*} 3$ |
|  | All strains | NA | MHR | NA | NA | NA |
| Mycoplasma flocculare | ATCC 27399 | CP007585 | MFL27399 | Commensal | Available in France | Calcutt et al., 2015 |
|  | ATCC 27716 | AFCG01000000 | MFL27716 | Commensal | Available in Brazil | Siqueira et al., 2013 |
|  | All strains | NA | MFL | NA | NA | NA |
| All species | All strains | NA | Pan-Network | NA | NA | NA |

Notes:
NA : Not available
*1: Strains available only in Brazil are protected strains which are property of the government and cannot be used outside authorised laboratories.

Strains available in France were purchased from the ATCC repository; these strains were also available for testing in the laboratory in Brazil.
*2: M. hyorhinis strain GDL-1 was retrieved from a contaminated cell line and, to our knowledge it has never been reported either as pathogenic or as an attenuated strain.
*3: The genome of M. hyorhinis strain ATCC 17981 genome is available online but not published in any paper up to now. (source: http://genomeportal.jgi.doe.gov/Mychy1/Mychy1.info.html).

Pathway Tools automatically associated genes with reactions, based on the annotation names, Gene Ontology (GO) terms and enzyme code (EC) numbers contained in the GenBank files. The software assembled reactions into pathways by comparing them with the reference database, MetaCyc. The software automatically added missing reactions from the reference database to the model, creating many orphan reactions (reactions that do not have an enzyme associated). The Pathway Hole Filler tool, included in the Pathway Tools software, attempted to identify genes associated with these orphan reactions. After this initial draft reconstruction, we deleted the remaining orphan reactions.
In our work, the refinement of the networks was made in a subsystems approach (Overbeek et al. 2005) simultaneously on all organisms. As a result of different genomic annotations, slightly different reactions arose throughout the automatic models. These reactions were checked for consistency, and after validation of only one of them, the duplicates were deleted. Gene-ProteinReaction (GPR) associations were systematically validated or included based on experimental data, a synteny analysis, the Pfam functional domain database (Bateman et al. 2004) and reciprocal sequence homology (BLASTp) searches, using E-value cutoffs of at least 10-5 (Altschul et al. 1990). Homologous proteins with equivalent functional domains were assigned as isozymes to a particular reaction, while proteins with distinct functional domains were assigned as subunits of a multi-protein complex.
Non-metabolic reactions such as DNA polymerization, protein synthesis and RNA synthesis were explicitly deleted from the network but implicitly included in the biomass assembly. Generic reactions were either specified or excluded from the models. The resulting models were further refined in accordance with a detailed protocol from Palsson and Thiele (Thiele and Palsson 2010). Reactions were computationally balanced for mass and charge, while cofactor usage was determined based on literature data for the studied species or for closely related ones. Reaction directionality was thermodynamically checked and validated. Reaction directionality was determined based on the component contribution method (Noor et al. 2012; Noor and Haraldsdóttir 2013), which extends the group contribution method (Jankowski et al. 2008) and achieves a significant improvement in the accuracy of the estimations of standard Gibbs energies. An online search and calculation interface called eQuilibrator along with metabolite and reaction thermodynamic databases are available at www.equilibrator.weizmann.ac.il (Flamholz et al. 2012). Heuristic rules were used to improve the directionality assignment.
Gaps in the metabolic network were filled only to allow known functionalities of the organisms or biomass production in the reconstructed models. We incorporated the minimal number of gap filling reactions into the models to avoid adding a behavior that is not described. We analyzed the network topology to identify compounds that were only produced or consumed in the network, the so-called dead-end metabolites (DEM). Whenever a DEM was found, we either (i) added a gap filling reaction to reconnect it to the rest of the network, or (ii) removed the reaction from the model when both substrate(s) and product(s) were disconnected from the network and did not affect the overall metabolism. Transporters were predicted at first from genome annotation using the

Transport Identification Parser, from Pathway Tools (Lee, Paulsen and Karp 2008). Transporters were predicted mainly based on sequence homology to other species, since experimental data for the three mycoplasmas were not available. Specific transport reactions and exchange reactions required for production of biomass components were manually added to the final version of the networks.

## Biomass Composition and Biomass Equation

In order to simulate growth, we had first to estimate the average cell composition of these mycoplasmas (biomass composition). To this end, we used a general macromolecule mycoplasma cell composition from Razin and collaborators (Razin, Argaman and Avigan 1963). Depending on the species, the authors showed that the solid residue contained $54-62 \%$ of proteins, $12-20 \%$ of lipids, $3-8 \%$ of carbohydrates, $8-17 \%$ of RNA, and $4-7 \%$ of DNA. The membranes comprised around $35 \%$ of dry weight of the organisms and contained $47-60 \%$ of proteins, $35-37 \%$ lipids, $4-7 \%$ carbohydrates and small amounts of DNA and RNA (Razin, Argaman, and Avigan 1963). Since no information on metal ions and cofactors was available, we included them quantitatively based on the metabolic networks reconstructed for related mycoplasmas (Wodke et al. 2013; Suthers, Zomorrodi, and Maranas 2009). Membrane and lipid components were added based on literature composition of the selected species (Chen et al. 1992; Maniloff 1992; Kornspan and Rottem 2012). Based on the previous information, we assumed the following fractions of macromolecules: 55\% proteins, $15 \%$ lipids, $6.88 \%$ carbohydrates, $12 \%$ RNA, $6 \%$ DNA, and $5.12 \%$ of ions and cofactors. To create a biomass elemental formula, we took into account the percentage contribution of each of the components to the overall cell. The biomass reaction represented the drain of these components into biomass production. It implicitly assembled DNA replication, RNA transcription, and protein synthesis into one single reaction. Amino acids were indirectly included in the biomass reaction: charged tRNAs were accounted as substrates and uncharged tRNAs as products. Growth and non-growth associated maintenance (GAM and NGAM) were estimated based on the literature and were manually added to the models (Feist et al. 2007; Neidhardt 1987).

## Model Validation and FBA Analysis

The metabolic networks were exported as a mathematical Systems Biology Markup Language (SBML) model (Hucka et al. 2003). They were uploaded to the COBRA-toolbox v2.0 Matlab extension (Schellenberger et al. 2011) for Flux Balance Analysis (FBA) testing. Minimum-maximum flux constraints were imposed based on literature information (Wodke et al. 2013). Growth simulations were achieved using biomass production as the FBA objective function.

## Experimental Methods

## Swine mycoplasmas cultivation

Complex media comprised (i) Friis media (Friis 1975) (available in Brazil), and (ii) a commercial mycoplasma
broth (provided by Indicia Biotechnology, available in France ). Defined media cultivation tests were performed in France and comprised (i) a medium described for M. pneumoniae strain 129 by Yus and collaborators (2009), and (ii) commercial media CMRL with no glutathione (Invitrogen). Since we had no information on metabolism of swine mycoplasmas, we decided to supplement the defined Yus medium with all amino acids (including aspartate and glutamate). We also supplemented the CMRL-1066 medium with peptone and/or other cofactors (Table S1 - Supplementary information). Cells were cultivated at 37 _C for different time periods, under gentle agitation ( 100 rpm ).

## Cell concentration estimation and viability by color changing units (CCU) measurement

Cell growth and viability was measured with triplicate time-matched samples of cells and culture media for CCU as described by Stemke and Robertson (1990). Viability of cells is visible by a change in medium color from red to yellow. For cell concentration measurements, the cultures were subjected to a series of 10 -fold dilutions in complex media and $1 \mathrm{CCU} / \mathrm{mL}$ was defined as the highest dilution of cells able to change color media (Poveda 1998).

## Samples for NMR spectroscopy

NMR was performed with complex Friis media (with strains MHP7448, MHPJ, MHR17981 and MFL27716) and defined Yus media (with strains MHPJ, MHR17981 and MFL27716). The medium was collected at the following time intervals: 0h, 8h, 10h, 24h, 32h, and 48h for Friis media and: 0h, 8h, 24h, 32h, 48h, 56h and 72h for defined Yus media. Cells were separated from growth media through sedimentation at 3360 g for NMR analysis. Samples consisted of biological triplicates in complex medium and biological duplicates in defined medium.
NMR Analysis
Sample preparation was as follows: $60 \mu \mathrm{~L}$ of a mixture containing 1.25 M KHPO phosphate buffer ( $\mathrm{pH}=7,4$ ) in D 2 O with 2 mM NaN and $0.1 \%$ trimethylsilyl propionate (TMSP) was added to $540 \mu \mathrm{~L}$ supernatant samples. Both solutions were mixed thoroughly and $550 \mu \mathrm{~L}$ were then transferred to 5 mm NMR tubes and sorted in 96 -tubes racks.
All NMR experiments were carried out on a Bruker 800 MHz NMR spectrometer equipped with a 5 mm TXI probe and a SampleJet autosampler, enabling high-throughput data acquisition for large collections of samples. The temperature was controlled at $27^{\circ} \mathrm{C}$ throughout the experiments, and the samples were kept refrigerated at $4^{\circ} \mathrm{C}$ during a waiting time of less than 24 h in the autosampler, before the NMR analysis. Standard H 1D NMR pulse sequence nuclear Overhauser effect spectroscopy (NOESY) with z-gradient and Carr-Purcell-Meiboom-Gill (CPMG) with water presaturation (Bruker pulse program noesygppr1d and cpmgpr1d) were applied on each sample to obtain the corresponding metabolic profiles. A total of 128 transient free induction decays (FID) were collected for each experiment with a spectral width of 20 ppm . The relaxation delay was set to 4 s . The NOESY mixing time was set to 10 ms . The total acquisition time of each sample was 12 min 34 sec . The CPMG spin-echo delay was set to 300 ms , for a
total filter of 77 ms , allowing an efficient attenuation of the lipid and protein NMR signals. The $90^{\circ}$ pulse length was automatically calibrated for each sample at around $10 \mu \mathrm{~s}$.
Data processing: All FIDs were multiplied by an exponential function corresponding to a 0.3 Hz line-broadening factor, prior Fourier transformation. H-NMR spectra were manually phased and referenced to the TSP signal ( $\delta=-0.016 \mathrm{ppm}$ at pH 7.4 ) using Topspin 3.1 (Bruker GmbH, Rheinstetten, Germany). Extraction of a data matrix for multivariate statistical analysis from the H NMR profiles was done using the Statistics toolbox of AMIX (Bruker Biospin). Spectra were integrated from 0.3 to 10 ppm at a step of 0.01 ppm but excluding the regions of residual water at $4.68-4.88 \mathrm{ppm}$. No normalization of the intensity was performed. The resulting data matrix contains 947 NMR variables.
Multivariate data analysis: Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were performed using SIMCA-P 13 (Umetrics, Umea, Sweden) with scaling based on the Pareto method.
Metabolites identification and quantification: Metabolite identification was achieved by comparing spectra with databases such as HMDB. Identification of the metabolites was further verified with homonuclear and heteronuclear 2D NMR experiments such as H${ }^{2} \mathrm{C}$ HSQC, $\mathrm{H}-\mathrm{H}$ TOCSY and J-resolved experiments. Absolute quantification of the metabolites was performed using Chenomx NMR Suite (Chenomx Inc., Edmonton, Canada).

## 3. Results

## Model Reconstruction and Refinement

Based on the published genomes of 6 strains of $M$. hyopneumoniae, 4 strains of $M$. hyorhinis and 2 strains of $M$. flocculare, we reconstructed 16 genome-scale metabolic models: one model for each strain separately, one for each species and a panreconstruction for all three species (Table 1). The semi-automated reconstructions were manually refined according to the description given in the Material and Methods Section.
The Pan-Swine Mycoplasma network (representing the merge of all strains, and called pan-network) was initially composed by 829 reactions, of which 269 consisted of orphan enzymes even after the Pathway Hole Filler step. These remaining orphan reactions were excluded from the models. Duplicate reactions arose from the fact that Pathway Tools is based only on the annotations contained in the GenBank files. We removed generic and duplicate reactions from the models and replaced them with the specific and validated ones. Non-metabolic reactions were also excluded at this point, along with absent capabilities of these mycoplasmas, such as heme, quinone or cytochrome dependent reactions (Razin, Yogev and Naot 1998). The networks were also tested for the presence of dead-end metabolites (DEM) and the ability to produce all biomass precursors. DEMs were analyzed on a case-by-case manner. From initially 157 DEMs, 124 disconnected metabolites (along with 58 reactions) were excluded from the models: 7 came from spontaneous reactions; 59 were carbohydrate substrates derived from wide range transport reactions and were not used by any other reaction in the network; 58 were excluded as they did not interfere with the overall metabolism and the enzyme had already been assigned to one or many other reactions. It is interesting to point out that other unknown enzymes (or even moonlight enzymes) from these organisms may indeed use some of the excluded DEMs; however, since we have no experimental evidence at present, we could not assess their interference in the metabolic models.
The remaining DEMs consisted mainly of cofactors and biomass precursors (such as nucleotides, amino acids, fatty acids) disconnected from the rest of the network. They were solved along with the biomass precursor check: 141 reactions were added to allow growth: the biomass reaction, 32 enzymatic reactions, 1 drain-synthetic reaction, 105 transport reactions and 5 spontaneous reactions.Transport reactions were considered as such even if an enzymatic activity was also present (i.e. the import of sugars with a concomitant phosphorylation of substrate). In order to correctly assign possible and specific transporters, we performed an extensive literature search and reciprocal blast alignments to characterized transporters in other species. These results have to be experimentally confirmed, since assignment of the correct substrate based only on sequence homology remains an open problem in genomic annotation.
We also changed inconsistencies of reversibility and cofactor usage. Even after all efforts, four DEMs still remained: TTP, hexulose6 -phosphate, deoxyinosine and xanthosine-5-phosphate. After the final addition of 101 exchange reactions, we ran FBA tests to check the consistency of all models. If a reaction was essential for biomass growth and no homolog gene was found in the genome, an orphan reaction was added to the reconstruction for modeling reasons only. The resulting refined models had a total of 457 reactions and 258 GPR associations (depending on the model). A comparison between the species models may be found in Figure 1. These results indicate that all strains from all species are indeed metabolically similar.


Figure 1. Venn diagrams representing the comparison of semi-refined networks (A) between species, and (B) between strains of $M$. hyopneumoniae. This analysis shows that most of the metabolism is common to all organisms. MHR: M. hyorhinis; MHP: M. hyopneumoniae; MFL: M. flocculare.

The overall characteristics of each reconstruction can be seen in Table 2. A list of all reactions and metabolites added to the models along with the corresponding genes can be seen in Table S2 (a and $\underline{b}$, Supplementary information).

Table 2: Characteristics of reconstructed models from different strains and species.

| Model |  | Genes |  | Reactions |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Organism | Name | Total | GPR*1 | Enzymatic*2 | Transport ${ }^{*} 2$ | Spontaneous *3 | Total ${ }^{4}$ |
| MHP232 | iMF170 | 170 | 233 | 208 | 111 | 9 | 426 |
| MHP168 | iMF172a | 172 | 234 | 209 |  |  | 427 |
| MHP168L | iMF172b | 172 |  |  |  |  |  |
| MHP7422 | iMF168 | 168 |  |  |  |  |  |
| MHP7448 | iMF171 | 171 |  |  |  |  |  |
| MHPJ | iMF172c | 172 |  |  |  |  |  |
| MHP | iMFmhp | $N A^{* 5}$ |  |  |  |  |  |
| MHRHUB1 | iMF177 | 177 | 239 | 209 | 111 | 6 | 423 |
| MHRGDL 1 | iMF175 | 175 | 242 | 210 | 113 |  | 426 |
| MHRSK76 | iMF181 | 181 | 243 | 211 |  |  | 427 |
| MHR17981 | iMF182 | 182 |  |  |  |  |  |
| MHR | iMFmhr | $N A^{* 5}$ |  |  |  |  |  |
| MFL27399 | iMF159 | 159 | 217 | 196 | 105 | 9 | 401 |
| MFL27716 | iMF157 | 157 |  |  |  |  |  |
| MFL | iMFmfl | $N A^{*} 5$ |  |  |  |  |  |
| Pan-Network | iMFpan | $N A^{* 5}$ | 258 | 230 | 117 | 9 | 457 |

*1: GPRs are gene-proteins-reaction associations present in each genome. For iMFmhp, iMFmhr, iMFmfl and iMFpan that account for more than one species, we added a GPR to the species model when any of the strains harbored a gene responsible for a specific activity.
*2: Reactions were considered as transport reactions even if the transporter was capable of performing a concomitant enzymatic activity.
*3: Spontaneous reactions included diffusion of small molecules and spontaneous conversions.
*4: Total number of reactions included also all exchange reactions needed for the mathematical modeling.
*5: Genes in the pan-network and species models were added synthetically (one per reaction), to enable a reaction essentiality analysis.

## Biomass Composition and Biomass Equation

The biomass equation drained all precursors (in their molar biological ratios) into biomass. Biomass composition according to the Material and Methods Section is found in Table S3 (Supplementary information). A detailed description of the assembly of the biomass reaction along with the biomass components can also be found in Text S1 (Supplementary information). Considering the percentage contribution of each of the components to the overall cell, an approximate biomass elementary composition for $M$.
hyorhinis was computed as follows: CH 1.57 O 0.36 N 0.21 P 0.02 S 0.02 , with traces of calcium, chlorum, cobalt, copper, iron, potassium, magnesium, manganese, molibdenium and zync and molecular weight (MW) on a C-mole basis of $23.89 \mathrm{~g} / \mathrm{C}-\mathrm{mol}$. We had a slightly different composition for $M$. hyopneumoniae and $M$. flocculare: CH1.59O0.34N0.21P0.16S0.02, with traces of calcium, chlorum, cobalt, copper, iron, potassium, magnesium, manganese, molibdenium and zync and MW on a C-mole basis of $23.31 \mathrm{~g} / \mathrm{C}-\mathrm{mol}$.

## Metabolism of M. hyorhinis, M. hyopneumoniae and M. flocculare

Similar to most mycoplasma species studied so far (Suthers, Zomorrodi, and Maranas 2009; Yus et al. 2009; Wodke et al. 2013), all reconstructed networks exhibit low connectivity due to the simplicity of the biological model. Out of the 457 reactions in the final pan-network, 258 had in at least one species a GPR association. From these, 212 were common to all species (Figure 1). The overall metabolism from the models reconstructed consisted of 11 distinct subsystems: amino sugar metabolism, amino acid metabolism, carbohydrate metabolism (further broken down into: glycolysis, pentose phosphate pathway, ascorbate degradation, myo-inositol degradation, general carbohydrate metabolism, and pyruvate metabolism), cofactor metabolism, lipid metabolism, and nucleotide metabolism (Figure 2 for iMFpan; GPR associations for each species can be seen in Table S4a, Supplementary information). While all enzymes were present in glycolysis, most metabolic pathways had major enzyme gaps (Figure S1, Supplementary information). In the product and cofactor metabolism, for instance, gaps accounted for up to $50 \%$ of the reactions. M. flocculare was the only species that did not show any exclusive metabolic activities in the models. Mycoplasma hyopneumoniae had 10 exclusive reactions, linked to the myo-inositol metabolism and alcohol dehydrogenase activity.M. hyorhinis had 24 exclusive reactions; most of them correspond to the carbohydrate metabolism (Table S4b, Supplementary information). A global model comparing each species enzymatic capabilities can be seen in Figure 3.


Figure 2. Distribution of the model reactions in the subsystems. The 457 reactions present in the model iMFpan were separated into (A) biological subsystems and (B) further into reaction types, with the exclusion of exchange reactions in this analysis.

Figure 3. (Next Pages) Global energy and carbohydrate model showing the enzymatic activities for $M$. hyorhinis, M. hyopneumoniae and $M$. flocculare. For zoomed images, see Figure S2 (a-h, Supplementary information).

Figure 4. (Next Pages) Lipid, amino acid, nucleotide and cofactor metabolism of M. hyorhinis, M. hyopneumoniae and M. flocculare. For zoomed images, see Figure S2 (j-r, Supplementary information)

## Transporters

Transporter assignment was made in silico based on sequence homology to known transporters. We were able to identify in all species complete putative $A B C$ transporters for multidrug/toxin efflux (ABC-Detox1 and ABC-Detox2) and for import of sugars (ABC-Sga, ABC-Fru), oligopeptides (ABC-Opp1, ABC-Opp2), peptides (ABC-Pep), spermidine/putrescine (ABC-Spd/Put), phosphonate/phosphate/thiamine (ABC-Pi), cobalt (ABC-Co), manganese/zinc (ABC-Mn/Zn) and glycerol (ABC-Gly). In the search of a possible nucleotide transporter, we came across with an unpublished work from Nakhyung (Nakhyung 2009) in which a system previously annotated as sugar ABC transport was experimentally validated as involved in nucleoside uptake in Mycoplasma bovis. These results may be highly speculative, and need further verification, but we were able to find homolog genes in the genomes of all species (ABC-Nucl). M. hyopneumoniae seems to have an extra ABC transporter proposed for myo-inositol (ABC-Myo) and M. hyorhinis has a unique ABC transporter for maltose/maltodextrins (ABC-Mal). Three complete phospho-transferase transport systems (PTS) were common to all species. Although specificity of PTS is difficult to ascertain, based on an in silico prediction from domain retrieval, we propose the following: one non-specific for sugar (PTS-Sga), one with specificity for fructose (PTS-Fru) and another for mannitol (PTS-MtI). Mycoplasma hyopneumoniae and $M$. flocculare also share two extra complete systems: one possibly for Ascorbate (PTS-Asc) and one for N -acetylglucosamine (PTS-GNAc). M. hyorhinis and M. hyopneumoniae seem to have an extra IIB component specific for glucose, and along with the other components of PTS-Sga were proposed to form a complete glucose PTS (PTS-Glc). The protein glpU, coded by gene MPN241 in M. pneumoniae, was recently described to act on the uptake of glycerophosphodiesters (Grosshennig et al. 2013). We found homologs to this gene in all strains of $M$. hyopneumoniae and $M$. hyorhinis adjacent to the gene coding for the enzyme responsible for the metabolization of these substrates (GlpQ) (Schmidl and Otto 2011). In M. hyorhinis strain HUB-1, this gene was annotated as a pseudo gene. All species also possess several unspecific amino acid permeases (Aa-F), a glycerol facilitator protein (Gly-F, which is less efficient than ABC-Gly (Hames et al. 2009)) and a major facilitator protein with unknown specificity (MIF). We could also find common transporters for cobalt and magnesium (CorC), chromate (Trans-Chr), magnesium (Trans-Mg), potassium (Trans-K), zinc (Trans-Zn) and a cation ATPase (Trans-Cation). M. hyorhinis has two extra transport systems: one for Sialic Acid (also known as N-Acetyl Neuraminate, Trans-SA) and one sodium phosphate co-transporter (Trans-Na/Pi). Details of the transport search throughout all species is summarized in Text S2 (Supplementary information).

## Carbohydrate and Energy Metabolism

Glycolysis: As previously described (Siqueira et al. 2013), all genes for the glycolysis exist in all strains of all species. M. hyorhinis may be able to convert dihydroxyacetone (DHA) directly to dihydroxyacetone-phosphate (DHAP) with the action of DHA kinase (2.7.1.29), but the mechanism of DHA uptake is not clear.
Pentose Phosphate: In agreement with previously described literature (Vasconcelos et al. 2005; Arraes 2007; Siqueira et al. 2013), we found no enzymes for the oxidative branch of the pentose phosphate pathway in the studied species, turning ribulose-5phosphate into a DEM. This problem was overcome by the assumption that the reaction ribulose-phosphate-3-epimerase (EC 5.1.3.1) can be reversible, as reported in Lactobacillus casei (Bourand et al. 2013). Out of the 11 reactions present in the final models, we found homologs for 9 in all species. Although a gene coding for transaldolase (EC 2.2.1.2) is missing in all species, we included this reaction in the model to prevent both sedoheptulose-7-phosphate and erithrose-4-phosphate from becoming DEMs. Ascorbate Metabolism:
The ascorbate metabolism alone could feed the pentose phosphate pathway successfully. Only one enzyme is missing from the typical pathwyay: L-ascorbate-6-phosphate-lactonase (ulaG). This enzyme is responsible for the turnover of L-ascorbate-6phosphate (A6P) to 3-keto-gulonate-6-phosphate (3KG6P, EC 3.1.1.-). From the gene context, MHP7448_0377 from the M. hyopneumoniae strain 7448 and its homologs from all other organisms may encode the missing enzyme. The gene was annotated as a conserved hypothetical protein with a proposed phosphotriesterase activity.
Uptake of other carbohydrates: While the uptake of fructose, mannitol and mannose is similar in all species and they all can be fed into glycolysis as fructose-6-phosphate (F6P) and DHAP, M. hyorhinis has an extra set of enzymes enabling the conversion of fructose-1-phosphate (F1P) into fructose-1,6-biphosphate (FBP, 2.7.1.56). Only in M. hyopneumoniae might the conversion of glyceraldehyde (GAld) into glycerol be possible through the moonlight activity of alcohol dehydrogenase (EC 1.1.1.372), although up to date no experimental evidence can sustain that assumption. There also seems to exist a b-galactosidase that converts lactose into glucose and galactose (EC 3.5.1.23) in $M$. hyopneumoniae and $M$. hyorhinis. The presence of a unique transcriptional unit (TU) with carbohydrate metabolism-related genes in $M$. hyorhinis probably enables the uptake and metabolization of isomaltose (EC 3.2.1.10), maltose (EC 2.4.1.8), trehalose (EC 2.4.1.64 and EC 3.2.1.93) and sucrose (EC 3.2.1.48) into either G6P or F6P. This may be related to the fact that $M$. hyorhinis can overgrow the other two species in several growth media.
Myo-inositol metabolism: A TU for the myo-inositol catabolism is present in all $M$. hyopneumoniae species, with the exception of the enzyme 5-dehydro-2-deoxyphosphogluconate aldolase (iolJ, EC 4.1.2.29). The gene encoding this enzyme in other organisms is similar to the fructose-biphosphate aldolase (Fba) from glycolysis (EC 4.1.2.13). Since there are two copies annotated for Fba in M. hyopneumoniae, we proposed one of them as candidate for this activity based on sequence alignments with both genes from Bacillus subtilis. Inositol can be used as a carbon source and also produces acetyl coenzyme-A (AcCoA), which can be a source of cofactor coenzyme-A (CoA).
Amino sugar metabolism: $M$. hyopneumoniae and $M$. flocculare can uptake and convert N-acetyl-glucosamine (GIcNAc, PTS-GNAc) to N -acetyl-glucosamine-6-phosphate (GlcNAc6P); M. hyorhinis imports Sialic Acid (NeuNAc, TRANS-SA) and converts it to GIcNAc6P in three steps that are unique to the $M$. hyorhinis models (EC 4.1.3.3, EC 2.7.1.60, EC 5.1.3.9).
Pyruvate metabolism: All enzymes for the conversion of pyruvate to acetate (EC 1.2.1.-, EC 2.3.1.8 and EC 2.7.2.1) and lactate (EC 1.1.1.27) were found in all species. From the literature data, $M$. hyopneumoniae is able to produce oxaloacetate (OXAc, EC 6.4.1.1), malate (MAL, EC 1.1.1.37) and 2-oxoglutarate (2KG, EC 2.6.1.1) (Manolukas et al. 1988; Constantopoulos and McGarrity 1987). Decarboxylation of 2-oxoglutarate to succinyl-coenzyme-A (SucCoA, EC 1.2.4.2) has been previously reported in M.
hyorhinis extracts (Tourtellotte and Jacobs 1960). Using gap-filling, we proposed that the activity 1.2.4.2 is performed by the pyruvate dehydrogenase complex. Moreover, Cordwell and collaborators (Cordwell et al. 1997) suggested that in Mollicutes lactate dehydrogenase (Idh, EC 1.1.1.27) could also function as malate dehydrogenase (mdh, EC 1.1.1.37). M. hyopneumoniae is the only organism among the three to have a gene coding for an alcohol dehydrogenase, probably enabling the turnover of acetyl-coenzyme-A (AcCoA) to acetaldehyde (AcAld, EC 1.2.1.1 0) and ethanol (EtOH, EC 1.1.1.1). Conversion of pyruvate to formate (EC 2.3.1.54), malate to fumarate (EC 4.2.1.2) and SucCoA to succinate (EC 6.2.1.5) were added to the models in a final step to accommodate the results from the metabolomics experiments.

## Lipid Metabolism

The lipid metabolism in the models of the three species included the uptake of glycerol, choline, glycerophosphodiesters and fatty acids to the production of the biomass precursors cardiolipin (CL), 1,2-diacyl-sn-glycerol (DAG), phosphatidyl-glycerol (PG), phosphatidyl-choline (PC) and galactosyl-diacylglycerols (MGDG and DGDG, except for M. hyorhinis). Extracellular cholesterol and sphyngomyelin (SPM) were incorporated unmodified directly into biomass. While M. hyopneumoniae has four different ways to uptake and metabolize glycerol, $M$. hyorhinis lacks one and $M$. flocculare lacks two (Figure 4). The turnover of glycerol-3-phosphate into DHAP (EC 1.1.3.21) allows the usage of glycerol as the sole carbon source, with the production of the highly toxic hydrogen peroxide. This is only possible in the $M$. hyorhinis and $M$. hyopneumoniae models.

The enzyme responsible for the turnover of glycerol-3-phosphate into acyl-sn-glycerol-3-phosphate (Acyl-G3P, EC 2.3.1.15) was found only in the genomes of $M$. hyorhinis. Although this can be seen as a possible difference between the species, we had to add the reaction to the models for the other species to enable growth. It is possible that the other two species import this metabolite directly from the media, however further experiments are required to confirm this hypothesis. No homologs for the enzyme that produces phosphatidylglycerol (PG, EC 3.1.3.27) were found; however, all Mollicutes definitely synthesize this metabolite, and hence must use a hitherto undetected enzyme for this step (Razin and Herrmann 2002). The ACP protein was only found in the genomes of $M$. hyorhinis and may act as a fatty acid and coenzyme-A (CoA) donor in these organisms. Orphan reactions for the production of glycolipids (EC 2.4.1.46 and EC 2.4.1.241) were added to models for $M$. hyopneumoniae and $M$. flocculare based on the presence of these metabolites in vivo (Chen et al. 1992).

## Amino Acid Metabolism

The import of amino acids was added in two forms: oligopeptide import and cleavage (ABC-Opp1/ABC-Opp2 and EC 3.4.24.-), and amino acid import (Unknown-ABC). tRNA charging accounts for most of the reactions in this pathway (23 reactions). Production of the biomass precursor S-adenosyl-methionine (SAM) from methionine is possible in all strains (EC 2.5.1.5). Moreover, since the enzyme adenosylhomocysteine nucleosidase (EC 3.2.2.9) is present in all species, we added two reactions to restore connectivity to this subpathway (EC 2.1.1.- and EC 4.4.1.21). This addition resulted in the production of two metabolites essential for quorum sensing and cell communication in other species: S-rybosyl-homocysteine (SRH) and autoinducer-2.

## Nucleotide Metabolism

The mycoplasmas in this study cannot synthesize de novo purines and pyrimidines; therefore, they have only salvage pathways and interconversions to supply the cell with nucleic acid precursors. The three species have the same enzymatic capabilities, except for the presence of thymidylate synthetase (EC 2.1.1.46) in M. hyorhinis, which is also important for cofactor metabolism and is responsible for the conversion of dUMP and 5,10-methylene-tetrahydrofolate (MeTHF) to dTMP and dihydrofolate (DHF). Overall, the nucleotide metabolism consists in the uptake of guanine, adenine, uracil, thymine and cytidine and produces all deoxyribonucleotides (dATP, dCTP, dGTP, TTP) and ribonucleotides (ATP, CTP, GTP and UTP).

## Cofactor Metabolism

Around $60 \%$ of the enzymes that comprise the cofactor metabolism of the reconstructed models were not found in any species. For instance, thiamine-pyrophosphate (ThiPP) is imported unchanged directly into biomass (ABC-Pi), while pyridoxal (PYX) is imported and converted to the biomass precursor pyridoxal-phosphate (PYP, EC 2.7.1.36 missing in all species). From the pantothenate/coenzyme-A metabolism, we only found homologs for the conversion of 4'-phosphopantetheine (4PPAN, EC 2.7.7.3) into dephospho-coenzyme-A (DPCoA). Three forms of folate are incorporated to the biomass: MeTHF, 5,6,7,8-tetrahydrofolate (THF) and 10 -formyl-tetrahydrofolate (10FTHF). We assumed that folate was imported and converted to these three biomass precursors by: dihydrofolate reductase (EC 1.5.1.3, only present in M. hyorhinis), serine hydroxymethyltransferase (EC 2.1.2.1, present in all species) and formate-tetrahydrofolate ligase (EC 6.3.4.3, missing in all species). This may also account for the better fitness of $M$. hyorhinis when compared to the other two species. Although no homologs for NAD kinase were found, two reactions were added (EC 2.7.1.23 and EC 2.7.1.86) to allow the presence of NADP and NADPH, which are cofactors for several essential reactions. The NADPH and NADH produced from the degradation of glucose and other carbohydrates are recycled by the action of two enzymes: NADH oxidase (hydrogen peroxide forming, EC 1.6.3.3) and thioredoxin disulfide-reductase (EC 1.8.1.9).

## Mycoplasmas Cultivation

We cultivated the three species of mycoplasmas in complex and defined media to gather experimental information and compare to in silico growth (Figure 5). In the CMRL media supplemented with cofactors (CMRL+), M. hyopneumoniae and M. flocculare remained viable only when peptone was present (CMRL+/Pep). Supplementation of peptone appeared to have a negative effect on $M$. hyorhinis growth, but more tests should be performed to verify this hypothesis. Defined Yus medium (Yus et al. 2009) did not allow proliferation in any species, but maintained cell concentration and viability even after 5 days of culture (if
inoculated afterwards in complex media). These results are in agreement with a previous work performed by Bertin and colleagues (2013) for M. mycoides subsp. mycoides that shows that CMRL-1066 contains all components to support cellular metabolism but not growth. Here, the supplemented versions CMRL+ or CMRL+/Pep allowed proliferation only for M. hyorhinis during the first 24 hours of growth. It seems that after this period of time, one or more essential metabolites initially present were no longer available.


Figure 5. Cultivation curves in defined and complex media by species. Cell concentrations were estimated by the CCU method and error bars were calculated as the standard deviation between triplicate time-matched samples. As expected, the three species had better growth rates in the complex medium than in defined media. M. hyopneumoniae strain J (MHP), the M. flocculare strain 27716 (MFL) and the M. hyorhinis strain ATCC17981 (MHR).

By comparing the reconstructed models and the media composition, we propose here that key cofactors are not delivered to mycoplasmas in the correct form. For instance, thiamine pyrophosphate should be directly delivered to all species instead of its precursor thiamine, and pyridoxal-5-phosphate should be the cofactor of choice in place of pyridoxal. A list of correct precursors for the models versus the actual cofactors found in the defined Yus medium can be seen in Table S5 (Supplementary material). We also propose a new defined medium Table S6 (Supplementary material) for M. hyorhinis, M. hyopneumoniae and M. flocculare.

## Metabolomics of swine mycoplasma

NMR analysis of the culture media was performed in order to detect possible differences in the metabolism of the three species. All data measured in defined and complex media can be seen in Table S7 (Supplementary information).
The major differences in both complex and defined media were related to the metabolism of pyruvate (Figure 6): $M$. hyorhinis produced higher quantities of pyruvate at the end of 48 hours in complex medium. As a result, pyruvate conversion to acetate was detected in low quantities for this species in both media. M. hyopneumoniae and M. flocculare, on the other hand, produced higher amounts of acetate (this was even more pronounced for the growth of $M$. hyopneumoniae in complex medium). Low amounts of formate, fumarate and succinate were also detected, and this indicates the presence of genes encoding the enzymes EC 2.3.1.54, EC 4.2.1.2, EC 6.2.1.5 in these genomes. All models were thus modified to accommodate these activities in a final step. Formate production seems to be independent from acetate production in M. hyopneumoniae and M. flocculare. However, the similar low concentrations of acetate and formate in both growth media from $M$. hyorhinis might be an indication that both are produced concomitantly by the action of the enzymes EC 2.3.1.54 (turnover of pyruvate to formate and AcCoA), EC 2.3.1.8 (AcCoA to Acetyl-P) and EC 2.7.2.1 (acetyl-P to acetate).
To check if the lower concentrations of acetate were a result of an impaired activity of the pyruvate dehydrogenase complex in $M$. hyorhinis, we compared the predicted proteins PdhA, PdhB, PdhC and PdhD from all species (Clustal W alignments available in Text S3, Supplementary information). The known active sites of ODP2 (homolog of PdhC) and OLDH1 (homologs to PdhC and PdhD respectively) from Bacillus subtilis were analyzed in search for a possible mutation. No active sites have been described for PdhA and PdhB so far, but the enzymes for all three swine mycoplasmas do not present significant differences. Although not all active sites from PdhD seem to exist in mycoplasmas, all predicted proteins from the species analyzed (including $M$. genitalium and $M$. pneumoniae) are similar in terms of active sites, when these are present. The only difference between M. hyorhinis and the other two species was found in PdhC, in the protein portion related to the binding to PdhA and PdhD. This portion is the most variable between the species. An enzyme characterization should be made in order to verify if this complex is not active in this species, or if it is responsible for another unforeseen activity.


Figure 6. Distinct products of the metabolism of pyruvate from growth in complex Friis medium and defined Yus medium of the $M$. hyopneumoniae strains 7448 (MHP7448) and J (MHPJ), M. flocculare strain 27716 (MFL) and M. hyorhinis strain ATCC17981 (MHR). In complex medium, we calculated the ratio between the peak signal in cultivated versus control medium and error bars were calculated as the standard deviation between triplicate time-matched samples. For defined medium, we detected the actual concentration for the metabolites and error bars were calculated as the standard deviation between duplicate time-matched samples. (A) In a complex medium, M. hyopneumoniae (both strains) and $M$. flocculare can produce high amounts of acetate; the yields are even higher from M. hyopneumoniae. M. hyorhinis, on the other hand, produces low concentrations of acetate in this medium. The final glycolysis product for $M$. hyorhinis is thus pyruvate. In defined medium, $M$. hyopneumoniae (strain J ) and $M$. flocculare produce similar amounts of acetate while $M$. hyorhinis contains only residual levels of this metabolite. The three species can produce low amounts of formate in both media; (B) shows zoomed image of formate production.

The presence of myo-inositol in swine serum (He et al. 2012) may be directly related to the higher production of acetate in the growth of $M$. hyopneumoniae in complex medium, if compared to $M$. flocculare. Since the myo-inositol pathway produces AcCoA, the recovery of a molecule of CoA from myo-inositol is possible and is directly linked to the production of acetate. Since the myoinositol catabolic pathway is one of the few distinctions between the metabolic models of $M$. hyopneumoniae and $M$. flocculare, it is possible to assume that the differences in acetate concentration in vitro may indeed arise from the ability of $M$. hyopneumoniae to uptake myo-inositol. Indeed, when no source of myo-inositol is present (defined medium), no difference in the concentration of acetate is observed for these two species.
The analysis of the amino acid uptake was not trivial since both media contained peptone, which at first was not measured through the CPMGexperiment but, in the course of time, was degraded into single amino acids, and thus changed the overall signal of the NMR spectra. To address this issue, we only took into account the defined medium and a single time point after 72 hours of growth (Figure S3, Supplementary information). M. hyorhinis seemed to have lower concentrations of most amino acids at the end of the growth curve if compared either to the control medium or to the other species. This may be related to several factors, such as lower rates (in comparison to the other two species) of peptone degradation by membrane proteases or higher uptake rates of amino acids by $M$. hyorhinis. At this point, we could no longer verify which hypothesis is more suitable for this particular distinction, however, since peptone was not necessary for the maintenance of the viability of $M$. hyorhinis in the defined medium, it is possible that this species may harbor transporters with higher specificity or effectiveness for single amino acids.
The results as concerns the other metabolites indicate for the three species the uptake of glucose and nucleotides.

## Flux balance analysis (FBA)

FBA was used to check the properties and capabilities to produce detected metabolites by NMR spectra of all models (also during the reconstruction and refinement process). FBA simulations were performed with an in silico medium that contained all biomass precursors. Similarly to the in vitro growth results, in the presence of oxygen, acetate was the main product and NADH was recycled
by the conversion of molecular oxygen into water. In the absence of oxygen (not tested in vitro), all models were able to grow but the main product became lactate. As expected, the availability of myo-inositol in the in silico medium allowed an alternative source of coenzyme-A and higher concentrations of acetate as a final product in the models for $M$. hyopneumoniae. Hydrogen peroxide production was linked to the metabolism of glycerol in both $M$. hyopneumoniae and $M$. hyorhinis, while the models for $M$. flocculare did not produce toxic levels of this metabolite. Since we have no information on gene essentiality for any species, we checked reaction essentiality in the pan-network. This means that we deleted individual reactions (all transport, enzymatic and spontaneous reactions) even if they did not have a GPR association. A total of 111 reactions ( 69 enzymatic, 37 transport, and 5 spontaneous reactions) were essential for growth in the pan-network (Table S8, Supplementary information).

## 4. Discussion

In this work, we created a metabolism as realistic as possible for the three known mycoplasmas present in the respiratory tract of swines. It is essential to point out that, although we had to include $30 \%$ of orphan reactions (including transporters, see Table S2) in order to allow growth, we only used about half of the genomes of these organisms. The other half consists either of hypothetical or of conserved hypothetical proteins (Siqueira et al. 2013), which may in part fill the missing gaps of the models. The number of essential reactions in the pan-network in this work is not directly comparable to the number of indispensable genes predicted for $M$. genitalium ( 382 genes) or M. pneumoniae ( 310 genes) (Glass et al. 2006,Hutchison et al., 1999). Gene essentiality accounts for more than metabolic enzymes which means that proteins not included in our models, related to protein synthesis, DNA polymerization or RNA turnover, are not accounted for in the essential reactions. If we added these proteins with an activity that is not metabolicrelated, we would arrive at numbers closer to those of $M$. genitalium and $M$. pneumoniae. The lack of experimental information on gene essentiality is also a setback for the validation of the models created here. Further experiments on this matter should help us better refine and complete the networks.
As for most of the mycoplasma species studied (Suthers, Zomorrodi and Maranas 2009; Yus et al. 2009; Wodke et al. 2013), all reconstructed networks exhibit low connectivity due to the simplicity of the biological model. We were able to show in this work that the three swine mycoplasma species have similar metabolic capabilities, except for the metabolism of myo-inositol, amino sugar, and carbohydrates and for the uptake of glycerol (Figure [Diff]). Overall, the methods used here enabled us to address some of the main problems caused by most automatic reconstruction methods which are the permissive inclusion of pathways and over prediction of capabilities (Suthers, Zomorrodi and Maranas 2009). Annotation errors arise with the attribution of ambiguous or partial EC numbers (Devos and Valencia 2001; Green and Karp 2005) and the propagation of these errors may then lead to many other ones. Thus, it is known that the prediction of GPR associations based only on name-matching and EC-codes is not sufficient to add confidence to a model (Green and Karp 2005). When we decided to simultaneously refine the 12 models, we enhanced the confidence of each GPR association, by adding information on the synteny between the genomes, protein sequence alignments and phylogenetic distance between orthologs.


Figure 7: Differential metabolism of $M$. hyorhinis, $M$. hyopneumoniae and $M$. flocculare.

Our main objective was not only to reconstruct the metabolic models for M. hyorhinis, M. hyopneumoniae and M. flocculare. We also wanted to compare the metabolism of the three species and find possible links to virulence, host-colonization capacity and life-style. M. hyorhinis, for instance, is not only a pathogen, but also a common contaminant in many mammalian cell cultures (Drexler and Uphoff 2002). This may be explained by the wide range of carbohydrates which this organism can uptake and metabolize. This may be a crucial factor to the ability of $M$. hyorhinis to grow in diverse sites inside the host (Kobisch and Friis 1996) and even to invade other hosts and potentially develop cancer in humans (Huang et al. 2001; Kornspan et al. 2011). M. hyopneumoniae has been detected in other sites such as brain, liver and spleen (Friis 1974; Marois et al. 2007), but M. flocculare has never been detected outside of the respiratory tract of swines. One may wonder what could explain this if both organisms are so similar. M. hyopneumoniae is the only Mycoplasma species with sequenced genome that has the genes for the catabolism of myo-inositol. Myo-inositol is readily abundant in the bloodstream of mammalian hosts, and can be used as a secondary carbon source for energy production (Reynolds 2009). Mycoplasma iguanae has been described to produce acid from inositol (Brown et al. 2006). However, there is no available complete genome sequence for this organism, making impossible any comparison of the genes involved in this pathway. Although at this point, we cannot confirm that this pathway is functional in $M$. hyopneumoniae, the NMR results we obtained suggest that myo-inositol might be directly related to the higher production of acetate in complex medium of $M$. hyopneumoniae if compared to $M$. flocculare. This assumption is based on two factors: first, a previous work has detected myo-inositol in swine serum, which is a component of the complex medium (He et al. 2012); and second, the only difference in the networks that could influence the acetate concentration is the myo-inositol catabolism. This pathway may also be involved with an alternative production of $A c C o A$, precursor to $C o A$, an essential cofactor for growth in all species. These metabolic distinctions may help M. hyopneumoniae to grow in diverse sites if compared to M. flocculare. Furthermore, the catabolism of myo-inositol has been experimentally described as a key pathway for competitive host nodulation in the plant symbiont and nitrogen-fixing bacterium Sinorhizobium meliloti (Kohler et al. 2010). Host nodulation is a specific symbiotic event between a host plant and a bacterium. Kohler et al showed that whenever the catabolism of inositol is disrupted (by single gene knockouts from the inositol operon), the mutants are outcompeted by the wild type for nodule occupancy. This means that the genes for the catabolism of inositol are required for a successful competition in this particular symbiosis. Myoinositol has been extensively described in several organisms as a signaling molecule (Downes and Macphee 1990; Gillaspy 2011) that moreover is important for pathogenicity (Reynolds 2009). Thus, it is possible that the myo-inositol catabolism in M. hyopneumoniae is one of the reasons for the high pathogenicity of this species when compared to $M$. flocculare andM. hyorhinis.
A striking result from the NMR results was the reduced capability of $M$. hyorhinis to produce acetate. This was not foreseen in the model reconstruction, but predicted protein sequences from the pyruvate dehydrogenase complex of the three species showed a particular distinction in PdhC, more specifically in the region responsible for binding to other complex components. We could not verify at this point if this complex is not active in the particular growth conditions of our work in $M$. hyorhinis or if it is responsible for another unforeseen activity. However, a transcriptome profiling of $M$. hyorhinis has detected both pdh $A$ and $p d h B$ in the pool of genes with the highest number of transcript reads (Siqueira et al. 2014), indicating that the complex might be translated in vivo. Moreover, pyruvate dehydrogenase activity has been previously detected in $M$. hyorhinis extracts (Constantopoulos et al. 1988), and it seems to be correlated to oxygen availability. In our cultivations, the cells are not grown in a complete aerobic system, which may explain the differences between our findings and those previously published.
Experimental growth tests also showed that neither species was able to grow in the defined media. This is probably due to a lack of the correct cofactors as previously mentioned. For instance, thiamine, pyridoxal/pyridoxine, pantothenate, spermine and folate were the actual media components tested, but the reconstructed models were not able to convert them to biomass precursors (thiamine to thiamine pyrophosphate, pyridoxal to pyridoxal-5-phosphate, pantothenate to 4-phospho-pantheteine, or spermine into spermidine). Only M. hyorhinis seems to be able to uptake folate directly while the other two might need intermediate metabolites. This might also explain why M. hyorhinis is extensively found as a cell culture contaminant. Even though CMRL-1066 was supplemented with all missing components from the defined Yus medium (Table S1), cells maintained viability for longer periods in the latter. Indeed, common cofactors (which were not added to CMRL-1066) existed in lower concentrations in the CMRL1066. This means that possibly one or more cofactors enriched in the defined Yus media are essential for the viability of the three species. The enriched cofactors included: choline, folate, pantothenate, pyridoxal, thiamine and spermine.
Other differences among the species seem to indicate that $M$. hyopneumoniae and $M$. flocculare lack one or more amino acid transporters. This is due to two things: (i) both remained viable for longer periods whenever CMRL+ was supplemented with peptone, and (ii) we detected a higher amino acid accumulation in the defined media if compared to M. hyorhinis. M. hyorhinis on the other hand may have all the transporters for single amino acids, but this hypothesis needs to be further verified. From the sequence analyses, we did not find significant differences among the species that would explain this distinct behavior. Moreover, since $M$. hyorhinis seemed to overgrow in the first 24 hours of culture in both CMRL media (CMRL+ and CMRL+/Pep), it is possible that one or more compounds were missing in the defined Yus medium in order to allow growth. Possible candidates for such (present in CMRL and absent in the defined Yus medium) would be: coenzyme A, nicotinamide, ascorbate and/or pyridoxine. Indeed, ascorbate seems to be essential for nucleotide metabolism, and its absence may be responsible for the impairment of the growth of these organisms.
Despite considerable evidence supporting the existence of a polysaccharide capsule in mycoplasmas, it is not yet known if the material is synthesized by the bacteria or imported from the medium for most species (Daubenspeck, Jordan and Dybvig 2014). Recent reports have even shown the capacity of species from the mycoides cluster to build a capsule using only phosphorilated glucose (Bertin et al 2013, Bertin et al 2015). However, no homolog proteins related to these activities were found in none of the species studied in this work. Although we were not able to introduce the capsule production itself in the models, we tried to relate its existence with the possible substrates used to produce it. The composition of capsular polysaccharides has been related to the level of pathogenicity in other bacteria. For instance, highly virulent Escherichia coli strains seem to have practically nonimmunigenic capsular material, due to the fact that these antigens are similar or identical to the ones found in the host (Boulnois
and Jann 1989). The structure of the K5 antigen from E. coli capsules is basically N -acetylglucosamine (GIcNAc) and glucuronic acid (GlcA) in a molar ratio of 1:1 (Vann et al. 1981). Erlinger and collaborators (1995) identified heparan sulfate (HS) as the predominant glycosaminoglycan in the porcine respiratory tract and the most common disaccharide unit within HS is GlcNAc linked to GlcA. Since $M$. hyopneumoniae and $M$. flocculare can import GlcNAc directly, they might use it for the composition of its capsule; M. hyorhinis, on the other hand, imports sialic acid, and possibly uses it directly to produce its own. Of course this is highly speculative, and we are not sure if all these hypotheses indeed happen in vivo.however this is particularly important because Tajima and Yagihashi (1982) already reported that capsular polysaccharides from $M$. hyopneumoniae play a key role in the interaction between pathogen and host. For several bacterial species, the amount of capsular polysaccharide is a major factor in their virulence (Corbett and Roberts 2009) and it decreases significantly with in vitro passage. This supports the idea that non-pathogenic strains of $M$. hyopneumoniae may have significantly less capsule material and, in this way, be more susceptible to the host defense.
The metabolism of glycerol is the final major difference between the pathogenic $M$. hyopneumoniae and $M$. hyorhinis and the nonpathogenicM. flocculare. Such metabolism and the production of hydrogen peroxide are essential for the cytotoxicity of $M$. pneumoniae (Hames et al. 2009) and M. mycoides Subsp. mycoides (Vilei et al. 2001). The enzyme responsible for this activity is absent in $M$. flocculare and present in the other two pathogenic species. This may explain why both $M$. hyopneumoniae and $M$. flocculare can adhere to the cilia of tracheal epithelial cells, but only the adhesion of $M$. hyopneumoniae results in tissue damage (Young et al. 2000). Moreover, while $M$. hyorhinis has in the reconstructed models 3 ways of uptaking glycerol, $M$. hyopneumoniae seems to have 5 , and this may reflect in its enhanced pathogenicity.
We presented in this work an overview of the differential metabolism of $M$. hyopneumoniae, $M$. hyorhinis and $M$. flocculare using different approaches. The reconstructed models showed some distinctions among the species, namely the myo-inositol metabolism for $M$. hyopneumoniae, the uptake of carbohydrates for $M$. hyorhinis and the usage of glycerol as a carbon source for the two pathogenic species. The models also served as a basis for all the assumptions made for the experimental data. Metabolic profiling of both complex and defined media pointed to new differences that we were not able to identify based solely on the sequenced genomes. The major ones were related to the pyruvate conversion rates to acetate, which appeared to be higher in M. hyopneumoniae and $M$. flocculare than in $M$. hyorhinis. Growth tests also allowed us to assign correct cofactors for biomass production in each species and may help formulate new defined media for the cultivation of swine mycoplasmas.
Whether the main differences among the species we reported here (summarized in Table S9). are related to virulence or pathogenicity have not yet been addressed experimentally, but it is tempting to speculate. The same factors that may enhance virulence of $M$. hyopneumoniae may help the commensal species $M$. flocculare to better survive inside the host.
All these in silico and in vivo metabolic differences among M. hyopneumoniae, M. hyorhinis and $M$. flocculare might influence the different levels of pathogenicity in each of them. However, it is highly likely that gene regulation may also interfere directly in the metabolism. This, in turn, may account for many aspects still unknown that influence directly the levels of pathogenicity in each of them. One of our future goals is therefore to understand and integrate gene regulation into the metabolic models. Upcoming experiments will aim at testing the hypotheses formulated here, particularly those related to the metabolisms of glycerol and myoinositol. We also intend to better understand the habitat of these species, and the possible metabolic and genetic dialogues with the host and other bacteria present in this environment. Either way, this work serves as a basis for the study of the differential metabolism and pathologies caused by the swine lung mycoplasmas and may help to propose ways to prevent disease development in the future.

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## TITRE en franćais

Invéstigation métabolique des mycoplasmes dans le tractus réspiratoire des cochons

## R'ÉSUMÉ en franćais

L'appareil respiratoire des porcs est colonisé par plusieurs bactéries, parmi lesquelles trois espèces de mycoplasmes : Mycoplasma flocculare, Mycoplasma hyopneumoniae et Mycoplasma hyorhinis. Lors de ce doctorat, notre principal objectif était de mieux comprendre le métabolisme différentiel dans chacune des espèces à l'aide de différentes approches. Nous avons reconstruit les réseaux métaboliques complets pour toutes les souches séquencées de ces trois espèces de mycoplasmes afin d'y détecter des caractéristiques distinctes. Nous avons pu montrer que, bien que les trois espèces de mycoplasmes du porc ont des capacités métaboliques semblables, certaines différences existent qui incluent, d'une part, le catabolisme de myo-inositol et un système plus complet pour l'absorption du glycérol, et d'autre part, une large gamme de moyens d'absorption de carbohydrates chez $M$. hyorhinis. L'utilisation de glycérol comme source de carbone, une activité qui est absente uniquement dans $M$. flocculare, produit du peroxyde d'hydrogène qui est toxique, ce qui peut expliquer l'absence de pathogénicité de cette espèce. L'absorption d'un plus large éventail de sources de carbone chez $M$. hyorhinis peut également expliquer pourquoi cette espèce est un contaminant largement connu des cultures cellulaires. Des expériences de croissance ont montré que les milieux définis décrits pour d'autres espèces de mycoplasmes ne sont pas appropriés pour la croissance de mycoplasmes du tractues respiratoire de porcs, et que la peptone est essentielle pour le maintien de la viabilité des cellules à la fois de M. hyopneumoniae et de M. flocculare dans des milieux définis. Dans ce travail, nous proposons également de nouveaux média définis qui, in silico, sont extrêmement appropriés pour les mycoplasmes du porc. Les données de métabolomique suggèrent que même si ces espèces sont extrèmement similaires du point de vue de leurs génomes et des métabolismes, les produits et les taux de réaction diffèrent et la régulation des gènes peuvent interférer directement dans le métabolisme. Pour expliquer ces différences ainsi que d'autres décrits dans la littérature qui suggèrent que certains types de régulation de l'expression du gène existent en effet dans ces espèces, nous avons également essayé de recueillir des informations sur de nouvelles séquences promotrices. Ainsi, cette thèse servira de base pour l'étude du métabolisme différentiel et des pathologies causées par les mycoplasmes du tractus respiratoire du porc et pourra aider à proposer des façons de prévenir à l'avenir le développement des maladies associées.

[^1]Title in English
Metabolic investigation of the mycoplasmas from the respiratory tract of swines

## Abstract in english

In this PhD thesis, we presented three main types of analyses of metabolism, and in most cases involving symbiosis: metabolic dialogue between a trypanosomatid and its symbiont, comparative analyses of metabolic networks and exploration of metabolomics data. The respiratory tract of swines is colonized by several pathogenic bacteria, among which are three mycoplasma species: Mycoplasma flocculare, Mycoplasma hyopneumoniae, and Mycoplasma hyorhinis. In this work, we created whole-genome metabolic network reconstructions for all sequenced strains from these three Mycoplasma species. Similar to other Mycoplasma models all reconstructed networks exhibit low connectivity due to the simplicity of the biological model. We were able to show that the three swine mycoplasma species have similar metabolic capabilities. Interesting metabolic differences include the myo-inositol catabolism and a more complete system for glycerol uptake in M. hyopneumoniae and a wide range of carbohydrate uptake in M. hyorhinis. Glycerol conversion to DHAP, a missing activity only in M. flocculare, produces toxic hydrogen peroxide and may explain the lack of pathogenicity of this species. The uptake of a wider range of carbon sources in M. hyorhinis may also explain why this species is a wide-known contaminant in cell cultures. Growth experiments showed that defined media described for other Mycoplasma species are not suitable for the growth of respiratory tract swine mycoplasmas and that peptone is essential for the maintenance of cell viability of both M. hyopneumoniae and M. flocculare in defined media. Metabolomic data suggests that even though these species are extremely similar from a genomic and metabolic point of views, the products and reaction rates differ and gene regulation may interfere directly in metabolism. This, in turn, may account for many aspects still unknown that influence directly different levels of pathogenicity in each of them.

Keywords in English
metabolic networks, pathogenicity, mycoplasmas.


[^0]:    This chapter discusses possible dialogue mechanisms intra and inter-species and tries to propose models for the interaction between host and pathogens, and between pathogens themselves. At first, we will discuss how these mycoplasmas are able to escape the host immune system and how they are able to communicate within the same species population. Then, we will discuss the possible metabolites available from the host metabolism in the environment. And at last, we will show how mycoplasmas and other species may exchange signals and metabolites in order to better survive inside the host. At the end, we will propose a model for intra and interspecies communication and exchange, based on the reconstructed models from Chapter 2.

[^1]:    MOTS-CLEFS en franćais
    réseaux métaboliques, pathogenicité, mycoplasmes.

