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Systems Analysis Of Minimal Metabolic Networks In Prokaryotes

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Systems Analysis Of Minimal Metabolic Networks In Prokaryotes

PhD Thesis in Chemical and Biological Engineering

This work was executed under the supervision of:
Professor Isabel Cristina de Almeida Pereira da Rocha
and
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STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, June 3rd 2016

Joana Rute Calça Xavier

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From the moment of birth every human being wants happiness and freedom and wants to avoid suffering. In this we are all the same; and the more we care for the happiness of others the greater our own sense of each other becomes.

—TENZIN GYATSO, THE 14TH DALAI LAMA *The Compassionate Life* (2001)

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Abstract

The complexity of living cells is staggering, as a result of billions of years of evolution through natural selection in constantly changing environments. Systems biology emerges as the preferred approach to the disentangling of this complexity by looking at living cells and their responses to environments in a holistic manner. Complete annotated sequences of genomes are now available for thousands of species of the simplest unicellular life forms known, the prokaryotes. Together with other large-scale datasets as proteomes and phenotypic screenings and a careful analysis of the literature, genome annotations allow for the reconstruction of large constraint-based models of cellular metabolism.

Here, genome-scale metabolic models (GSMs) of prokaryotes are used together with other disparate large-scale datasets and literature assessments to study and predict essential components in minimal metabolic networks. A conceptual clarification is presented in a review of systems biology perspectives on minimal and simpler cells. An assessment of the biomass compositions in 71 GSMs of prokaryotes was then performed, revealing heterogeneity that impacted predictions of reaction essentiality. The integration of 33 large-scale essentiality assays with other data and literature revealed universally and conditionally essential cofactors for prokaryotes. These were used to revise predictions of essential genes and in the prediction of one biosynthetic pathway in the GSM of *M. tuberculosis*.

Additionally, a large-scale assessment of essentiality of different metabolic subsystems was performed with 15 comparable GSMs. The results were validated with 36 large-scale experimental assays of gene essentiality. The ancestry of metabolic genes and subsystems was estimated by blasting representative genomes of all the phyla in the prokaryotic tree of life. Ancestry was correlated with essentiality in general but not with non-essentiality.

Finally, a method was devised to generate minimal viable metabolic networks based on a curated and diverse universe of prokaryotic metabolic reactions. Different growth media were tested and shown to generate different networks regarding size, cofactor requirements and maximum biomass production. The

results of this work are expected to contribute for fundamental investigations of core and ancestral prokaryotic metabolism and the design of modularized and controllable chassis cells.

Resumo

A complexidade das células vivas é surpreendente, como resultado de milhares de milhões de anos de evolução através de seleção natural em ambientes em constante mudança. A Biologia de sistemas surge como a abordagem preferencial para analisar esta complexidade por examinar as células e as suas respostas ao meio de uma forma holística. Estão hoje disponíveis sequências completas e anotadas de genomas para milhares de espécies das formas de vida unicelulares mais simples conhecidas, os procariotas. Juntamente com outros conjuntos de dados de larga escala como proteomas e triagens fenotípicas e uma análise cuidadosa da literatura, os genomas anotados permitem a reconstrução de grandes modelos do metabolismo celular baseados em restrições.

Neste trabalho utilizam-se modelos metabólicos à escala genómica (GSMs) de procariotas em conjunto com outros grandes conjuntos de dados díspares e avaliações da literatura para estudar e prever componentes essenciais em redes metabólicas mínimas. Um esclarecimento conceptual é apresentado numa revisão de perspectivas da biologia de sistemas sobre células mínimas e mais simples.

Segue-se uma avaliação das composições de biomassa em 71 GSMs de procariotas, revelando a heterogeneidade que afecta as previsões de essencialidade de reações. Com a integração de 33 ensaios em grande escala de essencialidade com outros dados e literatura, revelam-se cofactores essenciais universais e condicionais em procariotas. Estes foram utilizados na revisão de previsões de genes essenciais e na previsão de uma via biossintética no GSM de *M. tuberculosis*.

Adicionalmente, foi realizada uma avaliação em larga escala de essencialidade de diferentes subsistemas metabólicos com 15 GSMs comparáveis. Os resultados foram validados com 36 ensaios experimentais de essencialidade em larga escala. A ancestralidade de genes metabólicos e subsistemas foi estimada por blast a genomas representativos de todos os filos na árvore da vida procariota. A ancestralidade revelou-se correlacionada com a essencialidade em geral, mas não com a não-essencialidade.

Finalmente, concebeu-se um método para gerar redes metabólicas mínimas viáveis com base num universo curado e diversificado de reações metabólicas procariotas. Diferentes meios de crescimento foram testados, mostrando-se a geração de diferentes redes em relação ao tamanho, os requisitos de cofactores e a produção de biomassa máxima. Espera-se que os resultados deste trabalho contribuam para investigações fundamentais dos metabolismos essencial e ancestral de procariotas e para o desenho de células chassis modulares e controláveis.

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CHAPTER 1
General Introduction

The footsteps of Nature are to be trac'd, not only in her ordinary course, but when she seems to be put to her shifts, to make many doublings and turnings, and to use some kind of art in endeavoring to avoid our discovery.

—ROBERT HOOKE, *Micrographia* (1665)

In this chapter, a brief historical view of systems biology is presented to portray the scientific and technological context where this work develops. Genome scale metabolic models and the methods used to build and simulate them are introduced, with an emphasis on flux balance analysis, used thoroughly in this work. The potential of comparative systems biology leading ultimately to the inference of minimal metabolic networks is also presented. The research aims guiding the work reported in this manuscript are enumerated, and the outline of this thesis is presented with a short description of each chapter. Finally, the scientific output of this thesis is referenced.

1.1 Context and Motivation

1.1.1 A Short History of Systems Biology

In France, back in 1864, Claude Bernard insists that living creatures are bound by the same laws as inanimate matter, foretells the development of mathematical biology and formulates the principle of control of the internal environment, nowadays well-known as homeostasis (Bernard 1864). Ground-breaking views in the midst of a 19th century still roamed by vitalist theories, these can be sufficient-enough reasons to root back to Bernard the origins of systems biology (Noble 2008). In actuality, one of the insulators around the modern paradigm of systems biology lies beyond its look at biological systems as a whole (e.g. large sets of components, cells, organisms, or other levels of biological organization): the application of mathematics and physical principles to biological questions (Westerhoff et al. 2009). The seminal Hodgkin-Huxley model, a mathematical model of the neuron's axon potential, is a prime example (Hodgkin & Huxley 1952). With accurate measurements of ionic currents and a set of nonlinear differential equations, Hodgkin and Huxley approximated the electrical characteristics of excitable cells, for which they received the Nobel Prize. These characteristics would later be applied in modeling the electrical functioning of the heart (Noble 1962) in an elegant depiction of one of the assets of systems biology, one where it resembles physics more than traditional biology: the prediction of general principles, rather than being purely descriptive. Around the same time, Peter Mitchell enunciates his quantitative theory of chemiosmosis, stating that ATP synthesis is coupled with the electron transfer chain (Mitchell 1961). These quantitative and predictive approaches to biological entities constitute the 'systems root' of systems biology (Westerhoff & Palsson 2004).

In the antithesis to the system root of systems biology emerges the 'biology root', with its traditional, analytic reductionist approaches, cataloguing and exploring individual biological entities. Westerhoff and Palsson described how the scaling-up of molecular biology occurred from the discovery of the structure and information coding of DNA, restriction enzymes, cloning technology and automatic

DNA sequencing to the current stage of fully-sequenced and annotated genomes (Westerhoff & Palsson 2004). The stage where biology lies today, that of large-data enabled by the blossoming of experimental biotechnology (Joyce & Palsson 2006), is where it merges with systems theory in modern systems biology (Kitano 2002). The history of systems biology is somewhat overlapping with that of bioinformatics, as Paulien Hogeweg, credited with the coining of the term together with Ben Hesper, reviewed in her recent historical perspective (Hogeweg 2011). Genome-scale metabolic models (GSMs) are at the front of modern systems biology and are a crucial element in this overlap with bioinformatics (Hogeweg 2011, Kitano 2002, Westerhoff & Palsson 2004). The next section elaborates on this type of model and its simulation, used thoroughly in Chapters 3, 4 and 5 of this thesis.

1.1.2 Genome-scale Metabolic Models and Flux Balance Analysis

Genome-scale metabolic models (GSMs) are one of the most advanced and detailed efforts towards predictive, quantitative biological models available currently, allowing for the accurate estimation of growth rates under different conditions (Edwards et al. 2001) and even of the outcome of adaptive evolution of laboratory strains (Ibarra et al. 2002). While the first model was that of *Haemophilus influenza* (Edwards & Palsson 1999), *Escherichia coli* was exhaustively explored with different GSMs (Edwards & Palsson 2000, Feist et al. 2007, Orth et al. 2011, Reed et al. 2003) and several other species have been modeled in the last years with numerous applications, including antibiotic design and strain optimization (as reviewed in (Durot et al. 2009, Monk et al. 2014, Oberhardt et al. 2009)).

A GSM can be formally described as a system of linear equations derived from stoichiometry and a set of inequality constraints, which allows for quantitative simulations. Manually-curated GSMs are built in a four-step process (Oberhardt et al. 2009). The first involves an initial draft reconstruction built from a genome annotation to which information from databases is added, including various enzyme data such as ligand molecules (cofactors, substrates, products, inhibitors and activators), reaction formulae and metabolic pathways obtained e. g. from KEGG

(Kanehisa et al. 2014), EXPASY (Artimo et al. 2012), BRENDA (Chang et al. 2015) and Metacyc (Caspi et al. 2014). Secondly, an examination of the primary literature is performed to improve the initial reconstruction and a conversion to a mathematical model of all the knowledge achieved is performed. Thirdly, a validation of the model is attempted at through the comparison of its predictions to phenotypic information. Finally, the model is submitted to continued wet/dry lab cycles to improve its accuracy and test hypotheses.

Flux Balance Analysis (FBA) is one of the methodologies used to predict phenotypes with GSMs. Through stoichiometric and the reversibility constraints, it employs a linear programming (LP) strategy to generate a flux distribution that is optimized towards a particular objective or phenotypic goal, which is usually the production of biomass or cellular growth (Feist & Palsson 2010). FBA was introduced on the basis of the Darwinian principle that states organisms optimization during evolution (Ruppin et al. 2010, Varma & Palsson 1993).

In the last few years, FBA has been the most successful and widely used technique at a system level in metabolic engineering. *E. coli*, as the preferred model organism, was engineered to overproduce with high yields the amino acids threonine (Lee et al. 2007) and valine (Park et al. 2007), lactic acid (Fong et al. 2005) and succinic acid (Lee et al. 2005).

On its birth, FBA counted only with stoichiometric constraints; since then, other constraints were added to the standard method, as regulatory (Gustin et al. 1998) and thermodynamic (Beard et al. 2002). Also, a dynamic approach to FBA was developed, yielding temporal profiles of fluxes (Mahadevan et al. 2002). The applications that FBA developments have in the analysis of GSMs are vast, but several improvements still need to be done, not only to the simulation technologies, but also to the models themselves. The development of the several omics datasets makes promising statements, as with the integration of expression data that was already used to improve predictions of metabolic fluxes (Åkesson et al. 2004, Faria et al. 2014, Machado & Herrgård 2014). Other challenges are to be met in data integration, especially the extraction of biological meaning from these large datasets (Joyce & Palsson 2006, Saha et al. 2014).

1.1.3 Comparative Systems Biology and the Inference of Minimal Metabolism

The use of metabolic or protein networks in comparative biology can provide unique insights into the relationship and evolution of species. Metabolic phenotypes can be regarded as the result of several evolutionary processes, but these phenotypes do not emerge directly from the evolution of genomes. Non-orthologous gene displacement causes completely different genotypes to result on the same phenotype (Koonin 2003), and therefore comparing genomes is not the same as comparing the functionality of the cells that contain them. Comparative functional analysis can help to overcome this limitation and even identify some mistaken phylogenetic inferences that exist to date (Kuchaiev et al. 2010, Yamada & Bork 2009).

While genetic sequences provide insights on close phylogenetic relationships (suitable for more recent proteins) and protein sequences are used to make inferences about evolutionary trajectories of older proteins, the comparison of metabolic pathways can give us insight about even more ancient features, possibly existing since before the last universal common ancestor (LUCA). Metabolic network comparisons have been used and validated to make phylogenetic inferences (Kuchaiev et al. 2010, Ma & Zeng 2004, Oh et al. 2006), but not yet using GSMs. The comparative methodology using these models can, hypothetically, not only produce these scientific outputs, but also have applications in expediting model construction and improvement and strain optimization.

Ultimately, comparing metabolic networks at large-scale will lead to the identification of core functions common to all or most the networks analyzed that are hypothetical essential features of cells. These are assumed to be characteristics of LUCA, of theoretical minimal cells and of chassis cells for diverse applications. This motivation is further contextualized and explained in Chapter 2 of this thesis. The detailed research aims following this motivation are presented below.

1.2 Research Aims

In the general context of the current status of systems biology and of the potential of the comparison of genome-scale metabolism stated above, the main goal of this thesis was to infer viable minimal metabolic networks for cellular growth. In order to achieve this goal, the state of the art in minimal cells was reviewed extensively. Genome-scale metabolic models were collected, studied and chosen for further comparative work, with a special focus on comparability, validation and phylogenetic reach. A particular effort was put on understanding the impact of the biomass compositions used in GSMs in the prediction of essential metabolic functions. The GSMs chosen were then used in simulations of single knockouts with the aim of predicting essential reactions for prokaryotic metabolism. These predictions were compared to experimental data and large-scale sequence alignments to infer on the ancestry of specific metabolic functions. Finally, the universe of metabolic reactions obtained and a curated core biomass composition were used with the aim of predicting and generating viable minimal metabolic networks in different growth conditions.

1.3 Outline of the Thesis

This thesis has been structured addressing the above-stated goals in six chapters:

- In **Chapter 1**, the current chapter, this thesis was contextualized in the modern state of the field of systems biology, together with the motivation and aims of this work, its structure and scientific outputs.
- In **Chapter 2**, an extensive review of the broad and ambiguous field of minimal cells was conducted, with a special focus on systems biology conceptualizations and approaches. Partially overlapping concepts as minimal cell, minimal genomes, LUCA and chassis cells were clarified. Traditional reductionist, top-down approaches were contrasted with

bottom-up and integrative approaches to minimal cells. The different goals of the minimization of cellular components and the simplification of biological complexity were contrasted.

- A large-scale integration of disparate experimental data, literature and 71 GSMs was performed regarding biomass composition in **Chapter 3**, leading to the identification of universally and conditionally essential organic cofactors for prokaryotic metabolism. The effect of the absence of these core components in the biomass composition was studied, leading to the prediction of new essential genes and one experimentally validated biosynthetic route in two pathogens, *Klebsiella pneumoniae* and *Mycobacterium tuberculosis,* respectively.
- In **Chapter 4**, 15 highly curated and comparable GSMs were simulated in rich media conditions to predict highly essential metabolic functions for prokaryotic metabolism. The results were integrated at the level of metabolic subsystems and validated with experimental data. Ancestral metabolic subsystems were estimated from 79 manually selected genomes covering all the prokaryotic phyla in the tree of life with quality genome sequences. The subsystems of tRNA charging, Transport, and Cofactor and Prosthetic Group metabolism were identified as ancestral and highly essential.
- All the previous chapters are integrated in the work conducted in **Chapter 5**. A Universe of metabolic reactions was re-annotated and curated to serve as a pool to generate minimal viable and diverse metabolic networks. A curated core biomass reaction and three different growth media were used, including one theoretical rich medium and two common laboratory media. The networks obtained were compared and analyzed regarding content and capabilities.
- Finally, in **Chapter 6** the main conclusions of this thesis are recapitulated. Some perspectives on future research based on unanswered or new questions identified throughout this work are also presented.

- Supplementary Files are provided in http://darwin.di.uminho.pt/jcxavier/ and within the CD containing the digital version of this document

1.4 Scientific Output

The scientific output produced from the results obtained in this thesis is presented below.

1.4.1 Peer-reviewed Publications

Xavier JC, Machado D, Patil KR, Rocha I. Prediction of Minimal Metabolic Networks With Diverse Manually Curated Data (in preparation).

Xavier JC, Patil KR, Rocha I. Essential And Ancestral Metabolic Functions In Prokaryotes (in preparation).

Xavier JC, Patil KR, Rocha I. Integration of Biomass Formulations of Genome-scale Metabolic Models with Experimental Data Reveals Universally Essential Cofactors in Prokaryotes (submitted).

Xavier JC, Patil KR, Rocha I. Systems Biology Perspectives on Minimal and Simpler Cells. Microbiology and Molecular Biology Reviews 2014, 78:487–509.

1.4.2 Conference Presentations

Xavier JC, Patil KR, Rocha I. Universally Essential Cofactors in Prokaryotes. Oral presentation delivered at the IV Constraint-Based Reconstruction and Analysis (COBRA) Conference, 2015. Heidelberg, Germany.

Xavier JC, Patil KR, Rocha I. Integration of biomass functions of genome-scale metabolic models with experimental data reveals universally essential cofactors in prokaryotes. Poster presentation delivered at the Metabolic Pathways Analysis Conference, Biochemical Society, 2015. Braga, Portugal.

Xavier JC, Patil KR, Rocha I. Standardization and comparison of the biomass objective functions of manually curated genome-scale metabolic models. Poster presentation delivered at the III Constraint-Based Reconstruction and Analysis (COBRA) Conference, 2014. Virginia, U.S.A.

Xavier JC, Patil KR, Rocha I. Systematic comparison of essential reactions in manually curated genome scale metabolic models. Poster presentation delivered at the III Copenhagen Biosciences Conferences, Cell Factories and Biosustainability, Novo Nordisk Foundation, 2013. Hillerød, Denmark.

Xavier JC, Patil KR, Rocha I. Analysis of minimal metabolic networks through whole-cell *in silico* modelling of prokaryotes. Poster presentation delivered at the XI Jornadas de Bioinformatica, 2012. Barcelona, Spain.

1.4.3 Invited Talks

Xavier, JC. “From Bioengineering to Systems Biology and back: an insider’s perspective”. 5th Symposium on Bioengineering, 2013. Porto, Portugal.

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CHAPTER 2

Systems Biology Perspectives on Minimal and Simpler Cells

The true causes of natural effects and of the phenomena we observe are often so far from the principles on which we can rely and the experiments we can make that one is obliged to be content with probable reasons to explain them.

—ÉMILIE DU CHÂTELET, *Institutions De Physique* (1740)

The concept of minimal cell has fascinated scientists for a long time, from both fundamental and applied points of view. This broad concept encompasses extreme reductions of genomes, the last universal common ancestor (LUCA), the creation of semi-artificial cells and the design of protocells and chassis cells. In this chapter, with a focus on systems biology, these different areas of research are reviewed and common and complementary aspects of each are identified. The classical top-down and bottom-up approaches towards minimal cells are discussed together with the so-called middle-out approach, with its innovative mathematical and computational modeling contributions. The also-classical genomics view that emphasizes minimal genomes, or rather minimal gene sets, is contrasted with the recent fundamentally expanding views of the minimal gene set as a backbone of a more complex system - the progress being made in understanding the system-wide properties at the level of transcriptome, proteome and metabolome. Network modeling approaches are enabling integration of these different omics datasets towards understanding the complex molecular pathways connecting genotype to phenotype. The key concepts central to the mapping and modeling of this complexity are reviewed, which are at the heart of research on minimal cells. Finally, the distinction between minimizing the number of cellular components and minimizing cellular complexity is discussed, towards an improved understanding and utilization of minimal and simpler cells.

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2.1 Introduction

As recognized in the beginning of the current era of molecular systems biology, a cell could be as simple as we could define life in its simplest form (Szostak et al. 2001). Indeed, all known life forms have the cell as their basic unit. On the other hand, the cell is the most complex structure known to man in the micrometer size range (Fehér et al. 2007). Despite several achievements in identifying and characterizing the molecular constituents of life, we are far from understanding how these constituents interact with each other, giving rise to a robust and self-replicating system. Also, there is not a widely accepted theory of how the first cells arose on Earth or a complete synthesis from scratch of simpler living cells achieved in the laboratory. Therefore, at present, the minimal cell can only be defined at a semi-abstract level as a living cell with the minimal and sufficient number of components (Henry et al. 2010) having three main features: i) some form of metabolism to provide molecular building blocks and energy necessary for synthesizing the cellular components; ii) genetic replication from a template or an equivalent information processing and transfer machinery; and iii) a boundary (membrane) that separates a cell from its environment. To this definition it could be added the necessity of coordination between boundary fission and the full segregation of the previously generated twin genetic templates. Another fundamental characteristic that could be added to the essential features of a minimal cell is the ability to evolve, which is a universal characteristic among all known living cells (Umenhoffer et al. 2010).

From a physicochemical perspective, the minimal cell portrays the transition from non-living to living matter, which can refer to the transition that occurred during the origin of life that preceded the evolution of species on Earth, as well as the transition that is expected to be attained in the laboratory with the creation of an artificial living cell (Rasmussen et al. 2004). The result of the former transition, usually called the last universal common ancestor (LUCA), universal common ancestor, last common ancestor or cenancestor, roots the currently accepted tree of life from which all life forms are supposed to have evolved (Doolittle 1999, Theobald 2010a). The hypothetical laboratory transition forms the basis of the concept of artificial cells: minimal cells fully created in the laboratory from known parts. It is often difficult to separate the concept of artificial cell from that of semi-artificial cell that is, to some degree, built from biogenic parts. The pioneering work by J. Craig Venter's team is perhaps the best example of a semi-artificial cell, having reported the first functional cell with its genetic material being an artificial, *in vitro* synthesized chromosome (Gibson et al. 2010).

Because of its interdisciplinary nature, the work on minimal cells has been closely linked with several lines of research including minimal genomes, protocells, models of minimal cells, and chassis cells, as shown in **Table 2.1**.

Table 2.1 – Concepts relating to minimal or simpler cells.

Concept/Construct	**Short definition**	**Scientific landmarks**	**Reviews**
Minimal genome	A simplified genome without non-essential genes (given specific environmental conditions).	(Gil et al. 2004, Hutchison et al. 1999, Mushegian & Koonin 1996)	(Dewall & Cheng 2011, Fehér et al. 2007, Koonin 2000, Moya et al. 2009, Mushegian 1999)
LUCA (Last Universal Common Ancestor)	A life form commonly accepted to have existed before the divergence of Bacteria, Archaea and Eukarya domains. Hypothesized to have been inorganically hosted (Russell & Hall 1997).	(Harris et al. 2003, Mirkin et al. 2003, Theobald 2010a)	(Chen 2006, Delaye et al. 2005, Lazcano & Miller 1996, Morange 2011, Penny & Poole 1999, Zimmer 2009)
Chassis cell	A cell designed for use in industrial production processes, with a high degree of controllability and efficiency.	(Ara et al. 2007, Mizoguchi et al. 2007, Morimoto et al. 2008, Umenhoffer et al. 2010)	(Foley & Shuler 2010, Vickers et al. 2010)
Artificial/Semi-artificial cell	Cells built in the laboratory (at least partially) with resource to extant genetic and other biological material.	(Gibson et al. 2008, 2010)	(Jewett & Forster 2010, Murtas 2009, Pohorille & Deamer 2002, Porcar et al. 2011, Rasmussen et al. 2004)

Table 2.1 – Concepts relating to minimal or simpler cells (continued)

Minimal cell models	Protocells	*In vitro* models of a minimal cell, usually containing some kind of biological material encapsulated in liposomes or other lipidic vesicles.	(Chen et al. 2004, Hanczyc et al. 2007, Huang et al. 2013, Oberholzer et al. 1995)	(Solé 2009, Solé et al. 2007, Szathmáry et al. 2005)
	In silico minimal cell models	Virtual model/reconstruction of any of the possible constructs described above, or, any other model of a minimal "ome" relevant to the study of the minimal cell.	(Castellanos et al. 2004, Flamm et al. 2007, Gabaldón et al. 2007, Gánti 1975, Karr et al. 2012, Shuler et al. 2012, Surovtsev et al. 2009)	(Stelling 2004, Tomita 2001)

Minimal cell models, as the name indicates, refer to any construct that exhibits certain characteristics of biological cells while being considerably simpler in its nature. The simplicity of such constructs permits a detailed study of the biological characteristics of interest. Minimal cell models comprise physical constructs - protocells, as well as theoretical models, based on mathematical and/or computational descriptions that capture certain features of the living cells (Solé et al. 2007). Protocells are compartmentalized assemblies based on lipidic vesicles, polymeric or polypeptide capsules, colloidosomes, coacervates, and others, as reviewed in (Huang et al. 2013) that usually encapsulate biological material, such as organic chemicals, proteins or RNA. Considered as models of transition states towards fully functional living cells, protocells are mainly developed for studying the emergence of biological characteristics such as self-organization and replication in simpler assemblies of biochemical entities.

The concept that relates to the minimal cell from a more applied angle is that of the chassis or platform cell. The chassis cell can be defined as a cell with reduced complexity designed for one or several biotechnological applications, and that can be modified and controlled with precision and in a predictive manner (Vickers et al. 2010). Although studies towards minimal cells often have claimed both scientific and technological purposes, often the two aims are incompatible. For example, bacterial cells that have evolved the smallest genomes in nature show slower and less efficient metabolism with low division rates, features that are opposite to those desired in a chassis cell (Foley & Shuler 2010, Vickers et al. 2010). Thus, the chassis cell will need to achieve a trade-off between the simplicity or minimality needed for predictive manipulations and the complexity needed for robustness and efficiency.

In this review, the various concepts and approaches related to the research on minimal cells are further discussed from a systems biology perspective. The plural terms 'minimal cells' and 'simpler cells' are preferred, as many configurations of each seem to be possible, given the high functional redundancy observed in biological networks.

2.1.1 A Systems Biology Perspective on Minimal Cells

Besides being the focus of fundamental and applied research for a long time, minimal genomes have been quasi-synonymous of minimal cells since the sequencing of *Mycoplasma genitalium,* in 1995 (Fraser et al. 1995). *M. genitalium* is so far considered as the microbe with the smallest autonomously replicating genome (~580 kb) that can be grown in laboratory cultures (Fraser et al. 1995). Recently, the focus of the minimal cell research has been expanding beyond the genome, as high-throughput technologies are enabling system-wide quantification of other bio-molecules. These mainly include proteomics, lipidomics, metabolomics and fluxomics. The exponential growth of different omic datasets and computational models has been helping biologists to integrate those data and to predict the behaviour of whole cells. The study of life, and consequently, of minimal cells is thus facing a new paradigm, with systems biology starting to be accepted as an approach that puts biology closer to the other natural sciences, by establishing laws and making quantitative predictions (Westerhoff et al. 2009).

2.1.2 Minimal or Simpler Cells?

When discussing minimal cells there is frequently an association of two different concepts. The first relates minimal cells with the smallest number of components, implying cells with a small number of genes and expressed proteins.

The second concept centers on the smallest complexity and connotes so-called simpler cells, cells with a behavior easier to predict and easier to manipulate. While the minimality in terms of the number of components is relatively straightforward to measure with genome sequencing and other high-throughput technologies, the quantification of complexity is yet to be tackled. For example, the number and dynamics of the interactions between different bio-molecules can be regarded as indicators of a cell's complexity (Bonchev 2004). However, the technologies for mapping bio-molecular interactions in a system-wide manner are yet to mature (Bouveret & Brun 2012).

As the relationship between the number of components in a system and the system's complexity is often non-linear, the minimal cell may not necessarily be the simplest cell. Therefore here the literature is reviewed concerning both concepts. First, systems with a smaller number of components are reviewed – from the minimal genome to the minimal proteomes and minimal nutritional requirements. Next, the special cases of LUCA and chassis cells are analyzed. Following, different systems level approaches towards minimal and simpler cell-constructs are explored, namely Top-Down, Bottom-Up and the Middle-out/Integrative approach. The last section discusses the importance of considering complexity in a holistic approach to minimal cells and the contribution of systems biology to attaining this goal.

2.2 Towards the Smallest Number of Components

Finding the smallest number of components required to constitute a living cell is the classical approach to understand and create minimal cells. One of the fundamental distinctions to be made here from the systems biology perspective is between a minimal set of components and a minimal "ome". This distinction was introduced early in 1996, with the first comparative approach between two full genomes (Mushegian & Koonin 1996). A (minimal) genome, proteome or another ome, is the full, functional set of components within a (minimal) living cell – either sequenced, enumerated or even not fully accessible yet as the case of the metabolome (van der Werf et al. 2007). On the other end of the spectrum, a (minimal) set is theoretical, derived from comparative or analytical studies, and has not been proved to be functional in a living cell.

2.2.1 Minimal Genome

As the genome was the first available "ome" in cell-level systems biology, searching for the smallest functional genome represents most of the state of the art in minimal cells. One comprehensive definition of minimal genome was given by Koonin: "the smallest possible group of genes sufficient to sustain a functional cellular life form under the most favourable conditions imaginable, that is the presence of a full complement of essential nutrients and the absence of environmental stress" (Koonin 2000). The phrase "most favourable conditions" should be emphasized, which in practice indicates that one minimal cell may have extremely complex nutritional requirements. The smallest prokaryotic genomes sequenced to date belong to species not considered autonomously alive that, while missing essential genes, became entirely dependent on much more complex hosts – insects (McCutcheon et al. 2009a). "*Candidatus* Carsonella ruddii" has an impressive 160-kb genome (Nakabachi et al. 2006) and "*Candidatus* Hodgkinia cicadicola" an even smaller one with 144 kb, which leaves scientists at the edge of considering them organelles, as in the case of mitochondria and chloroplasts (Tamames et al. 2007). The genome of "*Candidatus* Carsonella ruddii" lacks genes involved in cell envelope biogenesis and metabolism of nucleotides and lipids (Nakabachi et al. 2006) and also in DNA replication, transcription and translation, essential for any bacterial cell to live autonomously (Tamames et al. 2007)**.** However, achieving a minimal genome implies that the microorganism containing it should be accessible to current isolation and cultivation techniques without the aid of another living host, as emphasized by Mushegian when defining a minimal genome as the "smallest number of genetic elements sufficient to build a modern-type free-living cellular organism" (Mushegian 1999). As mentioned above, the natural smallest genome capable of autonomous growth or laboratory cultivation in pure culture and also in a defined medium (Yus et al. 2009) is the one of *M. genitalium* with 580 kb (Fraser et al. 1995).

The first theoretical minimal gene-set was proposed by Mushegian and Koonin, based on a system-wide comparison of *Haemophilus influenzae* and *M. genitalium* genomes, consisting of 256 genes (Mushegian & Koonin 1996). Later one integrative study utilized a larger dataset, including results from both experimental and computational approaches to the minimal genome and predicting a set of 206 genes for a theoretical minimal gene set (Gil et al. 2004). This minimal gene set included genes for DNA replication, repair, restriction and modification; a basic transcription machinery; aminoacyl-tRNA synthesis, tRNA maturation and modification; ribosomal proteins, ribosome function, maturation and modification; translation factors; RNA degradation; protein processing, folding and secretion; cellular division; transport; and energetic and intermediary metabolism (glycolysis, proton motive force generation, pentose phosphate pathway, lipid metabolism, biosynthesis of nucleotides and cofactors). The authors did not include rRNA or tRNA genes, and they recognized that the basic substrate transport machinery could not be clearly defined, even though this minimal cell would rely highly on the import of several substrates, including all the 20 amino acids (for which it had no biosynthetic ability). Theoretical minimal gene sets will need to be tested *in vivo* to qualify as minimal genomes. The technology to synthesize full genomes has been developed only very recently and it has not yet been applied towards this goal (Gibson et al. 2010).

Determining a minimal gene-set is frequently associated with predicting which genes are essential for a species. *M. genitalium* was the first to be analysed in a large scale essentiality assay, with between 265 to 350 genes being identified as essential (Hutchison et al. 1999). Proof of gene dispensability, however, requires isolation and characterization of pure clonal populations, which was not done in this study. This gap was later filled by the same team, which identified 382 essential genes; the difference in the number of essential genes might have occurred not only due to mutant complementation in the previous approach, but also due to different media conditions (Glass et al. 2006). Several other prokaryotes were targets of genome-wide essentiality studies, either for antibiotic design or antimicrobial control, providing important datasets for benchmarking results. These include *Acinetobacter baylyi* (de Berardinis et al. 2008), *Caulobacter crescentus* (Christen et al. 2011) *Francisella novicida* (Gallagher et al. 2007), *Haemophilus influenzae* (de Berardinis et al. 2008) *Helicobacter pylori* (Salama et al. 2004) *Salmonella enterica* serovar Typhimurium (Langridge et al. 2009) *Staphylococcus aureus* (Chaudhuri et al. 2009, Forsyth et al. 2002) *Neisseria meningitidis* (Mendum et al. 2011) and *Vibrio cholerae* (Cameron et al. 2008). Both DEG (Zhang & Lin 2009) and OGEE (Chen et al. 2012) databases centralize much of these data.

Essential gene sets obtained by determining all viable single-knock-outs of a species are always a subset of a possible minimal genome, due to synergistic effects. In other words, these sets exclude genes that are not essential when deleted individually, but which cause cell death when deleted simultaneously, also termed synthetic lethals. Higher-structure chromosomal effects will also not be evident when deleting genes individually, as reviewed in (Fehér et al. 2007). Also, essential gene sets usually lack essential non-coding sequences that would be part of a minimal genome, as essential promoter regions, tRNAs, small non-coding RNAs and other non-coding sequences with unknown but essential function. A recent genome-scale essentiality study identified and described 130 essential non-coding elements of *Caulobacter crescentus*, including 90 intergenic segments of unknown function (Christen et al. 2011).

It is now commonly accepted in the scientific community that multiple minimal genomes can exist. Currently known prokaryotic genomes are complex and highly adapted, exhibiting functionally equivalent components with different evolutionary origins, named non-orthologous displacements (NODs). In order to reduce the number of potential combinations, one rational direction is to identify a minimal genome for a number of functional niches, or to determine which is the minimal gene set for a thermophilic autotroph, a mesophilic heterotroph, among others (Koonin 2000).

2.2.2 Other Minimal Sets of Components

The cell-level evaluation of components other than the genome includes functional inferences from the genome at the protein level, directly generating theoretical minimal proteomes by assuming a general translation from the genome. Recently, this functional inference has allowed other omic approaches that analyse whole sets of specific genetic sequences. One example is the comparison of the complete sets of tRNA isoacceptors (tRNomics) and tRNA/rRNA modification enzymes (modomics) in all sequenced Mollicutes, a class of bacteria that lacks cell wall and includes the genera Mycoplasma (de Crécy-Lagard et al. 2007). In this study, it was shown that the organisms have developed different strategies to minimize the RNA component of the translation apparatus. Even given a good representation of the RNA modification enzymes in the genomes of these bacteria (up to 6% in *M. genitalium*), only 9 enzymes were identified as more resistant to loss in Mollicutes (de Crécy-Lagard et al. 2007). This finding indicates that even in extremely reduced genomes, for the most basic processes of the cell, as translation and codification, different strategies can be adopted.

Recently, the whole methylomes of *M. genitalium* and *Mycoplasma pneumoniae* were analysed at a single-base resolution, suggesting a potential role for methylation in regulating the cell cycle and gene expression in these reduced bacteria (Lluch-Senar et al. 2013). On another study, the whole transcriptome of *Prochlorococcus* MED4 - the smallest known photosynthetic organism considering both genome and cell size – was analysed with a focus on the effects of the light cycle (Zinser et al. 2009). It was found that 90% of the annotated genes of this species were expressed in some condition, and 80% showed cyclic expression together with the light-dark cycle, including genes involved in the cell cycle, photosynthesis and phosphorus metabolism. While the measurements of the proteome and the metabolome are not available for *Prochlorococcus*, transcriptomics allowed *per se* the identification of specific metabolic transitions and possible regulatory proteins for these minimal photosynthetic bacteria (Zinser et al. 2009).

Minimal protein sets have recently begun to be inferred by integrating experimental data. This meant a step in moving from the functional inference from minimal genomes toward a real assessment of minimal proteomes. Pioneer works included the comparison of 17 prokaryotic genomes integrating a database of experimentally determined unique peptides to define a core proteome (Callister et al. 2008). The authors predicted 144 orthologs for the core genome, from which ~74% were actually expressed in all species. More than half of this core proteome was related with protein synthesis, but strikingly, 10 proteins had not been functionally characterized. This study also identified differences in the proteomes associated with the different lifestyles of the bacteria analysed, concluding that the phenomenon of phenotypic plasticity has an impact on the minimal proteome, which could not be accessed simply by comparing genomes (Callister et al. 2008). In another work, the proteomes of *Acholeplasma laidlawii* and *Mycoplasma gallisepticum* were analysed by 2D electrophoresis, matrix-assisted laser desorption/ionization (MALDI) and liquid chromatography/mass spectrometry (LC-MS) (Fisunov et al. 2011) and compared to the proteome of *Mycoplasma mobile* obtained in another study (Jaffe et al. 2004). Clusters of Orthologous Genes (COGs) were used to compare both genomes and proteomes of the three Mollicutes species (Fisunov et al. 2011). 212 COGs were identified as part of the core proteome, including DNA replication, DNA repair, transcription and translation and molecular chaperones. Some metabolic pathways were also represented in this core proteome, including glycolysis, the non-oxidative part of the pentose-phosphate pathway, glycerophospholipid biosynthesis, and the synthesis of nucleoside triphosphates (Fisunov et al. 2011). One surprising finding was the low conservation of proteins related to cell division, as only two proteins were conserved in the core: FtsH and a Smc-like protein. Strikingly, *M mobile* does not even contain FtsK or FtsZ in its genome, which indicates that the essential process of cell division has greater plasticity than other cellular systems (Fisunov et al. 2011). Building up on results of another study of the interactome of *M. pneumoniae* (Kühner et al. 2009), the authors concluded also that most COGs in the Mollicutes core proteome - 140 - are expected to associate in protein complexes, and 54 COGs are predicted to participate in more than one complex (Fisunov et al. 2011). Due to secondary functions of such complexes as the maintenance of overall cellular stability (and particularly genome stability) which could explain the maintenance of incomplete metabolic pathways in reduced genomes, the authors propose that the concept of minimal genome would be treated not as a set of essential functions but as a set of essential structures (Fisunov et al. 2011).

Another system that can be analysed at the cell-level is the metabolic network of an organism. Given that the whole metabolome is still not accessible due to technological limitations, studies in this area are mainly computational. A minimal metabolic network of 50 enzymatic reactions was derived from the theoretically-inferred minimal gene set of Gil et al. (Gil et al. 2004); it was shown that the encoded metabolism was consistent and the network's topological parameters were similar to others of natural metabolic networks (Gabaldón et al. 2007). Another work performed data-mining on the KEGG Pathways database, in an effort towards obtaining a minimal anabolic network and the correspondent minimal metabolome for a reductive chemoautotroph (Srinivasan & Morowitz 2009). The resulting metabolic network comprised 287 metabolites, more than half being intermediates in the biosynthesis of monomers.

Recently, a series of three papers reported a variety of analyses for *M. pneumoniae,* a genome-reduced bacterium. These include the determination of the proteome (Kühner et al. 2009), the transcriptome (Güell et al. 2009) and a metabolic network that allowed the identification of a minimal medium that supported growth of *M. pneumoniae* as well as of *M. genitalium* (Yus et al. 2009). This series was a pioneering step forward in the integration of omes other than the genome in the minimal cell panorama and also in using the power of a holistic, system-perspective in the study of one single species.

The work on minimal omes other than the genome facilitates the analysis of the impact of different environmental conditions in the minimal sets, mainly through transcriptomics and expression proteomics (Callister et al. 2008). Also, proteomics permits the insight into the spatial organization of minimal cells, by analysing which protein complexes are assembled and which structural functions these could have (Fisunov et al. 2011, Kühner et al. 2009). On the negative side, environmental-dependent cell-level analyses are more prone to errors than genome sequencing. The technology for expressional proteomics is still under development and proteins with extreme physical and chemical properties, as low mass and high hydrophobicity, including membrane-proteins, can be under-represented in these assays (Chandramouli & Qian 2009). Moreover, some proteins might be dispensable under optimal growth conditions and expressed only in specific stress conditions. This will decrease the size of core transcriptome and proteome if the experimental setup does not include sufficient diversity.

2.2.3 Minimal Environmental Conditions for Life

Evolution enabled many alternative ecological niches and nutritional pathways for prokaryotes, and there is no experimental or even conceptual support to the existence of just one form of a minimal prokaryotic cell from a metabolic point of view, as recognized by Szathmáry (Szathmáry 2005), Koonin (Koonin 2000) and Gil et al (Gil et al. 2004). Many minimal metabolic networks adapted to different habitats could sustain the universal genetic machinery – the translation and transcription apparatus that are usually more conserved and similar among distant prokaryotes. Depending on environmental conditions like temperature, pH and salinity, and especially on the nutrients available in a specific niche, organisms could differ substantially and still have a reduced number of genes. Here, an important minimal set, almost absent in the scientific literature, comes to scene as a major player in the study and design of minimal cells - the minimal, defined media able to sustain such cells. The minimal medium is not a biological component *per se*, but it is an emergent biological property that directly reflects the degree of dependency of the cell on the environment.

Currently there are no comprehensive comparative studies about the different minimal nutritional requirements of different prokaryotic organisms. However, there is a variety of old studies that seem to have been relatively forgotten. A good example is the extensive work started in the 50s by MacLeod and co-authors about minimal nutritional requirements of marine bacteria (Macleod et al. 1954, Wong et al. 1969). The authors explore and present several combinatorial possibilities for the composition of defined media, mentioning special needs for amino acids as sole carbon sources or as supplements in addition to non-amino acid sources of carbon and energy, and also identifying special needs for ions, vitamins and other growth factors (Macleod et al. 1954). Bryant and Robinson reviewed work on nutritional requirements of ruminal bacteria and corroborated in their study the conclusions that volatile fatty-acids are essential for the growth of several of these organisms, as well as ammonium, which is required regardless of the amount of amino acids and peptides present in the media (Bryant & Robinson 1962).

The study of mutations leading to specific auxotrophies in bacteria started also several decades ago, way before the DNA structure was discovered (Roepke et al. 1944). Fundamental for the identification of the different steps of metabolic pathways, the classical study of auxotrophies is also central for the study of minimal or simpler cells by identifying possible pathways for viability after gene inactivation.

Old studies on nutritional requirements also include the interesting finding that minimal nutritional requirements increase with extreme temperatures for strains of *Lactobacillus arabinosus* (Borek & Waelsch 1951), *Escherichia coli* (WARE 1951) and several strains of thermophilic *Bacilli* (Campbell & Williams 1953). This implies that genome reductions starting from those species will have to take into account the conditions the cells will face in artificial cultures.

Extensive nutritional requirements were predicted for the earlier theoretical minimal gene sets, including all amino acids, nucleotides, fatty acids and complex coenzymes (Mushegian & Koonin 1996). The size of a minimal medium is therefore not a limiting factor when designing and deriving theoretical minimal cells, as long as it does not require other living cells (it remains an axenic culture). However, it certainly becomes a limitation for industrially-relevant chassis cells, which shall be efficient and profitable (see section Chassis Cells). Both organisms most used in minimal cell studies for biotechnological applications - *E. coli* and *Bacillus subtilis* - are facultative anaerobes, highly versatile organisms with relatively simple nutrient requirements (Clements et al. 2002). Indeed, *E. coli* has probably the simplest growth requirements known so far: a medium composed of as little as seven substances corresponding to eight components - Disodium Phosphate, Monopotassium Phosphate, Sodium Chloride, Ammonium Chloride, Magnesium Sulphate, Calcium Chloride and one carbon source - can sustain growth (Joyce et al. 2006). However, it should not be put aside that some trace metals are also considered essential, although not added to the medium, as they are present in sufficient amounts in water: copper (Rensing et al. 2000), nickel and cobalt (Bleriot et al. 2011) molybdenum (McLuskey et al. 2003), iron (Semsey et al. 2006), manganese (Jakubovics & Jenkinson 2001) and zinc (Lee et al. 2005). All these components together make probably the simplest growth requirements known so far for prokaryotes. An extensive review about nutritional requirements of microorganisms used in fermentation processes covers interesting points, as why each of the principal elements is needed for cell's physiology, the major requirements (carbon, nitrogen, sulphur, trace elements, vitamins and other growth factors) and also physicochemical constraints to growth, such as pH, ionic strength and the effect of concentrations on growth rates (Kampen 1997).

Defining minimal media for minimal cells requires also a definition of a minimal threshold of growth rates. Achieving a clear exponential phase might not be a necessity for the fundamental pursuit of a minimal/simpler cell, while for biotechnological applications minimalism will have to cope, in a more complex trade-off, with a minimum yield in biomass and a minimum specific growth rate.

It is estimated that only approximately 1% of bacteria on Earth can be readily cultivated *in vitro* (Vartoukian et al. 2010). With this lack of technological capabilities regarding cultivation of prokaryotic cells, there is a great possibility that simpler organisms with more complex requirements might go unnoticed. Organisms that cannot be maintained in a Bacteriology Culture Collection, not even in the richest media known, are commonly named *Candidatus* (Murray & Stackebrandt 1995). This is a useful term that is not completely implemented within the scientific community. There are no reports of the cultivation of *Buchnera aphidicola* without insect cells (Douglas et al. 2010, Gosalbes et al. 2008), however as this genus was discovered before the implementation of this nomenclature and there is sufficient biochemical information available about it, it is not named as *Candidatus* (Gil et al. 2002). While in many cases unknown nutritional requirements are the reason for the impossibility of cultivating an organism *in vitro*, *Candidatus* species may also require their host's cells due to unknown physical constrains.

Until recently, *M. genitalium* was hard to grow in defined media and efforts were made with genome-scale metabolic modelling to calculate the best composition of such medium (Suthers et al. 2009a, Yus et al. 2009). Those system-level approaches are certainly a promising direction in the field of estimating prokaryotic minimal nutritional requirements.

2.3 LUCA and the First Cells

Since the first proposal of the common ancestry theory, described by Charles Darwin in his seminal book *On the Origin of the Species by Means of Natural Selection or the Preservation of Favoured Races in the Struggle for Life* (Darwin 1859), much has been debated and speculated about the origin of life, and the nature of a possible cell or set of cells that had preceded the evolution of the three main lineages of the life forms known today - Archaea, Bacteria and Eukarya. The strongest support for this theory comes from the shared biological features of the three domains, including double-stranded DNA to encode genetic information, transcription to RNA, translation to proteins that are the universal operators of cellular functions, lipidic membranes and primary metabolism, among others. Other evidence include the high homologies of biological structures with different functions, indicating divergent evolution from a common ancestor; the congruence of morphological and molecular phylogenies; the agreement between phylogeny, the paleontological record and biogeography; and the hierarchical classification of morphological characteristics (Theobald 2010a).

A recent theoretical work (Koonin & Martin 2005) goes through the subject of LUCA's appearance, making a vital connection between the theory of an inorganically hosted origin of cells (Russell & Hall 1997) and the origin of genomes. The hypothesis of the inorganically hosted LUCA was first posed in 1997 by Russel and Hall, with the premise that it was based on "what life does rather than what life is" (Russell & Hall 1997). It was a detailed, complex description of 17 stages of geochemical transformation in a submarine hydrothermal spring where iron monosulfide bubbles were the hatcheries for the first cells. In a later publication, Russel and Hall, together with Mellersh, developed significantly the geochemical details of the theory, specifically on the implications of temperature and energetics in the primitive origin of cells (Russell et al. 2003). In the same year, more biochemistry was incorporated in the theory, including a comparison of the amino acid sequences of the enzymes of glycolytic pathways in eukaryotes and prokaryotes and a simplification of the visual model of the origin of life in hydrothermal vents (Martin & Russell 2003). Claiming that the first free-living cells were eubacterial and archaebacterial chemoautotrophs that emerged more than 3.8 billion years ago from inorganic compartments (Martin & Russell 2003), this is probably the most accepted theory so far for the origin of life (Koonin & Martin 2005, Martin et al. 2008). The geochemical conditions of early Earth and those of other planets in the solar system where life might have originated are discussed comprehensively elsewhere (Nisbet & Sleep 2001).

It has been proposed that the universal ancestor should have been a fully DNA and protein-based organism with extensive processing of RNA transcripts, have had an extensive set of proteins for DNA, RNA and protein synthesis, DNA repair, recombination, control systems for regulation of genes and cell division, chaperone proteins, and probably lacked operons (Penny & Poole 1999). There is however still uncertainty in the literature on the question of LUCA's genetic machinery having been based majorly on RNA or DNA, and if it had DNA, how it was replicated (Becerra et al. 1997, Poole & Logan 2005). By comparing sequences of proteins involved in DNA replication, it has been proposed that LUCA had a genetic system that contained both RNA and DNA, but the latter was, at the time, produced by reverse transcription (Leipe et al. 1999).

Recently, the first formal tests of the LUCA hypothesis were performed by Theobald, with a statistical evidence corroborating the monophyly of all known life (Theobald 2010a). In his study, the author ignored the commonly assumed sequence similarity as a proof of common ancestry, as sequence similarity can be a result of convergent evolution due to selection, structural constraints on sequence identity, mutation bias, chance, or artefact manufacture (Theobald 2010a). Although this was the first formal attempt towards establishing the LUCA theory with a statistical basis, others claim that the tests performed were not sufficient to reject the alternative hypothesis of separate origins of life (Yonezawa & Hasegawa 2010). Theobald replied with improvements of the models used for the formal test, and emphasizing that his work did not provide an absolute proof for the theory of LUCA, but mentioning several strong arguments in favour of it, as the low sequence requirements for a specific fold and the enormity of the sequence space (Theobald 2010b). Although the alternative hypothesis of separate origins cannot be absolutely ruled out (111, 112), a single common ancestry is currently the best-supported theory for the origin of life. Several extended perspectives and reviews have been published focusing on the issue (**Table 2.1**), while the focus here is on systems approaches concerning LUCA.

A prominent systems biology initiative concerning LUCA is the LUCApedia, a recently-launched online database that integrates different datasets related with LUCA and its predecessors (Goldman et al. 2013). With this database, users working on the LUCA hypothesis have a tool to benchmark their results to other studies predicting the characteristics of LUCA, searching by protein name or id in datasets of COGs, protein domain folds, protein structures, cofactor usage, etc. (Goldman et al. 2013). Comparative studies make up the vast majority of the system-level approaches to LUCA, including a focus on genome sequences (Kyrpides et al. 1999, Mat et al. 2008), protein domains (Kim & Caetano-Anollés 2011, Wang et al. 2007, Yang et al. 2005) and proteome hydrophobicity (Mannige et al. 2012). A comprehensive review concerning comparative genomics and its role in defining LUCA's theoretical gene sets suggests 500-600 genes as an estimate of the genome size of LUCA (Koonin 2003). The comparison of the protein folds of all three domains of life found approximately 50 folds that are present in all three domains (Yang et al. 2005), and one study that used the COGs database obtained 80 COGs present in all organisms studied, across the three domains of life, 50 of which show the same phylogenetic pattern as rRNA (which the authors called three-domain genes) (Harris et al. 2003). From the 50 three-domain genes, 37 were associated with the ribosome in modern cells (Harris et al. 2003). Another interesting study looked at a large set of diverse predicted proteomes to infer on the evolution of hydrophobicity (Mannige et al. 2012). Using the percentage of most hydrophobic residues in proteins, an universal "oil escape" was observed, indicating that LUCA was more hydrophobic than modern cells (Mannige et al. 2012).

One of the major problems when comparing whole genomes or proteomes in order to infer about LUCA's composition arises due to the relatively unknown extents of horizontal gene transfer (HGT) and gene loss (Koonin 2003), which generate phylogenetic trees not compatible with the rRNA phylogenetic tree topology. Mirkin *et al.* analysed the extent of HGT using the COG database to construct trees for all the COGs, finding an approximately equal likelihood for HGT and gene loss events in the evolution of prokaryotic genomes (Mirkin et al. 2003). Although the authors state their intent was not to reconstruct the functional aspects of LUCA but rather to make a preliminary attempt at constructing evolutionary scenarios using comparative-genomics data, they support the plausibility of a set of ~572 genes to be sufficient to sustain a functioning LUCA (Mirkin et al. 2003). Even though this and other studies have approached HGT events and gene losses within the LUCA context (Mirkin et al. 2003, Pál et al. 2005), it is still relatively hard to estimate the extent of the bias they cause in comparative approaches. There might have been genes present in LUCA that were lost before all the major lineages diverged, so when genomes are compared nowadays, those ancestral genes do not appear in the common pool. Also, some genes may not have been present in LUCA but, after originating, spread fast by HGT, being present nowadays in all microorganisms known (Koonin 2003). The presence of *de novo* synthetic pathways in some, but not all prokaryotes, may therefore leave some uncertainty about which were the metabolic routes taken by the universal ancestor.

The transition from organic chemical compounds to cells is still an extremely delicate subject in Biology (Morange 2011). The vast amount of data that modern experimentalists face in a rapidly evolving technological scenario might be the causing agent for a seemingly increasing distance between experimental approaches and the theoretical work taking into account the geochemical context of early life. This gap can be diminished with approaches becoming more holistic. The search for LUCA's minimal omes using evolutionary perspectives will undoubtedly contribute to and benefit from the generic quest for the minimal cell, as the examples mentioned above illustrate. The theory of the inorganically hosted origin of life (Koonin & Martin 2005, Martin & Russell 2003) can shed light on the design of membrane-free minimal cell systems. Similarly, the current discussion on the basis of LUCA's genetic machinery (Poole & Logan 2005) opens a possibility for minimal cell design based solely on RNA genomes. Also, LUCA's studies directly benefit from those of minimal cells: while minimal gene-sets are theoretical and do not explicitly incorporate evolution, comparative genomics is based on orthology and should approximate the resulting minimal gene sets with those of ancestral life forms (Koonin 2003).

2.4 Chassis Cells

Probably the most proclaimed reason for the recent interest in minimal cells and the related minimal datasets (e.g. the minimal genome and minimal metabolic networks) has been the potential for biotechnological applications. When referring to a minimal cell that is intentionally simplified for use in industry, the terms platform cell or factory cell (Foley & Shuler 2010) or the term chassis cell (Vickers et al. 2010) are preferred. This conceptual construct is of extreme importance for biotechnology industries as it implies more specialized and more comprehensible cells for bio-based production of industrial chemicals and pharmaceuticals.

Microbial cells have shown to be extremely profitable in many applications, thanks to the catalytic power of enzymes and also the large panoply of products they can synthesize. Nevertheless, these cell factories still remain, to a large extent, black boxes that often surprise engineers. In industrial bioprocesses, as opposed to scientific discovery, no surprises are desired and total control over a specially designed and fully comprehensible chassis-cell is the ultimate goal. This fact has led some to argue that a minimal cell would be directly interesting for industry, due to its supposed simplicity; however, this is highly debatable as shown in **Box 2.1**, where the predicted requisites of a chassis cell are enumerated, based on two recent, comprehensive reviews (Foley & Shuler 2010, Vickers et al. 2010).

Box 2.1 – Requirements for an industrially relevant chassis cell

- Overall simplicity
- Minimal number of carbon sinks and other non-optimal flux paths
- Predictable metabolic and regulatory networks (more control over growth and production)
- Simplified translation code
- Reduced genetic drift and limited evolvability
- Robust mechanisms for genome replication, cytokinesis and coordination in between
- Robust cell membrane and cell wall that confers resistance to shear stress in bioreactors
- Efficient transcription, translation and regulation for optimization of cellular fluxes to desired goals
- Availability of predictive mathematical models that save expensive trial resources.
- Process-specific modules for implementation of different industrial solutions (particular for each process).
- Other stress tolerance mechanisms, as
  - Product tolerance
  - High-substrate tolerance
  - Tolerance to low O_2

One of the facts that can be controversial when comparing industrially driven to scientifically driven minimal cells is the necessity to evolve – some have argued that, ideally, no mutation would occur on a chassis cell (Umenhoffer et al. 2010). A recent study has proposed that evolvability is inevitable and can actually increase without any pressure for adaptation in a population model, given that it is the result of the exploration of the genetic space (Lehman & Stanley 2013). Evolution seems to be an inextricable process from DNA replication and it can also be seen as necessary to improve organisms through evolutionary engineering, which major achievements have been reviewed elsewhere (Johannes & Zhao 2006, Lee et al. 2012). In populations of chassis cells that maintain evolvability, optimized pathways and enzymes and better growth rates could be selected for in desired media, either complex or defined.

A chassis cell needs to work on a combination of factors that bounce between simplicity and complexity – precise control often requires simplicity, but energetic and nutritional efficiencies and productivity mean complex pathways within relatively large networks. Model organisms like *E. coli* and *B. subtilis*, which are well studied and display robust growth, have been preferred as objects of genome-reducing approaches for chassis cells (Ara et al. 2007, Mizoguchi et al. 2007, Pósfai et al. 2006, Umenhoffer et al. 2010). When speaking about an industrial biotechnology process, even the complexity of an eukaryote can be accepted as the minimum simplicity, e.g. if the synthesis of eukaryotic proteins is desired (Giga-Hama et al. 2007).

Several large projects of genome reduction of industrially relevant prokaryotes have achieved satisfactory results so far. *B. subtilis* MGIM, based on a ~1Mbp deletion from *B. subtilis* 168, showed little reduction in growth and comparable enzyme productivity (Ara et al. 2007). *B. subtilis* MBG874 was achieved after a depletion of 874 kb (20% of the original genome size), showing a reorganization of the gene expression network and productivities of extracellular cellulase and protease 1.7 and 2.5-fold higher than those of wild-type cells, respectively (Morimoto et al. 2008). *E. coli* MGF-01 was obtained after successive deletions of genomic fragments from *E. coli* K12 (a total deletion of about 1 Mbp or 22% of the genome) and showed improved growth and higher threonine productivity when compared to the wild-type strain (Mizoguchi et al. 2007, 2008). *E. coli* MDS42, obtained after a 14.3% reduction of the genome of *E. coli* K12, showed genome stabilization and high electroporation efficiency (Pósfai et al. 2006), reduced evolvability (Umenhoffer et al. 2010) and later an 83% increase in L-threonine production after metabolic engineering, comparing with an *E. coli* MG1655 strain engineered with the same modifications (Lee et al. 2009).

Interesting modifications and bottlenecks to be tackled in biotechnological production have been identified using genome-scale network reconstructions (GENREs) (Oberhardt et al. 2009) and future designs of chassis cells might emerge from these. Accurate sub-models of *E. coli* MG1655 have been derived for aerobic, carbon-limited growth on a chemically defined medium with glucose, glycerol and acetate as carbon sources (Taymaz-Nikerel et al. 2010). These models were created from subsets of reactions from the first *E. coli* GENRE (Reed et al. 2003) with the biomass composition as a function of the growth rate (Taymaz-Nikerel et al. 2010). Several other metabolic models have been developed and their applications reviewed elsewhere (Oberhardt et al. 2009). However, when it comes to modelling the dynamics of chassis cells in synthetic biology, the focus has been more on modelling individual modules than whole chassis systems (Andrianantoandro et al. 2006).

It seems evident that for chassis-cell design, an integrative and pragmatic approach is required (**Box 2.1**) along with the best understanding possible of the model organisms to use. Between the widely used *E. coli* and the minimal organism *M. genitalium* there are considerable differences that should be taken into account in time-constrained industrial projects. Even though *E. coli* has ten times more protein coding genes than *M. genitalium*, a search for the species names returns 276 times more abstracts on Medline for the former. The Species Knowledge Index (SKI) is a measure of the amount of scientific literature available for an organism, defined as the number of abstracts on Medline referring to the species, normalized by the number of genes in the genome (Janssen et al. 2005). The SKI index at the moment is 31 times larger for *E. coli* than for *M. genitalium* (**Table 2.2**). Although a larger amount of scientific literature does not necessarily imply more knowledge, it is certainly a good indication that more science exists for *E. coli* than for *M. genitalium,* which will provide a more solid basis for future interventions in the former species. However, it is not only the knowledge about the species that places *E. coli* as a more promising starting point for the development of chassis cells. *E. coli'*s versatility and network redundancy are interesting for industrial processes that often require back-up and alternative metabolic routes in cases of enzyme saturation or the ability to change between substrates. The two bacteria also differ strikingly in their doubling time (**Table 2.2**), which is often a determinant factor in industrial processes. *E. coli'*s fast doubling time has been shown to be related to post-transcriptional control of protein abundances and post-translational control of flux rates (Valgepea et al. 2013). Studies with *Mycoplasma smegmatis* concluded that the organization of regulatory operons involved in regulation of DNA replication and macromolecular synthesis in mycobacteria is very different from the majority of other bacteria, which can introduce problems when trying to control the regulation of these cells (Klann et al. 1998).

Table 2.2 – Comparison of relevant characteristics of *Escherichia coli* and *Mycoplasma genitalium.*

Characteristics of the species	***Escherichia coli***	***Mycoplasma genitalium***
ORFs	4325 (Orth et al. 2011)	482 (Suthers et al. 2009a)
NCBI COGs	2131	362
NCBI Structure direct links	1096	6
DNA content, per mL of cell volume (Chen et al. 2004)	13 mg	100 mg
Doubling time (h) (Vieira-Silva & Rocha 2010)	0.35	12
Species Knowledge Index (Janssen et al. 2005)	47.5	1.53
Characteristics of the *in silico* metabolic network reconstruction	**iJO1366** (Orth et al. 2011)	**iPS189** (Suthers et al. 2009a)
Genes	1366	189
Overall accuracy of gene essentiality predictions	91%	87%
Reactions	2251	262
Metabolic	1473	178
Transport	778	84
Unique metabolites	1136	274
Gene-associated reactions	1310	168
Spontaneous reactions	25	6
Non-gene associated reactions	133	88

2.5 Systems' Approaches for Understanding and Creating Minimal Cells

The relevant systems biology approaches towards the construction or definition of minimal cells can be divided into four broad categories. The first two are the traditional approaches of any systems science or technology, namely Top-down (analytic, deconstruction of systems) and Bottom-up (synthetic, construction of systems), referred in many reviews of the field (Foley & Shuler 2010, Henry et al. 2010, Jewett & Forster 2010, Luisi 2002, Luisi et al. 2006, Moya et al. 2009, Rasmussen et al. 2004, Stano 2011, Szathmáry 2005). Both of these classical approaches have comprised mainly physical or experimental studies, *in vivo* in the case of top-down or *in vitro* in the case of bottom-up. Here the Middle-out approach is introduced, which includes large-scale data integration, modelling and simulations, relevant to the study of minimal or simpler cells. Following Denis Noble's definition, the Middle-out approach considered here is the one that "starts at any level [...] at which there are sufficient data and reaches (up, down and across) towards other levels and components)" (Noble 2002). The fourth category is occupied by system-level comparative studies, the first to be used at a system-level towards minimal cells (Mushegian & Koonin 1996) – and probably still the most used approach today in systems biology of minimal cells (Gupta et al. 2008, Koonin 2003).

Almost a decade ago Eörs Szathmáry highlighted the importance of bridging the gap between both the bottom-up and top-down approaches, but also between experimental and theoretical studies (Szathmáry 2005). In an attempt to organize the sparse and diverse knowledge in the long pursuit of minimal life, the diversity of relevant studies is reviewed, as depicted in **Figure 2.1.** The classification "experimental" vs "theoretical/computational" is considered to be independent of the 4 major categories presented above. In the following sections, there is also an attempt to associate each approach with the technologies associated and the disciplines it has majorly served, such as the association of the top-down approach with molecular biology, and bottom-up with biophysics and biochemistry. This is a different view from other authors' that associate the minimal cells' quest only with synthetic biology, for instance (O'Malley et al. 2008).

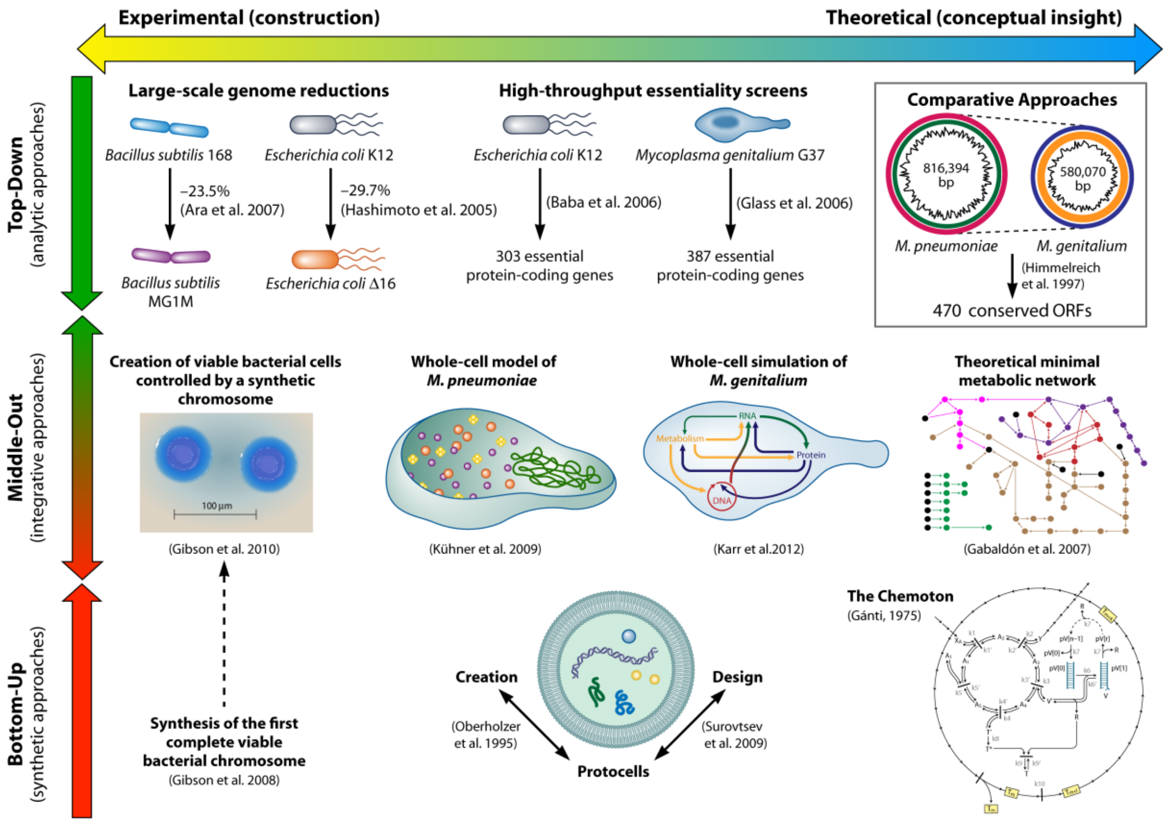

Figure 2.1 – Systems approaches and relevant results towards understanding and designing minimal or simpler cells.

2.5.1 Top-Down Approach

Broadly, top-down implies the removal of non-essential components of the system studied until it is not functional anymore, understanding in this manner each part's individual function within the whole system. Traditionally, it has also been referred to as reductionism and, in minimal cells studies, it has involved mainly trying to define minimal gene sets and minimal genomes (see section Minimal Genome), which was achieved by knocking-out genes to find which were non-essential.

Several techniques have been developed to perform large-scale knock-out studies, as reviewed elsewhere (Gil et al. 2004), including antisense RNA to inhibit gene expression, systematic inactivation of individual genes and massive transposon mutagenesis strategies (the most widely used approach). The recent technological capacity to study synthetic lethality at a genome-scale in *E. coli*, taking advantage of conjugation of deletion or hypomorphic strains to create double mutants (Butland et al. 2008), promises important datasets for the design of reduced strains. As conjugation occurs in other bacteria, it is expected that it will be applied to other organisms (Butland et al. 2008). Metabolic modelling has already been performed to predict synthetic lethals for *E. coli* at a genome-scale, not only for pairs of genes, but also triplets, some quadruples and higher-order lethal combinations (Suthers et al. 2009b).

Simultaneous deletions of large parts of the chromosome were done mainly for model bacteria that are at the same time industrially relevant (see section Chassis Cells). Reductions up to 29.7% of the genome of *E. coli* (Hashimoto et al. 2005) were achieved using the red recombination system of phage lambda (Murphy 1998). Another more recent large-scale deletion technique merged Tn5 transposon mutagenesis with the Cre/loxP excision system and phage P1 transduction (Yu et al. 2002). This method has the advantage of not requiring the construction of genetic vectors or performing complex PCR experiments for each deletion, but so far it only reached a reduction of 7% of the genome of *E. coli* MG1655.

The reduction of genomes occurs naturally in specific habitats, where bacteria adapt drastically to a specific niche, losing several unnecessary genes usually related to the biosynthesis of amino acids and other essential metabolites they can uptake from the stable niche. The natural top-down reduction of the genome of *B. aphidicola* has been raising interest, as this bacterium kept the biosynthetic ability for most amino acids that are provided to the insect host (van Ham et al. 2003). An innovative study analysed the dynamics of natural genome reduction in *Salmonella enterica,* by an experimental evolution procedure of serial passages (Nilsson et al. 2005). The authors obtained deletions of up to 200 kb (approximately 4% of the WT genome), and impressively, two of the large deletions isolated included several genes that were previously identified as being individually essential for growth (Knuth et al. 2004). These results reinforce the need to perform single-deletion studies in different experimental conditions and ultimately, to conduct large-scale simultaneous deletions when studying genome reduction.

Being based on existing natural genomes, top-down approaches can be limiting for drawing universal conclusions about minimalism and simplicity. It has been recognized that, as each study starts with a specific organism, it arrives at a specific minimal gene set (Huynen 2000). Finally, it seems that simplifying existing genomes will always lead to a complex cell with complex means of transcribing and translating its genetic code, and there is a general discussion about if that is indeed the simplest living system possible (Szathmáry 2005).

Table 2.3 enumerates the most relevant species used within the top-down or analytic approach to obtain or understand minimized cells.

Table 2.3 – Prokaryotic species with relevance to top-down, system-level studies towards minimal or simpler cells

Category	**Species**	**Genome Size**	**Special features and studies performed**
Mollicutes Usually parasites, without cell wall. First genomes to be analyzed by global transposon mutagenesis (*M. genitalium* and *M. pneumoniae* (Hutchison et al. 1999)). The same methodology was applied to *Mycoplasma pulmonis* (French et al. 2008). Defined media described for both *M. genitalium* and *M. pneumoniae* (Yus et al. 2009) Different species have been compared at systems-level for genome (Himmelreich et al. 1997), proteome (Fisunov et al. 2011), RNome (de Crécy-Lagard et al. 2007) and methylome (Lluch-Senar et al. 2013).	*Mycoplasma genitalium* G37	580 Kbp	Second genome to be fully sequenced (Fraser et al. 1995), still the autonomously replicating culturable species with the smallest genome. Full genome early analysed by global transposon mutagenesis for essential genes (Hutchison et al. 1999), an experiment re-assessed later with the conclusion that 387 protein-coding and 43 structural RNA genes were essential (Glass et al. 2006). Genome-scale metabolic reconstruction (Suthers et al. 2009a) and integrative whole cell computational model (Karr et al. 2012) available.
	Mycoplasma pneumoniae M129	816 Kbp	A genome-scale *in vivo* assay was performed for this bacterium to determine essential genes for mouse infection, identifying 194 (Sassetti & Rubin 2003). The proteome (Catrein & Herrmann 2011, Kühner et al. 2009), transcriptome (Güell et al. 2009), and metabolic network (Yus et al. 2009) have been analyzed at cell-level. It seems to have a higher fraction of multifunctional enzymes compared to other bacteria (Yus et al. 2009). The transcriptome was shown to be remarkably dynamic and complex (including antisense transcripts, alternative transcripts, and multiple regulators) and more similar to that of eukaryotes than to other bacteria (Güell et al. 2009).
	"*Candidatus* Phytoplasma mali AT"	602 Kbp	Insect-transmitted plant pathogen, represents an economically important disease of apple (Baric 2012). One of the most distinctive characteristics is the linear chromosome (Kube et al. 2008).

Table 2.3 – Prokaryotic species with relevance to top-down, system-level studies towards minimal or simpler cells (continued)

Obligate endosymbionts of insects Usually, the smallest and most GC-poor genomes yet reported, with the exception of *Hodgkinia* (McCutcheon et al. 2009b) The genomes indicate functional convergence during evolution (McCutcheon & Moran 2010)	*"Candidatus* Tremblaya princeps PCVAL"	138 Kbp	Smallest genome of an endosymbiont. Genes for synthesis of nucleotides and cofactors, energy production, transport, and cell wall biogenesis are absent; only part of the replication machinery is preserved (López-Madrigal et al. 2011). The ability to synthesize most of the amino acids is still encoded. It is a primary insect endosymbiont with a secondary endosymbiont (López-Madrigal et al. 2011).
	Buchnera aphidicola APS	656Kbp	Model bacteria for extremely reduced prokaryotic genomes of obligate endosymbionts of insects (Gil et al. 2002, Pál et al. 2006, Prickett et al. 2006, van Ham et al. 2003, Yizhak et al. 2011). There are no reports of its culture without insect cells (Douglas et al. 2010, Prickett et al. 2006).
	"Candidatus Hodgkinia cicadicola Dsem"	144Kbp	An unprecedented combination of an extremely small genome (144 kb), a GC–biased base composition (58.4%), and a coding reassignment of the UGA codon from Stop to Tryptophan (McCutcheon et al. 2009b).
	"*Candidatus* Carsonella ruddii PV"	160Kbp	Symbiont that appears to be present in all species of phloem sap-feeding insects; more than half of the ORFs are devoted to translation and amino acid metabolism (Nakabachi et al. 2006).
	"*Candidatus* Sulcia muelleri" DMIN GWSS	244Kbp (Woyke et al. 2010) 246Kbp (McCutcheon & Moran 2007)	The most ancient and widely distributed of insect nutritional symbionts, these can be very large cells with an elongated shape, often more than 30 μm in length (Moran et al. 2005). Present in a large group of related insects, which supports the ancient acquisition of the symbiont by a shared ancestor, dating the original infection to at least 260 million years ago (Moran et al. 2005). Together with other endosymbionts, they form dual symbiont systems that allow a collective production of the ten amino acids not synthesized by the host (McCutcheon & Moran 2010).

Table 2.3 – Prokaryotic species with relevance to top-down, system-level studies towards minimal or simpler cells (continued)

Other obligate endosymbionts	"*Candidatus* Vesicomyosocius okutanii HA"	1.02Mbp	Thioautotrophic primary endosymbiont of a deep-sea clam, this is the smallest reported genome in autotrophic bacteria (Kuwahara et al. 2007). It contains genes for thioautotrophy and for the synthesis of almost all amino acids and various cofactors, but apparently lacks several transporters for these substances to the host cell and several other genes that are essential in *E. coli,* mainly the ftsZ and related genes for cytokinesis (Kuwahara et al. 2007).
Free-living prokaryotes with the smallest genomes	*Pelagibacter ubique* SAR11 HTCC1062	1.31Mbp	Heterotrophic prokaryote, supposed to be the most abundant species on Earth (Carini et al. 2013). Smallest genome encoding the smallest number of predicted ORFs of all free-living microorganisms (Giovannoni et al. 2005). Contrasting with other genome-reduced prokaryotes, it has complete biosynthetic pathways for all 20 amino acids and all but a few cofactors; no pseudogenes, introns, transposons, extrachromosomal elements, or inteins known; few paralogs and the shortest intergenic spacers yet observed for any cell (Giovannoni et al. 2005). Non-canonical metabolic rearrangements reported in defined media (Carini et al. 2013). An analysis of the proteome covering 65% of the ORFs confirmed the remodelling of the expression during adaptation to stationary phase (Sowell et al. 2008).
	Prochlorococcus *marinus* MED4	1.66Mbp	Smallest genome and cell size of an oxygenic phototroph, believed to be the most abundant photosynthetic organism on Earth (Giovannoni et al. 2005). The two genomes spanning the largest phylogenetic distance in the genus were compared revealing genomic dynamics and low proportions of regulatory genes (Rocap et al. 2003). The number of non-coding RNAs relative to the genome size is comparable to that found in other bacteria (Steglich et al. 2008). A simplified regulation of nitrogen utilization was reported (García-Fernández et al. 2004).

Table 2.3 – Prokaryotic species with relevance to top-down, system-level studies towards minimal or simpler cells (continued)

Model bacteria relevant to Industry	*Escherichia coli* K-12 MG1655	4.64Mbp	The model Gram-negative bacteria (highest species knowledge index for a prokaryote (Janssen et al. 2005)). Different genome-scale gene essentiality assays concluded on 620 (Gerdes et al. 2003) and later 303 (Baba et al. 2006) essential genes. Using the lambda Red recombination system, genome reductions of up to 15% (Pósfai et al. 2006), 22% (Mizoguchi et al. 2007, 2008) and 29.7% (Hashimoto et al. 2005) of the original genome size were reported. Another procedure combining Tn5 transposon mutagenesis with the Cre/*loxP* excision system and phage P1 transduction achieved a smaller but faster reduction of ~7% (Yu et al. 2002).
	Bacillus subtilis subtilis 168	4.21Mbp	Model Gram-positive bacteria. An early estimation of the essential genes based on 79 chromosomal deletions extrapolated that 562 Kbp would be sufficient to sustain a minimal cell based on this species (Itaya 1995). A later assay concluded on 271 genes indispensable for growth (Kobayashi et al. 2003). 7.7% of the genome was deleted by removing prophages and AT-rich islands using plasmid-based chromosomal integration-excision systems, which resulted in the strain *B. subtilis* Δ6 (Westers et al. 2003). Another project, the MG1M strain, deleted about 25 % (991 Kbp) of the genome (Ara et al. 2007). Later, the strain MBG874 was reported, with a deletion of 874 kb (20%), showing enhanced protein productivity; this was the first report demonstrating that genome reduction could contribute to the creation of a bacterial cell with an application in industry (Morimoto et al. 2008).
Archaea	*Nanoarchaeum equitans* Kin4-M	491Kbp	The single known archaeal parasite, it is an obligate symbiont of another archaea (*Ignicococcus sp*.). Unlike the small genomes of bacteria undergoing reductive evolution, *N. equitans* has very small regions of noncoding DNA (Waters et al. 2003). The genome encodes the machinery for information processing and repair, but lacks genes for lipid, cofactor, amino acid, or nucleotide biosynthesis.

2.5.2 Comparative Approach

Comparative approaches applied to the minimal cell have been mainly those of comparative genomics, involving whole genomes and inferred proteomes. Usually, conserved genes have a higher probability of not only being essential (and therefore part of a possible minimal genome) but also ancient (possibly part of LUCA's genome). The best-known of these genes is the 16S rRNA, traditionally used for phylogeny. Comparative studies serve in this manner mainly Evolutionary Biology and the quest for LUCA's constitution (Delaye et al. 2005).

The referred early comparison of the genomes of *M. genitalium and Haemophilus influenzae* was the first system-level comparative approach towards a minimal genome (Mushegian & Koonin 1996). Although only 240 genes were conserved between both genomes, 22 cases of NODs were identified. Depending on the conceptual or practical cellular construct being pursued, choosing the simplest, most ancient or most economic protein when facing a NOD will be crucial in the search for a minimal cell. An analysis of possible functional redundancy and presence of parasite-specific genes in this study resulted in a final set of 256 as the hypothetical gene number capable of sustaining a cell (Mushegian & Koonin 1996).

A new wave of comparative studies integrates proteogenomics to validate genetic conservation, using high-throughput tandem mass spectrometry to verify the expression of predicted conserved coding regions (Ansong et al. 2008). Firstly used by Gupta *et al*. to compare the expression of orthologous genes across three *Shewanella* species (Gupta et al. 2008), not much later comparative proteogenomics was used in the referred quest for the core proteome of a minimal cell (Fisunov et al. 2011) (see Section Other Minimal Sets of Components).

Computational comparative proteomic approaches can be performed outpacing sequence comparison. One example includes the annotations of curated domain structures, which has been done in a referred phylogenomic study with 420 free-living organisms trying to define the proteomic content of LUCA (Kim & Caetano-Anollés 2011). Others have compared protein folds across Bacteria and Archaea, indicating a possible set of top 30 most conserved folds (Wolf et al. 1999).

When jumping from comparing genomes to comparing proteomes, transcriptomes or fluxomes, experimental conditions are an additional but indispensable layer of information. The results in these cases are influenced by the media and conditions provided to the cells, which must be kept constant to allow for comparative studies to be performed. The comparison of several omic datasets is highly promising, although it can be a challenging task, as many of the studies available in the literature were not done under the same experimental conditions. Even the same complex media can have small variations that will impair comparisons (Pavankumar et al. 2012) so ultimately defined media should be preferred for comparative analysis. This will require the generation of new, controlled, experimental data for future comparative studies.

Not only omic-level comparisons (arriving at a minimal set) can be relevant for the study of a minimal cell, but also the organelle-level can be targeted for relevant comparisons. The comparison of the sequences of modern ribosomes identified the most conserved regions from the three domains of life, which were then mapped onto determined structures of 30S and 50S subunits of ribosomes (Mears et al. 2002).

In silico system-level comparative studies include the comparison of biological networks using graph-theory based algorithms to perform topology-based-only comparison of biological networks (protein-protein or metabolic) at a global scale (Kuchaiev et al. 2010).

Arriving at minimal theoretical sets through comparative and top-down approaches is not sufficient to achieve minimal cells. After the 1000th prokaryotic genome was made available, the striking discovery that not one single protein-coding gene is conserved across all prokaryotic genomes shocked biologists (Lagesen et al. 2010). Moreover, if Archaea are excluded, only two protein-coding genes – a translation-elongation factor and a ribosomal protein – plus the two rRNA genes are conserved across all Bacteria (Lagesen et al. 2010). These facts imply that systematic comparative approaches will gain from focusing on functional differences at other levels than the genome. Ultimately, by recognizing that the comparative and top-down approaches are insufficient to reduce complexity to the level of a full comprehension of the cell, one would build or synthesize that minimal cell from its parts. That is what the bottom-up approach intends to achieve.

2.5.3 Bottom-Up Approach

The bottom-up – synthetic – approach is the one aimed at assembling a minimal or simpler cell in the laboratory, i.e., constructing minimal cells from non-living material (Rasmussen et al. 2004). Bottom-up studies have concerned mainly physical and chemical properties and the dynamics of the building blocks of life. Focus has been placed on inserting genetic material (RNA or DNA) or enzymes inside lipidic vesicles, creating what is often named as protocells (see section 2.1). Properties such as stability, permeability and self-reproduction, together with the dynamics of eventual biochemical reactions can be studied in these constructs (for a detailed compilation of the work of biophysics in this area, see (Luisi et al. 2006, Stano 2011)). More complex biological properties can also be analysed in protocells. For example, in a pioneer study it was shown that Darwinian competition emerges in populations of vesicles with encapsulated genetic material (Chen et al. 2004). The competition arose simply due to the physical principle of osmotic-driven vesicle growth. Others studied enzymatic RNA replication (Oberholzer et al. 1995) and the movement of vesicles resembling bacterial chemotaxis (Hanczyc et al. 2007) based on different protocells assembled in those studies.

Solé *et al*. (Solé et al. 2007) make a distinction between the major achievements in bottom up studies that would lead to building completely artificial cells, and those of reconstruction studies (Luisi et al. 2006) which use components from biological origin to produce what is here named as semi-artificial cells.

One innovative bottom-up project involves the idea of creating a minimal cell based on purified proteins. The authors intend to identify the necessary genes for a minimal cell, and after preparation of the purified biochemical molecules, to encapsulate those within membranes, possibly rendering an artificial cell (Forster & Church 2006, Jewett & Forster 2010). Another system of the kind is Cytomin, a cell free translation system that has revealed promising results in protein synthesis and energy efficiency (Jewett & Swartz 2004, Jewett et al. 2008).

Probably the major landmark that should also be included in the bottom-up approaches is the synthesis of the first artificial bacterial chromosome (Gibson et al. 2008). Although it is not the creation of a cell *per se*, it established the technology for the creation of the code for an entire cell. Nevertheless, although the creation and assembly of fully-artificial cells is one of the ultimate goals of bioengineering and would help understanding biosystems deeper, it seems part of science fiction, for now.

It might appear that bottom-up starts from a privileged position to the study of LUCA and prebiotic chemistry compared with the top-down approach, as the creation of artificial cells in the laboratory and the creation of ancestor cells in nature both constitute transitions from non-living to living entities (Rasmussen et al. 2004). However, the connection between both areas of research should be handled with care (Rasmussen et al. 2004, Szostak et al. 2001). While fully tracking the history of life until its origins could, in principle, allow the replication of the process in the laboratory, the opposite cannot be assumed. Any artificial cell to be created in the laboratory based on modern genes, modern proteins and modern membranes can be far from resembling what LUCA was. It has been argued that the origin of genetic and enzymatic machineries must have happened within some inorganic scaffold, with LUCA not being free-living at first (Koonin & Martin 2005), while common bottom-up studies use vesicles to build protocells (see section LUCA and the first cells). In this manner, classical bottom-up work, regarding the current state of the art, might not be directly associable to LUCA's study, as discussed elsewhere (Luisi et al. 2006, Stano 2011). Moreover, stating that a protocell would be a good model of a chassis cell would require protocells to be experimentally validated for chassis cell design. Within the state of the art, protocells are still, unfortunately, a meagre model of such constructs.

2.5.4 Middle-Out Approach

Kohl and Noble attribute the term middle-out originally to Sydney Brenner (Kohl & Noble 2009), who coined it during a discussion in a Novartis Foundation Symposium on "Complexity in Biological Information Processing" (Brenner et al.

2001). For the purposes of this review, given that the focus is on prokaryotic systems, Noble's definition (Noble 2002) was adapted to "the approach which starts at any level (gene, RNA, protein, metabolic or regulatory pathways) at which there are sufficient data and reaches (up, down and across) towards other levels and components". The middle-out approach is often difficult to distinguish from the classical approaches. In this review those studies that integrate different layers of information in a final holistic model or construct mentioned in **Table 2.1** are classified as middle-out.

Gil *et al*. did a large-scale work of integration of several minimal gene sets and generated probably the most comprehensive and accepted theoretical minimal protein-coding gene set for prokaryotic life (Gil et al. 2004) (See section Minimal Genome for the composition of this minimal gene set). The study integrated the orthologous genes resulting from the comparison of five endosymbionts' genomes (Gil et al. 2003) with functional equivalents without sequence similarity. After, the results were integrated with several datasets: a list of *B. subtilis* essential genes (Kobayashi et al. 2003); proposed essential genes for *E. coli* from different sources (Gerdes et al. 2003, Kang et al. 2004, Kato & Hashimoto 2007); the proposal of a computationally-derived minimal gene set by Mushegian and Koonin (Mushegian & Koonin 1996); the results of global transposon mutagenesis for mycoplasmas (Hutchison et al. 1999); a list of essential genes identified in *S. aureus* (Forsyth et al. 2002, Ji et al. 2001) and the reduced genome of the plant pathogen *Phytoplasma asteris* (Oshima et al. 2004). To identify corresponding orthologous genes and protein functions and reconstruct the metabolic pathways, the authors used a comprehensive variety of online databases and resources (Gil et al. 2004). The final functional classification of the gene set was done with the categories used in the sequencing work on *Aquifex aeolicus,* one of the earliest diverging bacteria known (Deckert et al. 1998), and the resulting minimal metabolic network was analysed for detecting gaps in essential pathways. The proposed minimal gene set reflects a rational integration that is described in detail in (Gil et al. 2004).

Another example of an integrative approach resulting in an original construct is the whole-cell tomogram of *M. pneumoniae*, which includes individual heteromultimeric protein complexes represented to scale within one bacterial cell, obtained using electron tomographies of 26 entire cells (Kühner et al. 2009). A combination of pattern recognition and classification algorithms allowed the positioning of identified protein complexes in a whole-cell illustration of the spatial organization of the proteome of this reduced bacteria (Kühner et al. 2009) (**Figure 2.1**).

A major achievement that so far represents the climax of integrative experimental projects towards the creation of artificial cells came two years after the creation of the first synthetic artificial genome (Gibson et al. 2008). The Venter Institute announced the successful transplantation of an artificial chromosome - *Mycoplasma mycoides* JCVI-syn1.0 genome - to another recipient cell, a *Mycoplasma capricolum*, creating new cells controlled by the synthetic chromosome (Gibson et al. 2010). This represented a stretching of the boundaries of biotechnology, opening doors to new work with semi-artificial bacterial cells.

2.5.5 Models and Simulations of Minimal and Simpler Cells

Because minimal or simpler cells are still conceptual constructs, theoretical representations and mathematical models are crucial for the advancement of the field. Theories (like the one of the hydrothermal origin of life, mentioned in section 'LUCA and the first cells' (Russell & Hall 1997, Russell et al. 2003)) and models (e.g. physical, experimental protocells or virtual, *in silico* simulation models) are the minimal or simpler cell-related constructs closer to being holistically understood, among those represented in **Table 2.1**, given the complexity of prokaryotic cells.

Theoretical or virtual protocell systems include a vast array of representations of self-replicable systems, some explored mathematically. A pioneering protocell model is the so-called chemoton, by Tibor Gánti (Gánti 1975). The chemoton consists of three functionally dependent autocatalytic subsystems: the metabolic network, the template polymerization and the membrane subsystem enclosing the previous. All three subsystems are precisely coupled by stoichiometry, which ensures the correct functioning. The chemoton is considered as an elegant platform to support different protocell models (Szathmáry & Griesemer 2008). Physical protocells as minimal cell models and theoretical models of protocells have been reviewed comprehensively elsewhere (Solé et al. 2007).

On the other hand, the field of modelling whole cells is still very scattered, and a variety of different modelling approaches have been used so far. In general, a whole-cell simulation requires modelling different biological networks at an appropriate scale. The existing models can be broadly categorized in three classes: interaction models or network representations; constraint-based models (e.g. stoichiometric models) and mechanistic models (e.g. kinetic models) although these are still far from being holistic (for a review see (Stelling 2004)). Among these, the constraint-based models have played a major role in the contemporary attempts of modelling minimal life, mainly because of the simplicity or abstraction they allow. Genome-scale network reconstructions (GENREs), which have been increasingly used in metabolic modelling, are one example with several practical applications discussed elsewhere (Oberhardt et al. 2009). GENREs require the integration of experimental data in a middle-out manner (Durot et al. 2009, Oberhardt et al. 2009). The minimal requirement for reconstructing a GENRE is the annotated genome sequence of the organism of interest. The resulting basic framework can be further refined and expanded with the incorporation of experimental data at the cell-level (mainly transcriptomics and proteomics) and manual curation based on the literature available. These models allow assessing the biosynthetic capabilities of a species in a systematic manner. Furthermore, they also enable the simulation of intra-cellular metabolic fluxes, as well as the effects of genetic modifications, such as gene knockouts (Orth et al. 2010, Price et al. 2004). So far, a large number of prokaryotic manually-curated GENREs have been published (Oberhardt et al. 2009). These models are promising for studies of prokaryotic simplification and even for comparative studies that will allow the definition of common and different metabolic features. A couple of studies with GENREs have been done relating to minimal or simpler cells. Pál *et al*. used one *E. coli*'s GENRE to analyse the reductive evolution from the network of *E. coli* toward the small networks of *B. aphidicola* and *Wigglesworthia glossinidia*, achieving a remarkable accuracy of 80% (Pál et al. 2006). GENREs have also been used to predict gene essentiality in different organisms, and theoretical compositions of minimal media (Gianchandani et al. 2010).

Other work in modelling minimal cells has been done regarding mechanistic cell-level models, focusing on different features such as cell geometry and division (Surovtsev et al. 2009), macromolecular interactions (Flamm et al. 2007) and also metabolism (Castellanos et al. 2004), with the last study interested in modelling a minimal cell from the knowledge of *E. coli* 's metabolic kinetics (Browning & Shuler 2001). Another comprehensive ongoing whole-cell simulation project is running in Japan, based on *M. genitalium* and including 127 genes – the E-CELL model (Tomita 2001). More recently, Shuler *et al.* developed probably the most comprehensive and abstract minimal cell model to date (Shuler et al. 2012), based on the minimal gene set derived by Gil et al. (Gil et al. 2004). The authors added genes for 3 rRNA products, 20 tRNA species and transport systems for amino acid and inorganic ions that were missing in the source gene set. This minimal cell model has 241 genes in total, represented in a 233-kb chromosome, coding for all the functions supposedly required for a chemoheterotrophic bacteria to grow and divide (Shuler et al. 2012). The model formulation consists of a differential algebraic equation system, which includes the DNA replication process, as well as cytokinesis and the coupling between cell physiology and cell growth. It is also able to output several parameters as partition factors, chromosome replication and cell division parameters (Shuler et al. 2012).

Recently published, the whole-cell model of *M. genitalium* was an important advance, not only for the modelling field, but also for the biological study of prokaryotes, allowing for accurate phenotypic predictions (Karr et al. 2012). This model integrates 28 essential cellular processes that were represented in different submodels; these fall into five main categories – DNA, RNA, Protein, Metabolism, and Other (cytokinesis and host interaction) including over 1,900 quantitative parameters. Each of the 28 submodels was simulated with an appropriate mathematical representation – for instance, metabolism was modelled using a constraint-based approach, while RNA and protein degradation used mechanistic Poisson processes (Karr et al. 2012). This integrative strategy makes the assumption that the submodels are approximately independent on short timescales, so that at each time step the submodels depend on the values of variables determined by the other submodels at the previous time step (Karr et al. 2012). This formulation of independent and decoupled modules allowed the most complete simulation of *M. genitalium* so far, not only providing insights on the simulated cellular functions, but also directing experimental assays that identified kinetic parameters and details on the biological function of metabolic genes.

2.6 Towards the Lowest Complexity

Both for fundamental science and for the design of better platform cells with applications in industrial biotechnology, one of the major concerns is the complexity of the cells used, rather than the number of components those cells have, and how precisely these cells can be understood and engineered in a predictive manner. Therefore, at this point it can be argued that, for the study of the minimal cell, the focus shall become minimizing complexity and not the number of components. Complexity is often related with the number of interactions patent in the interactome – all the interactions linking biological molecules in a cell (Kiemer & Cesareni 2007). Once the interactome is known, and the complexity of the system is understood, this complexity can be reduced by a rational deletion of some elements – single genes or even whole metabolic or regulatory modules that are not essential and that represent a considerable increase of complexity of the system. One example is the work by Trinh *et al*., where by knocking out only 8 genes the authors reduced the functional space of the *E. coli*'s central metabolic network from 15000 pathway possibilities to only six growth-supporting pathways (Trinh et al. 2008).

2.6.1 Interactomes and Network Biology

Network biology explores the connectivity of molecular elements in biological networks, which can change dramatically for different proteins (Bolser et al. 2003, Ravasz et al. 2002, Rives & Galitski 2003). It has been suggested that the complexity of the network of protein-protein interactions in a cell can be reduced to and represented by a small number of highly connected hubs or protein units of structure and function (Rives & Galitski 2003). Network biology also specializes in applying graph theory to biological systems and revealing universal features of cellular networks (Barabási & Oltvai 2004). One of the major discovered features was that biological networks follow a hierarchical organization (Ravasz et al. 2002), in a modular manner, a feature that, from a holistic perspective, can facilitate interventions and predictions in the network. Recently, the hierarchical organization of biological networks has been highlighted as vital for the reduction of complexity of bacterial cells for biotechnological applications, but under another nomenclature (Mampel et al. 2013). The authors emphasize the need to introduce in biology the concept of orthogonalization, a classical notion in engineering and mathematics that represents the ability of subsystems of a higher system to function independently (Mampel et al. 2013).

The analysis of different prokaryotic networks has suggested that more environmental variability is related to more network modularity and therefore more orthogonalization (Parter et al. 2007). It was demonstrated that *E. coli*'s metabolic modules are functionally uniform, with each metabolic class assignable to one specific structural module, while *B. aphidicola*'s reduced network modules show a larger mixture of different functions (Parter et al. 2007). Another interesting conclusion on biological complexity was that the transition to the largest and more complex metabolic networks was dependant on the presence of oxygen (Jason Raymond and Daniel Segre 2006).

2.6.2 Genome Size and Cellular Complexity

The results of high-throughput interactome studies permit a first glance at the relationship between the genome size (in terms of number of ORFs) and the number of interactions identified (Bouveret & Brun 2012), showing that no correlation exists between the two variables (**Figure 2.2**). The total of interactions exhibits a disperse distribution, but when normalized by the number of baits tested in each study, the ratio between interactions identified and number of baits situates between 2 to 8 fold, with the exception of *Campylobacter jejuni* in which the interactome size is 18 times larger than the number of baits tested (**Figure 2.2**). This indicates that the interactome size might be independent of the genome size, although the available data are still very incipient.

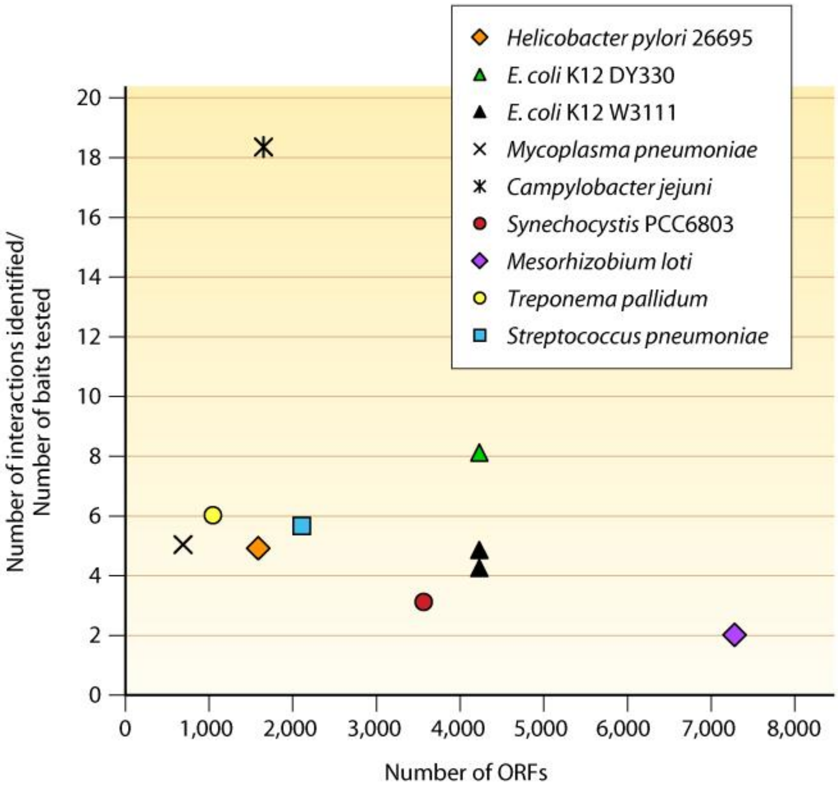

Figure 2.2 – Results from high throughput interactome studies of different prokaryotic species. Data from (Bouveret & Brun 2012).

A general lack of strong correlations between genome size and several other cellular features, inferred from annotation data (Markowitz et al. 2012), corroborates the notion that the genome size (in kb) is a poor indicator of complexity (**Figure 2.3**A). Of the recent annotation data, the worst correlation occurs for the number of predicted HGT events, accompanied closely by the number of pseudogenes and number of rRNA copies per genome. The absence of a correlation between the genome size and the copy number of small subunit rRNAs was also suggested by other authors (Fogel et al. 1999), as is the case for pseudogenes. It was shown that the vast majority (90%) of prokaryotic genomes contain <18% of non-coding DNA, but this value can go up to 50% in parasites which are enriched in pseudogenes (Rogozin et al. 2002). Interestingly, eukaryotic-like kinases are present in the genome of *M. genitalium* and *M. pneumoniae* (two and one kinases in each genome, respectively) but not in *E. coli* (Pérez et al. 2008).

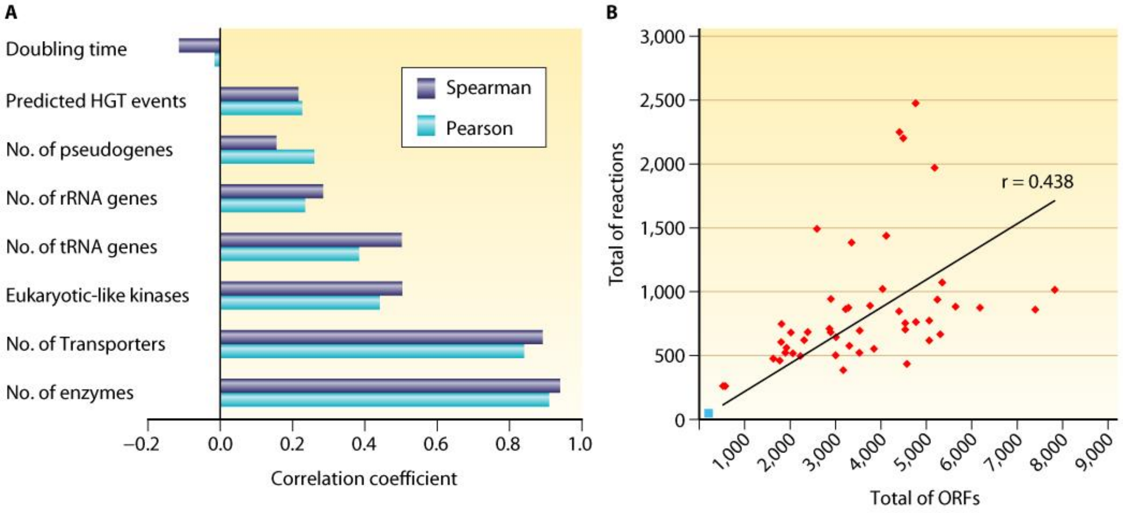

Figure 2.3 – Correlations between genome-sizes of prokaryotes and some genomic and phenotypic features. A – Correlation coefficients for annotation data and doubling times; data for eukaryotic-like kinases from (Pérez et al. 2008); data for doubling times from (Vieira-Silva & Rocha 2010); remainder data from the IMG database (Markowitz et al. 2012). All p-values below 0.001 with the exception of the correlations for doubling times (non-significant p-values). B – Pearson's correlation between number of reactions and number of ORFs for 49 manually curated metabolic network reconstructions (full list and references available in Supplementary Table 2.1); p-value = 0.001637; blue marker representing a theoretical minimal metabolic network (Gabaldón et al. 2007).

The lack of correlation between genome-size and doubling time is another interesting point to consider (**Figure 2.3**), from both evolutionary fitness and industrial application points of view. Indeed, codon usage bias is a much better indicator of growth rate (Vieira-Silva & Rocha 2010, Vieira-Silva et al. 2010) in comparison to the genome size. Another interesting feature, the CRISPR (clustered regularly interspaced short palindromic repeats) defence mechanism has been indicated as a complex feature of prokaryotes in which both the number of loci and size of the sequences do not correlate with genome size (Sorek et al. 2013).

The best correlations with genome size occur for metabolism-related features such as the number of predicted enzymes and the transporters assigned by the transporter classification system (TCs) (**Figure 2.3**). The correlation is weaker when considering manually curated GENREs only (**Figure 2.3**B; Supplementary Table 2.1). Manually curated GENREs are available for a significantly smaller number of species than those with sequenced genomes; however, the previous include a rigorous process of validation and a supervised procedure of gap filling of the network. Overall, it seems plausible to say that genome size reflects fairly well the metabolic capability of an organism. Metabolic networks are among the most studied and manipulated of all prokaryotic features (Durot et al. 2009, Oberhardt et al. 2009, Orth et al. 2011) and it has been suggested that the complexity of metabolism lies mostly in the regulation imposed on the metabolic network (Gerosa & Sauer 2011), which can occur at a large scale with the intervention of a single ubiquitous transcription factor (Brand & Curtis 2002), making it difficult to infer biological complexity based on the metabolic network size alone.

Complexity of transcriptional regulatory networks, e.g. through transcription factor-gene interactions, can be seen as another metric of overall cellular complexity. Although the number of transcription factors seems to increase with genome size, the number of regulatory sites per intergenic region is independent of it (Molina & van Nimwegen 2008). On another side, *M. pneumoniae*'s genome*,* despite having only 0.81 Mb, contains frequent antisense transcripts, alternative transcripts and multiple regulators per gene that make these bacteria's regulation and transcriptome highly dynamic and somehow similar to eukaryotes (Güell et al. 2009). *M. genitalium* lacks two-component regulatory systems with histidine kinase sensors and response regulator domains that are widespread in *E. coli* and *H. influenzae* (Fraser et al. 1995) which lead to the anticipation that its regulatory circuits would be less responsive to environmental signals (Koonin et al. 1996) and therefore less controllable in industrial scenarios.

The minimal nutritional requirements of a species summarize its biosynthetic capabilities and hence can be used as a metric of its metabolic complexity. Based on nutritional information for 15 species (Supplementary Table 2.2), there seems to be a non-linear relationship between the number of media components and genome size, with an apparent stabilization of a minimal media size between 7 and 8 components after the 3 Mb mark (for heterotrophic growth) (**Figure 2.4**). The underlying negative correlation is in accord with the expectation that the nutritional requirements of smaller genomes would be higher, reflecting evolutionary adaptations that have implications for the design of chassis cells.

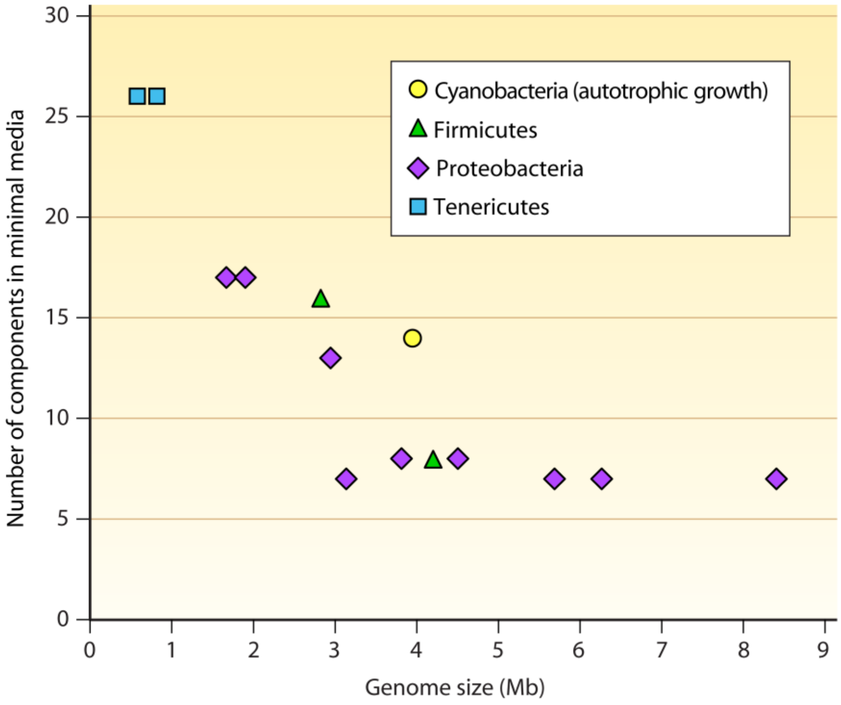

Figure 2.4 – Relation between the size of the minimal media and genome size for different prokaryotes of different phyla (media composition and references in Supplementary Table 2.2).

The number of genome copies per cell is another feature that defies genome size as an appropriate measure of complexity. Surprisingly, until recently, insect obligate endosymbionts detained the records for larger number of copies of genome per cell, with the average ranging from 20 to several hundred genome copies in *Buchnera* cells and between 200 and 900 in "*Candidatus* Sulcia" (Woyke et al. 2010). Moreover, it was shown that the number of copies of genomes of intracellular symbionts would vary as a response to the developmental stage of their host, increasing during post-embryonic development of insects to adulthood, and decreasing during ageing (Komaki & Ishikawa 2000). It is reasonable to think that endosymbiosis transforms these prokaryotes into cell factories more active in providing the host the “agreed nutrients” by an increase in genome copy number, which can be exploited for more profitable biotechnological applications of minimal cells.

2.7 Sub-cellular Architecture

Highly organized sub-cellular architecture has increasingly become an object of attention and brings a whole new perspective to the biology of prokaryotes (Gitai 2005, Minton & Rivas 2011), which have been until recently regarded as simple membrane-bounded cells with an uniform cytoplasm and one circular genome. It has been shown that even enzymes thought to have only specific chemical roles can have well-defined structural roles in a prokaryotic cytoplasm. The CTP synthase of *Caulobacter crescentus* forms filaments that help define the characteristic curvature of these bacteria, and these filaments are formed in *E. coli* as well (Ingerson-Mahar et al. 2010). *M. pneumoniae* also displays highly ordered structural features (Güell et al. 2009, Kühner et al. 2009) including a complex terminal structure that directs human respiratory tract colonization and is considered an organelle *per se*, with the function of promoting attachment (Popham et al. 1997). Although this bacterium is among the simplest prokaryotes with an extremely reduced genome, and without cell wall, its subcellular architecture shows that smaller genomes can translate into complex cellular structures.

2.8 Conclusions and Future Perspectives

The genome, as the first ome made accessible by the technological advances, has received most of the attention so far in the field of minimal or simpler cells. The efforts towards minimal genomes mainly include large-scale identification of non-essential genes, relatively few experimental genome reductions and an outstanding example of the construction of a bacterial cell harbouring a synthetic genome. On another line of research, comparative approaches have identified the core, conserved gene sets that were thought at first to constitute the minimal genome. With the sequencing of more and more genomes, nowadays this core is practically reduced to zero as no protein-coding gene is universal across the prokaryotic domain (Lagesen et al. 2010). This outstanding discovery has reshaped the way the field of minimal cells is viewed from a systems biology perspective. The genome is not seen any more as the static core-identity of the cell, but more as a backbone or a database of tools pertaining to a complex and dynamic system. Technologies complementary to genomics are thus entering the main stage, such as transcriptomics, proteomics and metabolomics, as well as computational tools for simulating the dynamic behaviour of the cell. The minimal cell can be seen nowadays as a broad concept that does not apply to one genome composition only. It seems that a panoply of different small genomes could exist, being regulated differently, expressed in different proteomes, and being strongly dependent on the available media and environment.

In parallel to the omics-oriented research, the study of the last universal common ancestor has been integrated with the geochemical context of the early earth, which is crucial to the re-constitution and understanding of the genetic and metabolic capabilities of this minimal cell. Furthermore, the design of chassis cells is becoming more and more targeted on specific needs like product and culture conditions, expanding on the previous notion that a general minimal cell with a reduced genome would fit industrial needs. Overall, it has become clear that both fundamental and applied goals of the research on minimal cells can only be achieved through a system-level analysis encompassing bottom-up, top-down and middle-out approaches.

The need for taking a holistic approach in the design of minimal cells is underlined with the necessity of complementing experimental approaches with mathematical modelling. Mathematical models can aid in the interpretation and integration of large omics datasets, hypothesis generation, uncovering general principles underlying the operation of complex cellular machinery, and eventually in designing the network modules for the minimal cells. One of the foremost tasks will be to devise metrics for assessing the minimality and simplicity of a biological system – features that may not necessarily go hand in hand. Although minimality can be defined in a relatively straightforward manner, e.g. in terms of genome size, to date there are no explicit metrics of complexity available. Several recent studies providing insight into the cellular interactome (Bolser et al. 2003, Bouveret & Brun 2012, Rives & Galitski 2003) indicate that the topological and functional features of these networks may be used for devising suitable complexity metrics.

A cell factory viewpoint of minimal and simpler cells can provide useful insights into the relationship between simplicity and complexity. A cell factory to be used in biotechnological applications will be required to strike a balance between various contrasting features (**Figure 2.5**A). For example, while the minimality implies a smaller genome size, it undesirably increases the requirements for nutritional supply. Similarly, minimal complexity and optimal local control may require a certain degree of orthogonalization between the functions of different components or functional modules, while some crosstalk between these will be essential to achieve globally optimal control and a high metabolic efficiency. Indeed, cellular metabolic networks are featured by both orthogonalization (e.g. distinct biochemical pathways) and crosstalk (e.g. through the use of universal redox and energy co-factors). Furthermore, metabolic efficiency and rates often counter each other (Bachmann et al. 2013), prompting another balance for the system as a whole. These different trade-off considerations clearly suggest that 'minimal' cells for an industrial purpose will have to be tailored to a particular need - the complexity of the desired phenotype and the economy of the overall process dictating the balancing point. It will be interesting to extend these engineering viewpoints to evolutionary considerations for LUCA. For example, the theoretical/experimental LUCA models could be refined so as to strike a balance between the number of components and the level of complexity that would likely represent optimal fitness under the postulated environmental conditions.

Research from diverse fields, ranging from fundamental biology to LUCA to chassis cells, is providing a clearer picture of the workflow that will most likely lead to the reconstruction of simple and minimal cells for basic research as well as for industrial applications. This will imply an iterative process building upon top-down studies generating omics datasets, bottom-up, mechanistic studies generating biochemical and biophysical data and middle-out integrative modelling allowing some degree of abstraction together with important predictions (**Figure 2.5**B).

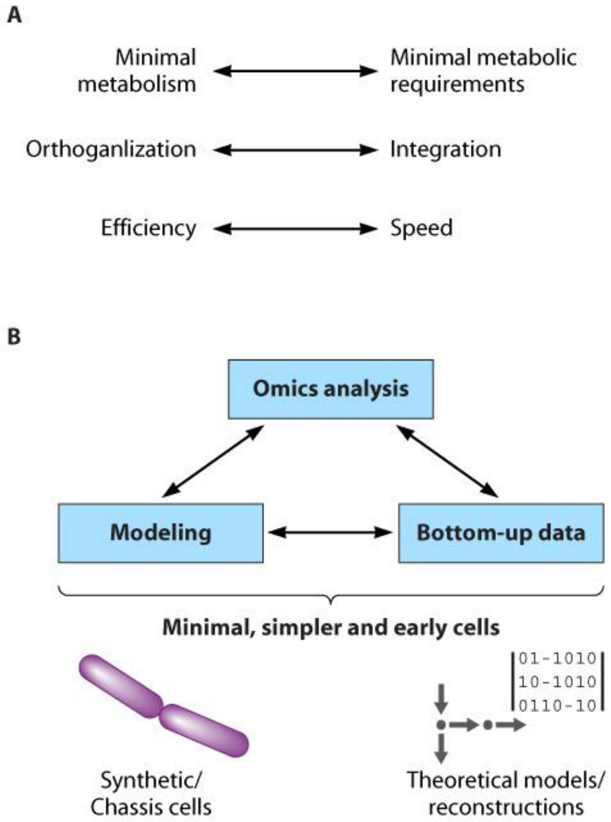

Figure 2.5 – A - Open questions and B - practical objectives in systems biology towards the design and creation of minimal or simpler cells.

Ultimately, all approaches towards minimal or simpler cells are systems biology approaches, as the goal is to achieve a whole system – the whole minimized or simplified cell - even though these have much to gain from non-systematic studies. Examples of these could include the studies of a specific protein or regulatory module for cell division of a minimal cell (Jonas et al. 2011, Lluch-Senar et al. 2010); the phylogenetic study and even reconstruction of ancient enzymes, tracing their chemistry back to the context of ancient life (Perez-Jimenez et al. 2011); the study of a specific pathway that could later be optimized in a chassis cell (Zhang et al. 2007), etc. The merger between such non-systematic studies, systematic approaches and synthetic DNA technology is expected to lead to exciting achievements towards minimal cells. This combination will be the key for answering the long sought questions on the origin and nature of life, and for improving our ability to rationally design minimal or simpler cells.

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CHAPTER 3
Essential Cofactors in Prokaryotes Revealed by Genome-scale Models and Large Data Integration

It is by avoiding the rapid decay into the inert state of 'equilibrium' that an organism appears so enigmatic; [...]. How does the living organism avoid decay? The obvious answer is: By eating, drinking, breathing and (in the case of plants) assimilating. The technical term is metabolism. The Greek word means change or exchange.

—ERWIN SCHRÖDINGER, *What is Life?* (1944)

The composition of a cell in terms of macromolecular building blocks and other organic molecules underlies the metabolic needs and capabilities of a species. Although some core biomass components such as nucleic acids, proteins and lipids are evident for most species, the essentiality of the pool of other organic molecules, especially cofactors and prosthetic groups, is yet unclear. Here 71 biomass compositions from manually curated genome-scale models are integrated with 33 large-scale gene essentiality datasets, enzyme-cofactor association data and a vast array of literature publications, revealing universally essential cofactors for prokaryotic metabolism. The results revise predictions of essential genes in *Klebsiella pneumoniae* and identify missing biosynthetic pathways in *Mycobacterium tuberculosis*. This work provides fundamental insights into the essentiality of organic cofactors and has implications for minimal cell studies as well as for modeling genotype-phenotype relations in prokaryotic metabolic networks.

The contents of this chapter were prepared and submitted as a research article to a peer-reviewed journal:

Xavier JC, Patil KR, Rocha I. Integration of Biomass Formulations of Genome-scale Metabolic Models with Experimental Data Reveals Universally Essential Cofactors in Prokaryotes (submitted).

3.1 Introduction

The biomass composition of a cell reflects the genetic repertoire necessary to synthesize, salvage, or uptake the necessary constituents for growth and maintenance. Indeed, it can be used in taxonomical classification (De Ley & Van Muylem 1963, Hiraishi 1999, Hoiczyk & Hansel 2000, Muto & Osawa 1987, Rosselló-Mora & Amann 2001, Schleifer & Kandler 1972) and is intimately related with the species' growth rates (Bremer & Dennis 1996, Kemp et al. 1993). Consequently, biomass composition is strongly linked to drug sensitivity, nutritional requirements, and the biosynthetic potential for industrial applications of a species.

Genome-scale metabolic models (GSMs) have underscored the need to reduce the knowledge gap in biomass compositions. GSMs have systematized metabolic knowledge on dozens of microorganisms, with applications in diverse areas, from industrial biotechnology to medical microbiology (Kim et al. 2012, Monk et al. 2014). Biomass composition is a critical element of these models, allowing the representation of cell growth *in silico*. This is realized through a growth reaction wherein necessary constituents are combined in stoichiometric amounts producing new biomass. Maximization of the flux through this reaction, the so-called Biomass Objective Function (BOF), is the most commonly used method for simulating growth phenotypes. The utility of metabolic models is tied to the accuracy of the biomass composition used (Feist & Palsson 2010, Feist et al. 2007, Mendum et al. 2011). Yet, most GSMs adapt the biomass composition from few well-studied organisms due to the lack of standardized protocols, both experimental and computational. Here, this problem is addressed by bringing together evidences for cofactor essentiality hidden in disparate data sources – manually curated GSMs, biochemical and bioinformatics databases, literature and genetic screens. These small molecules, although not consumed in metabolism, are essential for catalysis and need to be distributed in sufficient amounts among the daughter cells (Zhao & van der Donk 2003). The analysis performed revealed several essential organic cofactors for archaeal and bacterial metabolism.

3.2 Results

3.2.1 The Universe of Biomass Constituents in Prokaryotic GSMs Is Large and Heterogeneous

First, biomass compositions in published prokaryotic GSMs were extensively assessed. In total, 71 detailed biomass compositions were gathered, covering 9 phyla with 5 classes of Proteobacteria and one phylum of Archaea (Supplementary Table 3.1). To enable comparison across different models, diverse nomenclatures and representation styles were reconciled, ranging from lumped stoichiometry to reaction-level inclusion (e.g., coenzyme A is represented in isolation in some models while only in conjunction with lipids in others). This exercise resulted in 551 unique metabolites (nomenclature as per BiGG database (Schellenberger et al. 2010)) that are used as biomass constituents, including 20 charged tRNA molecules, 12 inorganic ions and water (Supplementary Tables 3.2, 3.3 and 3.4). Of these, more than half – 261 – are present in only one BOF. Clustering of these diverse BOFs revealed large discrepancy between biomass compositions used by models of species in the same phyla (e.g. four species of cyanobacteria) or even between different versions of models of the same species (**Figure 3.1**a). The clustering appears to be affected by the template biomass composition used in reconstruction. For example, one of the clearly separated clusters groups the BOFs based on the BOF of iJR904 (Reed et al. 2003), a 2003 model of *E. coli*. The detail of biomass compositions was found not to be correlated with the year of publication, and the majority of BOFs have a lower number of components than those indicated as core for *E. coli* in 2011 (Orth et al. 2011) (**Figure 3.1**b). Furthermore, none of the BOFs of the manually curated models included all biomass components deemed universal in the ModelSEED biomass template(Henry et al. 2010a) (**Figure 3.1**c). The least comprehensive BOF excludes 29 components and the most comprehensive excludes 6, amidst which well-known entities such as acyl carrier protein (ACP), AMP and GDP (**Figure 3.1**d). Although the overlap between the BOFs and the ModelSEED template increases considerably when excluding inorganic ions from the analysis, there is still no BOF with 100% overlap (**Figure 3.1**c; Supplementary Tables 3.5 and 3.6).

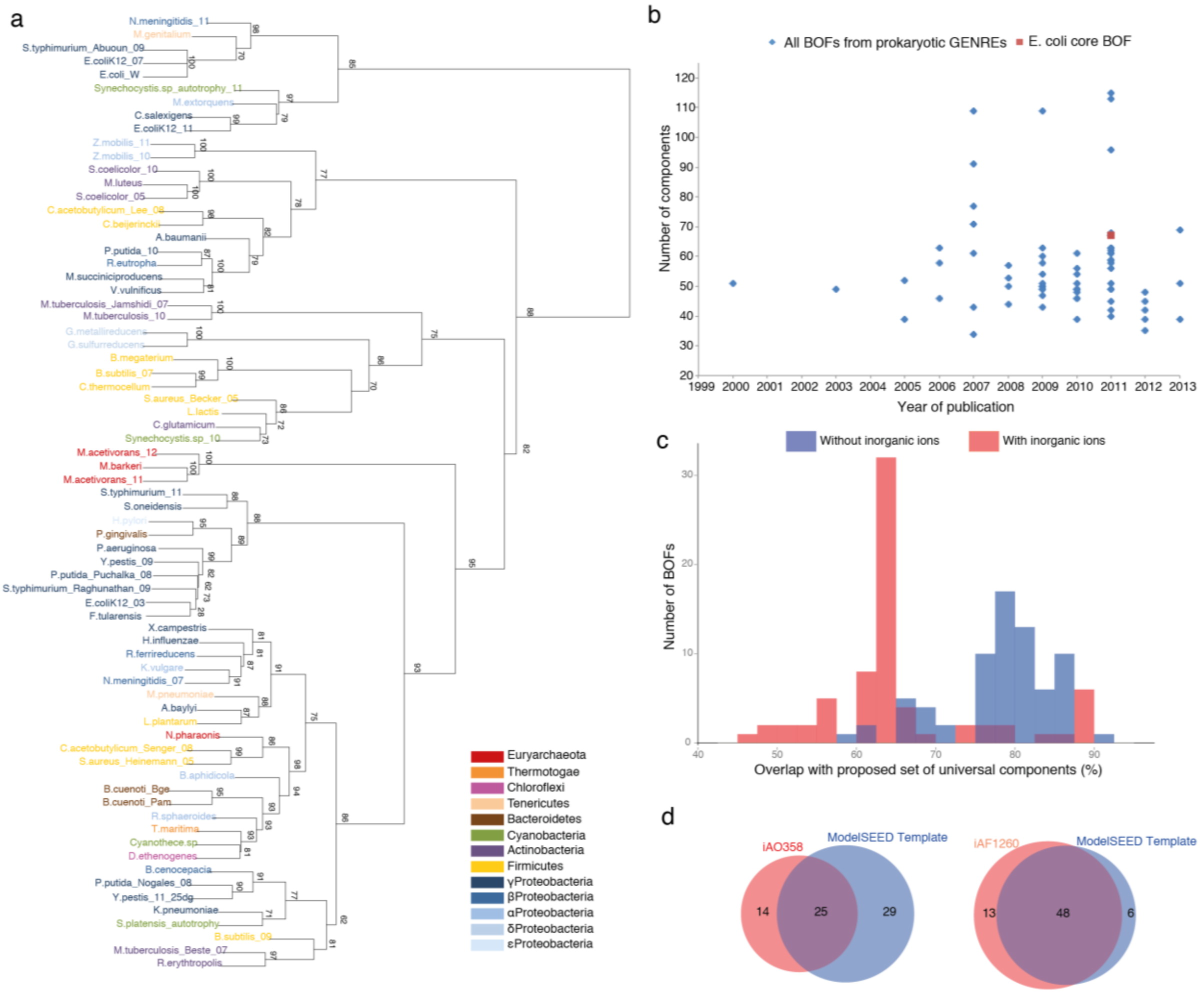
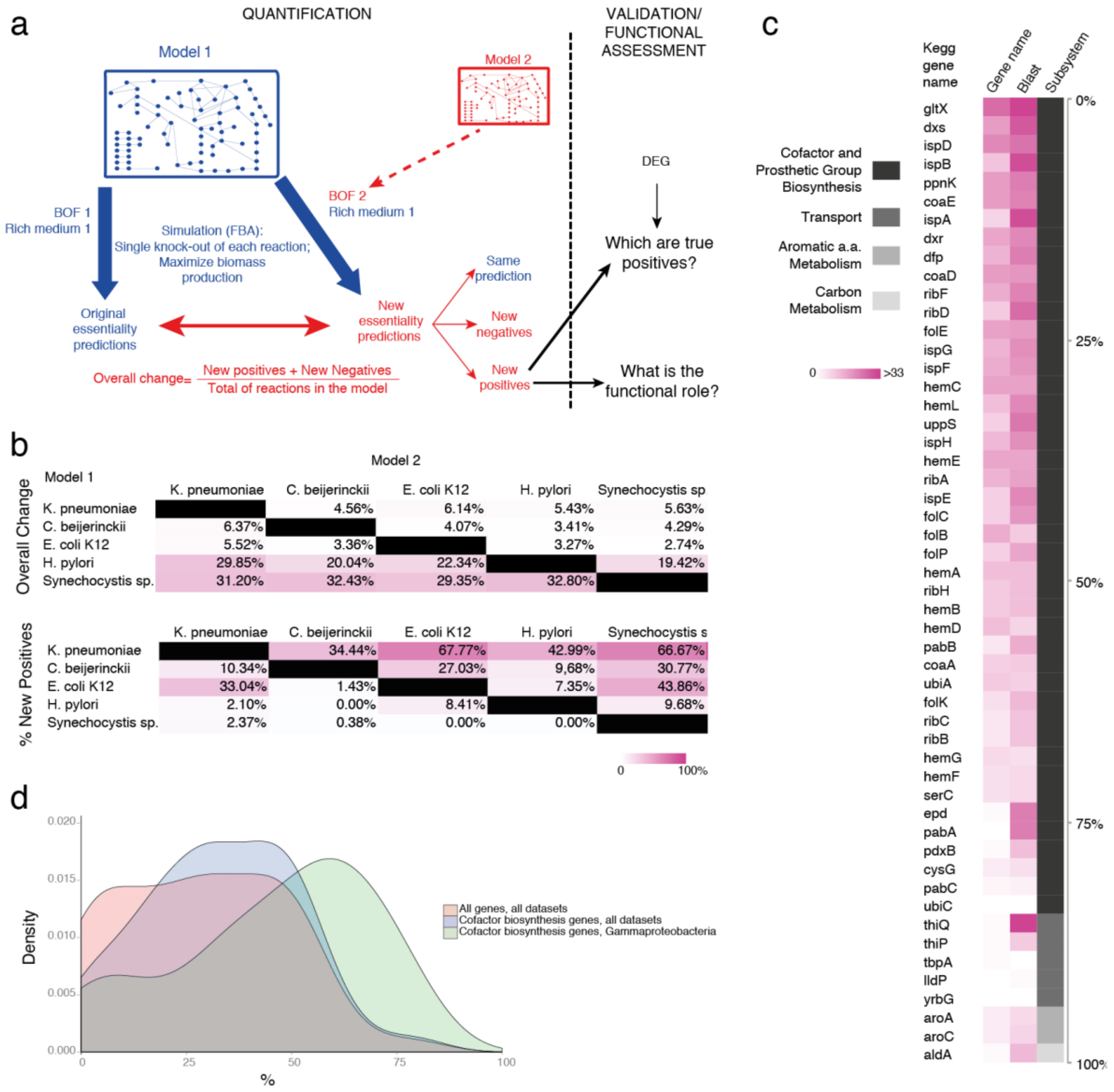


Figure 3.1 – Comparison of biomass compositions in prokaryotic genome-scale metabolic models. (a) Cluster dendrogram for qualitative biomass compositions of 71 manually curated GSMs. Numbers on branches show multi-scale bootstrap resampling probabilities (approximately unbiased p-values, %). (b) Qualitative dimension (number of components) of biomass objective functions (BOFs) of manually curated GSMs by year (blue dots) compared with the dimension of the core BOF of *E. coli* published in 2011 (red dot). (c) Distribution of overlaps of the biomass constituents of GSMs with the ModelSEED's proposed set of universal biomass components. In red, overlaps including all components; in blue, overlaps excluding inorganic ions from all compared sets. (d) Venn diagrams depicting GSMs with smallest and highest overlaps with the ModelSEED template (inorganic ions included), iAO358 (*Lactococcus lactis*) and iAF1260 (*E. coli*) respectively.

3.2.2 Qualitative Biomass Composition Drastically Impacts Essentiality Predictions

To assess the impact of the qualitative composition of BOFs on gene and reaction essentiality predictions, five GSMs representing phylogenetically diverse species were selected. Flux Balance Analysis (FBA) (Savinell & Palsson 1992) was used to predict single reaction essentiality. Then, for each model, the simulations were repeated after swapping the original BOFs with those from the other four models (**Figure 3.2**a; Supplementary Tables 3.7 and 3.8). Even under the rich media conditions used (all transport fluxes unconstrained), wherein the number of essential reactions would be the smallest, considerable changes in essentiality predictions were observed. The impact varied from 2.74% to 32.8% of the reactions changing status from essential to non-essential or vice-versa (**Figure 3.2**b) attesting the fundamental role of biomass composition in the applicability of GSMs.



Model 1	K. pneumoniae	C. beijerinckii	E. coli K12	H. pylori	Synechocystis sp
K. pneumoniae		4.56%	6.14%	5.43%	5.63%
C. beijerinckii	6.37%		4.07%	3.41%	4.29%
E. coli K12	5.52%	3.36%		3.27%	2.74%
H. pylori	29.85%	20.04%	22.34%		19.42%
Synechocystis sp.	31.20%	32.43%	29.35%	32.80%	

	K. pneumoniae	C. beijerinckii	E. coli K12	H. pylori	Synechocystis s
K. pneumoniae		34.44%	67.77%	42.99%	66.67%
C. beijerinckii	10.34%		27.03%	9,68%	30.77%
E. coli K12	33.04%	1.43%		7.35%	43.86%
H. pylori	2.10%	0.00%	8.41%		9.68%
Synechocystis sp.	2.37%	0.38%	0.00%	0.00%	

Figure 3.2 – Impact of biomass composition on predictions of reaction and gene essentiality (a) Outline of the *in silico* procedure used. Blue and red correspond to original and new model, constraints and predictions, respectively. (b) Number of reactions changing essentiality status after swapping biomass composition among five GSMs of different prokaryotes. Color scale according to normalized percentages: upper panel – overall change normalized by total of reactions in the model; bottom panel – percentage of new positives in the overall change. (c) Number of mappings – by gene name annotation and protein sequence – of 52 new essential genes predicted for *Klebsiella pneumoniae* (model iYL1228), against all experimentally determined essential genes for 33 bacterial genome-wide essentiality datasets in the database of essential genes (DEG). (d) Percentage of large-scale essentiality datasets in which new essential genes for K. pneumoniae show up as essential (density per number of genes). In orange, presence of all new essential genes in the whole DEG database; in light-blue, the subset of new essential genes annotated as involved in cofactor metabolism against all essentiality datasets; in green, new essential genes annotated as involved in cofactor metabolism against datasets of Gammaproteobacteria only.

To gain further insight into the biomass-dependency of essentiality predictions, the altered predictions were classified as new dispensable (negatives) or new essential (positives) reactions, i.e. essential with the original BOF, but not with the new BOF, or vice-versa. In the case of *Synechocystis sp.*, between 29.4 and 32.8% of essential reactions were different when using an alternative biomass composition (**Figure 3.2**b). Most of these new predictions, however, (from 97.6 to 100%) were reactions that became dispensable (new negatives) due to essential components for photosynthesis being removed with the swap (**Figure 3.2**b; Supplementary Table 3.9). Interestingly, in some swaps, new essential reactions were a larger proportion of the overall change. The extreme case was that of iYL1228 (*Klebsiella pneumoniae)* with the BOF of iAF1260 (*E. coli*), wherein 82 (67.7%) of the predictions were new essentials. The BOF of iAF1260 brings 19 new components that iYL1228 can produce (Supplementary Table 3.7; **Figure 3.2**b). Both species are closely related, belonging to Enterobacteriaceae, a common family of Gammaproteobacteria that includes known pathogens causing concerns due to multidrug-resistance (Pitout & Laupland 2008), which indicates that the biomass compositions of the two species might be similar and hints at possible gaps in the BOF of iYL1228.

3.2.3 Newly Predicted Essential Genes Have Essential Orthologs in Multiple Species And Are Related With Cofactor Metabolism

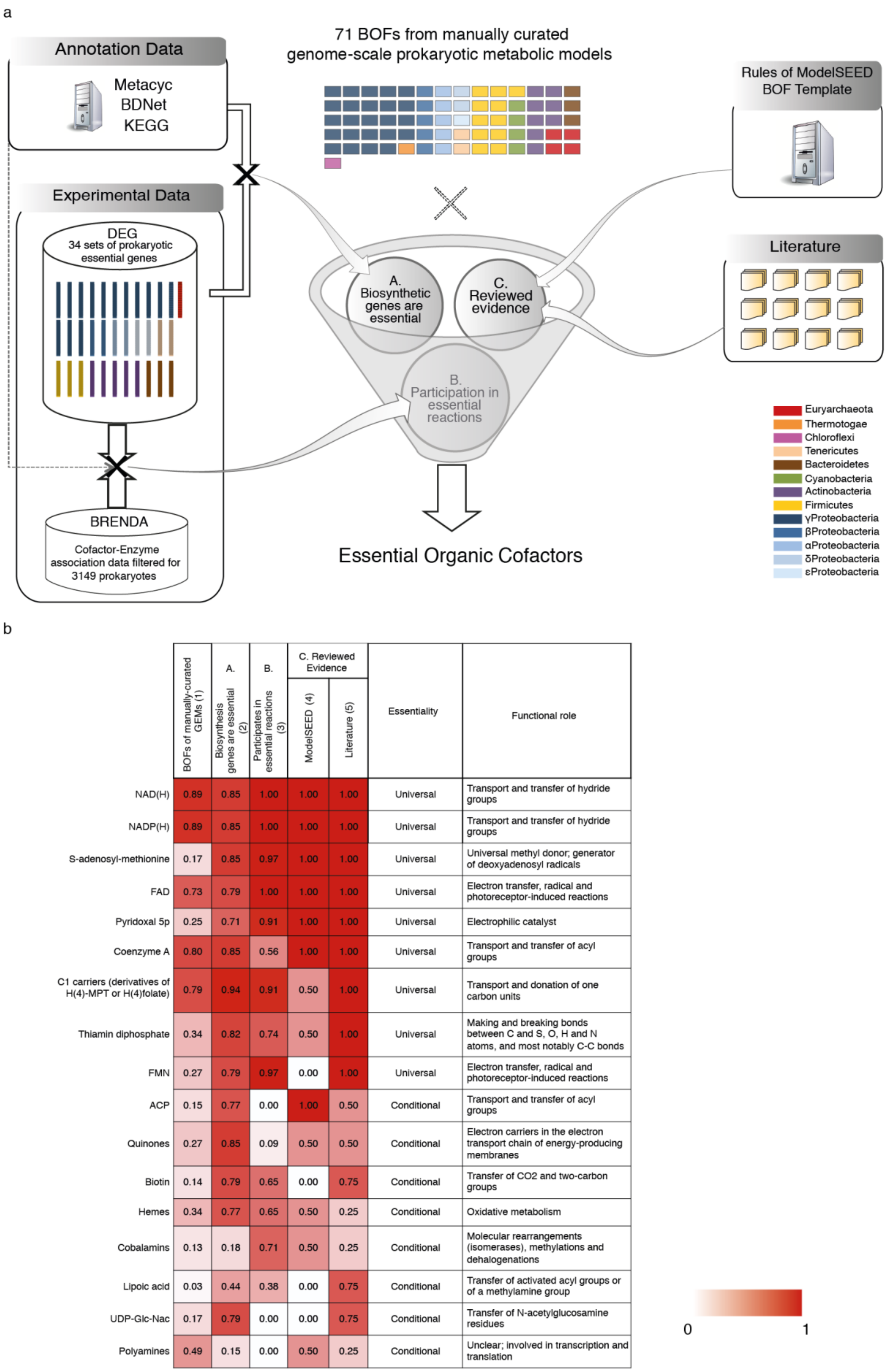
To investigate the essentiality and the biological role of the predicted new essential genes of iYL1228, given that there is no large-scale experimental assay of gene essentiality for *K. pneumoniae,* these genes were checked for whether they map to known essential genes in other bacteria. To this end, 33 gene essentiality datasets were used, covering 24 bacterial species, as available in the Database of Essential Genes (DEG) (Luo et al. 2014) (**Figure 3.2**a). The 52 new essential genes from *K. pneumoniae* (Supplementary Table 3.10) were mapped to DEG essential genes by using functional annotation and protein sequence comparison (BLASTP). 38 of the genes mapped to essential genes in at least 5 experimental datasets with both BLASTP and functional annotation. Similarly, 21 genes mapped, with both of the searching methods, to 11 or more datasets (1/3rd of the total datasets, spanning 8 or more different species) where these genes were experimentally determined as essential (**Figure 3.2**c, Supplementary Table 3.11).

The vast majority of the new essential genes (44) are annotated to functions related with biosynthesis of cofactors and prosthetic groups (**Figure 3.2**c). Moreover, all of the 21 genes found in at least one third of the datasets belong to this metabolic subsystem. For the subset of 44 cofactor-associated new essential genes, the median presence of a gene in DEG datasets is 31.8%; when additionally narrowing the searched DEG datasets for γ-Proteobacteria only (the class of *K. pneumoniae*), the median presence of a gene increases to 50% (**Figure 3.2**d).

3.2.4 Integration of Multiple Data Sources Reveals Universally Essential Cofactors

The true-positive rate of cofactor-related essential genes of iYL1228 in γ-Proteobacteria when using the biomass composition of iAF1260 indicates organic cofactors as crucial but missing biomass components in prokaryotic GSMs. To close this gap, the research proceeded to identify universally essential cofactors (or classes thereof) for prokaryotes that will improve accuracy and comparability of GSMs. For this, multiple large-scale datasets were integrated (**Figure 3.3**a). The compositions of cofactor pools of GSMs (Supplementary Table 3.12) were not used as evidence due to the lack of biological consistency and standards mentioned above. Three levels of evidence were used. A: the essentiality of genes involved in the biosynthesis of the cofactor(s) (Supplementary Tables 3.13 and 3.14). B: the participation of the cofactor(s) in reactions catalyzed by essential enzymes as per the enzyme-cofactor association data from BRENDA (Chang et al. 2015) (Supplementary Tables 3.15, 3.16 and 3.17). C: reviewed evidence, including the ModelSEED template (Supplementary Table 3.5) and an extensive review of publications on prokaryotic organic cofactors (Supplementary Tables 3.18 and

3.19). Each level of evidence was scored on a scale from 0 to 1 (details in figure caption). The results indicate 8 universally essential cofactors – nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), S-adenosyl-methionine (SAM), flavin adenine dinucleotide (FAD), pyridoxal 5-phosphate (P5P), coenzyme A (COA), thiamin diphosphate (THMPP) and flavin mononucleotide (FMN) plus one class of cofactors, which were identified as C1 carriers (includes tetrahydrofolates for bacteria and tetrahydromethanopterins for most Archaea), results summarized in **Figure 3.3**b. Highly essential cofactors with less evidence and for which there are some known exceptions were classified as conditionally essential cofactors, in which case either the phylogenetic branch not requiring this cofactor (e.g. most archaea do not use ACP) or metabolic modes in which it is not essential were identified. In the Supplementary Discussion and Supplementary Table 3.18 this classification is discussed and metadata on functional role, alternative nomenclature, related compounds, known transport systems and specificities that illustrate the complexity of the cofactor usage in prokaryotes is summarized.


a
Annotation Data
Metacyc
BDNet
KEGG
Experimental Data
DEG
34 sets of prokaryotic essential genes
BRENDA
Cofactor-Enzyme association data filtered for 3149 prokaryotes
71 BOFs from manually curated genome-scale prokaryotic metabolic models
Rules of ModelSEED BOF Template
Literature
A. Biosynthetic genes are essential
C. Reviewed evidence
B. Participation in essential reactions
Essential Organic Cofactors
Euryarchaeota
Thermotogae
Chloroflexi
Tenericutes
Bacteroidetes
Cyanobacteria
Actinobacteria
Firmicutes
γProteobacteria
βProteobacteria
αProteobacteria
δProteobacteria
εProteobacteria
b
0
1

	BOFs of manually-curated GEMs (1)	A. Biosynthesis genes are essential (2)	B. Participates in essential reactions (3)	C. Reviewed Evidence: ModelSEED (4)	C. Reviewed Evidence: Literature (5)	Essentiality	Functional role
NAD(H)	0.89	0.85	1.00	1.00	1.00	Universal	Transport and transfer of hydride groups
NADP(H)	0.89	0.85	1.00	1.00	1.00	Universal	Transport and transfer of hydride groups
S-adenosyl-methionine	0.17	0.85	0.97	1.00	1.00	Universal	Universal methyl donor; generator of deoxyadenosyl radicals
FAD	0.73	0.79	1.00	1.00	1.00	Universal	Electron transfer, radical and photoreceptor-induced reactions
Pyridoxal 5p	0.25	0.71	0.91	1.00	1.00	Universal	Electrophilic catalyst
Coenzyme A	0.80	0.85	0.56	1.00	1.00	Universal	Transport and transfer of acyl groups
C1 carriers (derivatives of H(4)-MPT or H(4)folate)	0.79	0.94	0.91	0.50	1.00	Universal	Transport and donation of one carbon units
Thiamin diphosphate	0.34	0.82	0.74	0.50	1.00	Universal	Making and breaking bonds between C and S, O, H and N atoms, and most notably C-C bonds
FMN	0.27	0.79	0.97	0.00	1.00	Universal	Electron transfer, radical and photoreceptor-induced reactions
ACP	0.15	0.77	0.00	1.00	0.50	Conditional	Transport and transfer of acyl groups
Quinones	0.27	0.85	0.09	0.50	0.50	Conditional	Electron carriers in the electron transport chain of energy-producing membranes
Biotin	0.14	0.79	0.65	0.00	0.75	Conditional	Transfer of CO2 and two-carbon groups
Hemes	0.34	0.77	0.65	0.50	0.25	Conditional	Oxidative metabolism
Cobalamins	0.13	0.18	0.71	0.50	0.25	Conditional	Molecular rearrangements (isomerases), methylations and dehalogenations
Lipoic acid	0.03	0.44	0.38	0.00	0.75	Conditional	Transfer of activated acyl groups or of a methylamine group
UDP-Glc-Nac	0.17	0.79	0.00	0.00	0.75	Conditional	Transfer of N-acetylglucosamine residues
Polyamines	0.49	0.15	0.00	0.50	0.25	Conditional	Unclear; involved in transcription and translation

Figure 3.3 – Essential cofactors for prokaryotic metabolism. (a). Data integration pipeline used towards the identification of universally and conditionally essential cofactors. Color-code of BOF and DEG datasets according to phyla. (b) Scores of prevalence of high-ranking prokaryotic essential cofactors, or classes thereof, in different analyses. Cofactor classes were defined after data integration as sets of functionally related molecules for which at least one representative should be chosen for simulations of biomass production. Capital letters A, B and C refer to the levels of evidence shown in (a). (1) Fraction of manually-curated GEMs in which the BOF contained the cofactor. (2) Fraction of DEG datasets in which there was at least one essential biosynthetic gene for the cofactor. (3) Fraction of DEG datasets in which there was at least one essential gene coding for a reaction in which the cofactor participates. (4) ModelSEED classification of essentiality: 1 - universal; 0.5 - conditional; 0 - not in the template. (5) Literature rational score: 1 - no exception found in the literature; 0.75 - several essentiality cases reported but at least one exception found; 0.25 - several exceptions found. See Supplementary Discussion and Supplementary Tables 3.18 and 3.19 for full descriptions of exceptions.

3.2.5 New Pathways and Improved Gene Essentiality Predictions for *Mycobacterium tuberculosis*

To substantiate the proposal of essential cofactors for prokaryotic life, the genome-scale model of *Mycobacterium tuberculosis* iNJ661v (Fang et al. 2010) was chosen, for this is a species for which there exists comprehensive experimental data for validations of predictions (Sassetti et al. 2003). Furthermore, although several GSMs have been built and improved for *M. tuberculosis* (Beste et al. 2007, Fang et al. 2010, Jamshidi & Palsson 2007), none of the BOFs include all of the here-proposed universally essential cofactors (conditionally essential cofactors were excluded from this analysis). In iNJ661v, although the BOF was missing NAD, NADP, COA, FAD, FMN, SAM and P5P, the network was able to produce all of these cofactors with the exception of P5P. To resolve the latter, the literature was searched for the known biochemistry regarding P5P in *M. tuberculosis.* Indeed, experimental evidence was found not only for a *de novo* pathway for P5P production that was missing in the model, but also for the essentiality of P5P for growth, survival and virulence of *M. tuberculosis* (Dick et al. 2010). After completing the BOF with all the mentioned universal cofactors that were missing, the new biosynthetic reaction of P5P was added to the model together with the two biosynthetic genes associated. This completed picture of P5P biosynthesis in *M. tuberculosis* is shown in **Figure 3.4**. The experimental study by Dick et al., that validated the P5P *de novo* pathway, reports that the growth of a mutant in this pathway could be rescued when providing pyridoxine in the medium (Dick et al. 2010). This indicates that one or all of the phosphorylations of pyridoxine, pyridoxamine or pyridoxal for which there is no genetic evidence must occur, and the gene(s) encoding them remain to be discovered. To test the modified model for its ability to predict gene essentiality, single gene knockouts were simulated in an *in silico* medium mimicking Middlebrook media (used in the experimental assay for validation of the predictions (Sassetti et al. 2003); Methods). Indeed, the gene essentiality predictions improved for the cofactor metabolic pathways, with 7 new true positive predictions (Supplementary Tables 3.20 and 3.21). The corresponding proteins are also expressed in *M. tuberculosis* (Schubert et al. 2013), adding more evidence to the findings of this work.

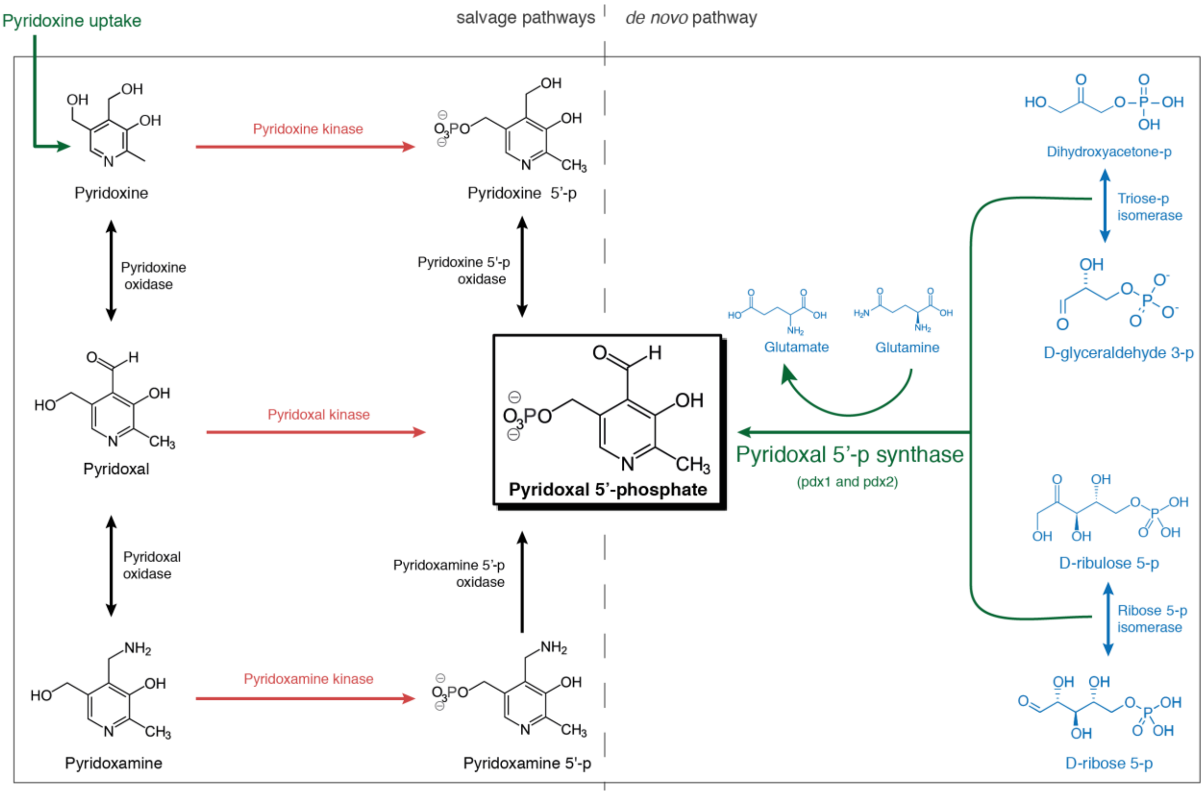


Figure 3.4 – Pathways related with pyridoxal 5'-phosphate (P5P) in different genome-scale models of *Mycobacterium tuberculosis* and additions of this work that allow production of P5P. In black, the compounds and reactions just present in iNJ661, iNJ661m and iNJ661v. In blue, reactions and compounds present in these models and also in GSMN-TB. In green, additions of this work to iNJ661v that permit the de novo production of P5P, which was not possible with any of the existing models. In red, reactions for which there is indirect biochemical evidence and no genetic evidence for *M. tuberculosis*, requiring further studies for their introduction in a model.

3.3 Discussion and Conclusions

Answering the question of what to include in the core of a biomass objective function is not always straightforward. One example is different nucleotide forms, which, although inter-convertible, are essential for cellular chemistry. Here it is proposed that all essential and irreplaceable molecules for metabolism should be included in the biomass functions of genome scale metabolic models. In the special case of cofactors, when two forms of the same cofactor take part in the same reactions (such as NAD and NADH), one form only could be included for the sake of simplicity. When a class of cofactors includes active and non-active interconvertible forms, the active forms should be preferred. A simple example case is the representation of flavins: FAD and FMN are the preferred active forms to be included in the BOF, oppositely to riboflavin, the non-active precursor.

A standardized and detailed core biomass composition for prokaryotes is proposed in **Figure 3.5**. This is a conservative proposal and thus includes only the three most prevalent lipid components in bacteria as representative species (phosphatidylglycerol, phosphatidylethanolamine and cardiolipin) and excludes non-universal macromolecules such as cell wall peptidoglycans (more details can be found in the Supplementary Discussion).

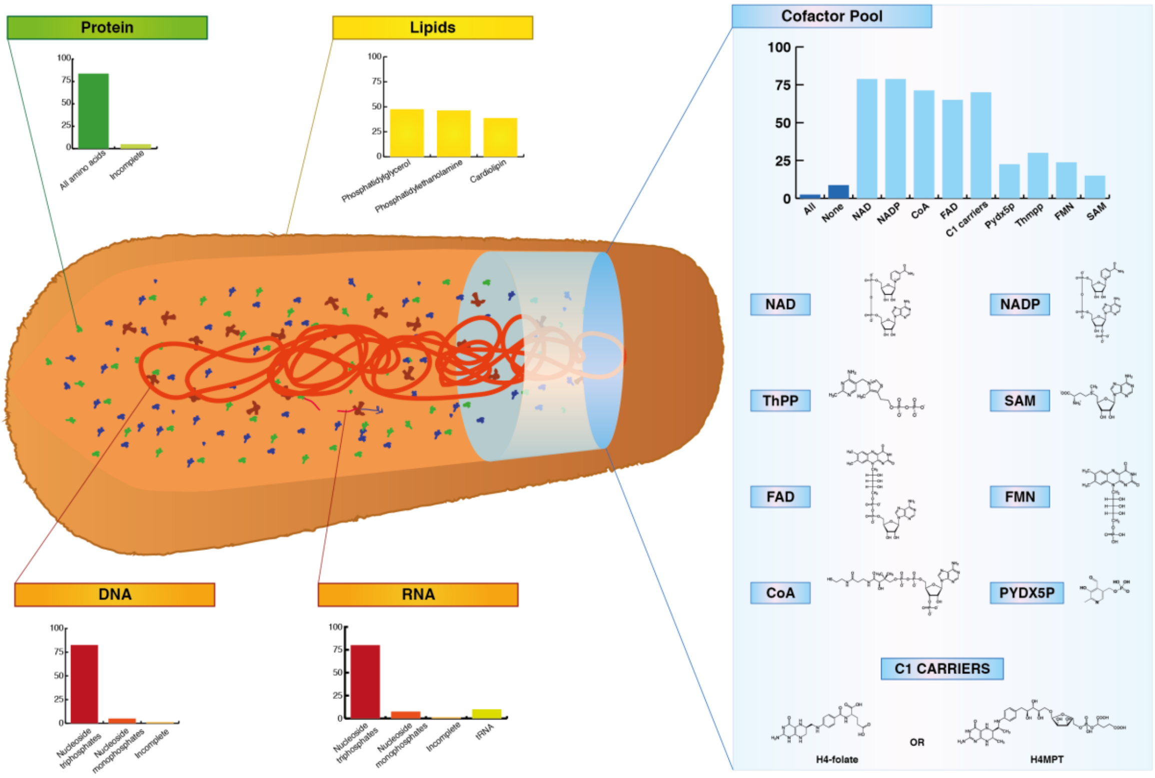


Figure 3.5 – Most prevalent components in the biomass composition of manually curated genome-scale metabolic models of prokaryotes and a proposal of universally essential organic cofactors. Prevalence in bar plots is quantified as the percentage of the 71 models analyzed in which each specified component or set of components is present: red – nucleic acids; green – protein; yellow – lipids; blue – organic cofactors. Highlighted set of organic cofactors summarizes the findings of this work.

The cofactors here identified as universally essential play fundamental roles in biochemistry. In most cases, they are related with the transfer of small units: hydride groups for NAD(P)(H), methyl groups for SAM, electrons for FAD and FMN, acyl groups for CoA and one carbon units in C1 carriers. The two special cases of P5P and THMPP correspond to direct intervention in catalysis, which stabilize intermediate metabolites and assist in the formation of new chemical bonds, respectively. The classification of universally essential is conservative, excluding cofactors for which minor exceptions were found in the data analyzed, e.g. biotin (**Figure 3.3**b; Supplementary Table 3.18). Such exceptions could be false negatives due to incomplete data or biases in databases, e.g. interactions in BRENDA may exclude carrier cofactors like CoA, ACP and quinones (more details in the Supplementary Discussion).

Updating the biomass composition in metabolic models allowed for the identification of new candidate essential genes for *K. pneumoniae,* backed by experimental genetic evidence for orthologues of related species. These could serve as potential drug targets for *K. pneumoniae,* a pathogen causing urgent concerns regarding antibiotic resistance (Kontopidou et al. 2014, Snitkin et al. 2012). The importance of using a comprehensive biomass composition for *M. tuberculosis* is also demonstrated. The modifications done to iNJ661v successfully led to the identification of a previously validated pathway for vitamin B6 biosynthesis, which was missing in the current model, and improved gene essentiality predictions.

When a new (essential) component is included in the BOF, it implies that this component needs to be provided, either through the biosynthetic pathway or via transport reactions. The construction of more complete and standardized BOFs will thus have a great impact not only in the predictions of essential genes but also in the construction of minimal media required for growth. Both applications are of utmost importance for pathogens, being in fact the most common motivations to construct GSMs for those organisms.

Overall, this work lays foundations for improving the definition of biomass composition in the current and future metabolic reconstructions – an important step towards biochemically more accurate models with higher predictive power. Moreover, it is the first large-scale systematization of essential metabolic organic cofactors for prokaryotes, which will be central in several fundamental and applied studies.

3.4 Supplementary Discussion

3.4.1 Sensitivity to Errors and Incompleteness in Databases

The deduction of essentiality of cofactors in this study is directly related to the results of several genome-scale assays of gene-essentiality stored in the database of essential genes (DEG) (Luo et al. 2014). On a first level, although most of the experiments are performed under rich media conditions, which benefits the conservative deduction of universal essentiality done here, the heterogeneity of experimental conditions of the assays should be noted, with one dataset of *Salmonella enterica* with conditional essential genes only (determined under different selective conditions of temperature and nutrients) (Khatiwara et al. 2012). Secondly, not all the datasets in DEG are exhaustive genome-scale assays of essentiality, for example with one dataset of *Pseudomonas aeruginosa* PAO1 consisting of antibiotic resistance genes (Gallagher et al. 2011).

DEG datasets indicate non-essentiality for some cofactors that are classified as universally essential. Some specific cases are noted below. Generally, even without the above-mentioned limitations, error-free datasets would not be guaranteed (as reviewed in (Gil et al. 2004) and (Yang et al. 2014)). Transposon mutant libraries, used in the majority of datasets in DEG (Luo et al. 2014), can overestimate essentiality through the misclassification of very slow growth mutants as lethal phenotypes. Reversely, essential genes might be classified as dispensable if they tolerate transposon insertions being nevertheless transcribed and translated to functional proteins. Even if consisting only of true classifications, single gene knockout mutant libraries are usually not sufficient for the deduction of functional essentiality. This occurs due to the well-known redundancy that is hard-wired in bacterial metabolism, where alternative pathways allow many times for the biosynthesis, salvage and import of several important molecules, including organic cofactors. These pathways are not always known, especially in the case of less studied species. Multiple simultaneous gene-knockouts would be required to confirm some cases of essentiality classification. Nevertheless, the approach here is conservative as it integrates gene essentiality data with different other types of data, deducing as universally essential only the cofactors with a high confidence level from the integrated data.

Regarding BRENDA, only full EC numbers were mapped to essential genes. Also, some cofactors considered here (e.g. ACP and polyamines) are not present in the cofactor-enzyme association datasets in the database. This decreases the level of evidence for some cofactors, and therefore the confidence of the deduction here would probably increase with the addition of this information.

3.4.2 Universally Essential Cofactors

Here some details on the classification of universal essentiality are discussed for the different cofactors based on the integration of databases described in section 3.2. For further details, refer to Supplementary Tables 3.18 and 3.19.

3.4.2.1 NAD(H) and NADP(H)

The common redox cofactors are accepted as universally essential without controversy; there are no reports in the literature of the dispensability of these cofactors. Several transporters exist for precursors. The dataset of essentiality of *Pseudomonas aeruginosa* PAO1 in DEG, mentioned already as a set of resistance-related genes, is the only dataset integrated with BRENDA in the current work where no essential gene dependent on NAD(H) was found.

3.4.2.2 S-adenosyl-methionine (SAM)

As a universal methyl donor and a key element in the "methylation cycle", SAM plays a fundamental role in metabolism. It is also a generator of deoxyadenosyl radicals, a regulator of transcription (McDaniel et al. 2003) and a direct intervenient in the assembly of the septal ring in cytokinesis. Integration of DEG and BRENDA always reveals essential genes depending on this cofactor, with the exception of one dataset of *Salmonella*, although others for the same species disagree. The integration of biosynthetic annotation data with essentiality provides moderate evidence for essentiality, probably due to the known existence of several transporters for this vitamin which surpass the necessity of a biosynthetic route in some species or environments (Binet et al. 2011, Haferkamp et al. 2013, Tucker et al. 2003).

A study with *Escherichia coli* claims depletion of SAM to very low levels using a SAM hydrolase (Posnick & Samson 1999). Others reported temperature-sensitive mutants of metK (a gene involved in SAM biosynthesis) that were genetically unstable and required methionine for growth (Satishchandran et al. 1990). A later study reviewed metK mutants as leaky, resulting in phenotypes as diverse as overproduction of methionine, methionine auxotrophy or complete inability to grow on defined media; however, all of these phenotypes included a residual SAM synthetase activity (Newman et al. 1998).

Contradicting these results, metK is classified as essential by at least 23 prokaryotic datasets of genome-scale essentiality in rich media, in DEG. Moreover, El-Hajj and colleagues reported in 2013 that the isolation of mutants totally deficient in SAM synthase became possible only with the isolation and cloning of a SAM transporter from *Rickettsia prowazekii* (Tucker et al. 2003) into an *E. coli* plasmid, allowing the metK mutant to grow in rich medium with an exogenous SAM supply (Driskell et al. 2005). El-Hajj and colleagues used this transporter to study SAM metabolism in further detail (El-Hajj et al. 2013).

3.4.2.3 FAD and FMN

Flavins are accepted as the universal currency for electron transfer, radical and photoreceptor-induced reactions. Riboflavin is commonly represented in the biomass objective functions (BOFs) of genome-scale metabolic models (GSMs) even though it is biologically inactive; there are known transporters for this precursor (García Angulo et al. 2013, Vogl et al. 2007). All datasets in DEG show at least one essential enzyme depending on FAD (Supplementary Table 3.16).

3.4.2.4 Pyridoxal-5-phosphate (P5P)

Several reviews indicate P5P as universal and essential (Christen & Mehta 2001, Fitzpatrick et al. 2007, Percudani & Peracchi 2003), even though only 25% of GSMs include it in the BOF. It binds covalently to its substrates, which can hinder measurements of the free vitamin available in the cell. There are known alternative pathways to the production of this vitamin, which also hinder inference of essentiality from single gene knockout studies (Kim et al. 2010).

Further experimentation would be required with *Campylobacter jejunii,* as the dataset from DEG used here (Metris et al. 2011) indicates non-essentiality when crossed with BRENDA, but another study indicates possible essentiality of pdxA, involved in P5P biosynthesis (Stahl & Stintzi 2011). A recent study reported non-essentiality of pdxA and a full depletion of P5P production, achieved with that single deletion (Asakura et al. 2013), although several questions can be posed regarding the use of those results for the purpose of this work: the use of a rich, undefined medium which most probably contains the vitamin or other vitamers or the more than two fold increase in the direct precursor of P5P in an alternative biosynthetic pathway (pyridoxamine 5 phosphate). Moreover, the residual amounts reported in the mutants could be sufficient for growth, as an amount of 6.0 ng/mL of pyridoxal has shown to be the growth-limiting concentration for mutants of *E. coli* (Scott & Hockney 1979).

3.4.2.5 Coenzyme A (CoA)

As the universal carrier of acyl groups in cells, reported as used by 4% of all known enzymes, CoA is commonly accepted as universally essential (Begley et al. 2001). Most species in the datasets used here have essentials genes involved in the biosynthesis of this cofactor, with the exception of the species of the genus *Mycoplasma*. It has been postulated that, along with other pathogens as *Rickettsia* and *Chlamydia*, these species can uptake dephospho-CoA (Spry et al. 2008).

3.4.2.6 C1 Carriers

Tetrahydrofolates in bacteria and some Archaea, and tetrahydromethanopterins in some other Archaea play the essential role of transport and donation of one-carbon units in metabolism (De Crécy-Lagard et al. 2012). The data in this study supports the essential biosynthesis of the active forms of these cofactors for all species analysed in at least one dataset. Regarding essential enzymes in BRENDA depending on these cofactors, all datasets in DEG show at least one, with the exception of the incomplete datasets referred in section 3.4.1 and the dataset for the archaea, which depends on methanopterins not included in BRENDA.

3.4.2.7 Thiamin diphosphate

Thiamin diphosphate assists in making and breaking bonds between several atoms in metabolic reactions, most notably C-C bonds (Frank et al. 2007). As there are transporter systems identified and assayed, especially for Salmonella (reviewed in (Begley et al. 1999)) the essentiality of biosynthetic genes alone is inconclusive. However, in three cases where the integration of DEG with BRENDA does not reveal essentiality of thiamine, the annotation of essential biosynthetic genes gives evidence of it: *Burkholderia, Campylobacter* and *Mycoplasma*. In the case of *S. aureus*, the requirement of this cofactor has been shown experimentally (Gretler et al. 1955, Mah et al. 1967), as for *H. pylori* (Nedenskov 1994) and *Streptococcus sanguinis* (Carlsson 1972).

3.4.3 Conditionally Essential Cofactors

Cofactors in this section showed a lower average level of evidence for universal essentiality (see **Figure 3.3**b in Results section and Supplementary Tables 3.14 and 3.17). Here the role of these cofactors in prokaryotic metabolism that can justify these results is discussed. The known cases where they are known to be not essential are mentioned.

3.4.3.1 Acyl-carrier protein (ACP)

ACP shares with CoA the 4-phosphopantetheine moiety, performing the same function as the latter cofactor as a carrier of acyl groups. It does not have the characteristics of most other cofactors, which can justify its absence from BRENDA cofactor association data, but it is considered a cofactor protein, essential for the synthesis of new membrane in all bacteria. It is currently believed that Archaea carry out fatty acid synthesis in an ancient ACP-independent manner, and most species lack ACP and its related enzymes (Lombard et al. 2012). The other species for which there was no evidence from essential biosynthetic genes related with ACP were *Burkholderia pseudomallei*, for which there is experimental evidence of essentiality (Cummings et al. 2014), and for *Bacteroides thetaiotaomicron*. However, in a close relative to the later, *Bacteroides fragilis*, the gene putatively encoding for ACP is essential and thus further experimentation is required regarding that organism.

3.4.3.2 Quinones

Quinones are essential for all chemiosmotic (respiratory or photosynthetic) energy-converting systems, allowing for electron movement across membranes, with the exception of those of methanogenic organisms (Schoepp-Cothenet et al. 2009). Strict fermentative metabolism does not require quinones, but even though some bacteria that are obligatorily fermentative have lost their ability to synthesize quinones (being the best studied *Lactobacillus*, *Streptococcus* and *Bifidobacterium* (Walther et al. 2013)), some of the species still retain the biosynthetic pathway (Nowicka & Kruk 2010) and the electron transport chain and respiratory metabolism can be induced by the presence of both environmental quinones and heme (Brooijmans et al. 2009, Yamamoto et al. 2005). Interest has been raising by the fact that cultures of other anaerobes do produce several menaquinones, in sufficient amounts to provide dietary requirements (e.g. *Lactococcus lactis* and *Brevibacteirum* (Walther et al. 2013)). Given that quinones play other functions in prokaryotic cells, they can actually be essential in some types of fermentative metabolism (Kato et al. 2010). The phylogenetic distribution of quinones is widespread across prokaryotes (Collins & Jones 1981) and the data in this study indicates essentiality of biosynthetic genes for many of the species in DEG, even those with a versatile metabolism. More studies are required to analyse the essentiality of quinones in versatile conditions.

3.4.3.3 Biotin

Biotin plays a crucial role in the transfer of CO_2 and two-carbon groups, although the data in this study fails to provide evidence of essentiality of this cofactor in several species. It has been reported that *Buchnera sp.*, *Borrelia burgdorferi*, *Aeropyrum pernix*, thermoplasmas and mycoplasmas have neither the biotin biosynthetic genes nor birA, a bifunctional protein which acts both as a biotin–protein ligase and as a transcriptional repressor of the biotin operon

(Rodionov et al. 2002). For Mycoplasma, it was also reported earlier that some strains have a biotin requirement, and others do not (Smith 1991). In *E. coli* and other species, it was shown that there is a strict requirement for this cofactor (Finkenwirth et al. 2013).

3.4.3.4 Hemes

Heme situation in metabolism is very similar to that of quinones, in that it is essential for both the aerobic and anaerobic respiration, and it has also been shown that its biosynthesis is coupled to it (Möbius et al. 2010). In the absence of exogenous heme or the ability to produce it, some species can live on fermentative metabolism. These include lactic acid bacteria and some opportunistic and endosymbiotic species (Gruss et al. 2012, Lechardeur et al. 2011). Other species however have been shown to have a strict requirement for heme, including *Porphyromonas gingivalis, Bacteroides fragilis* and *Haemophilus influenzae* either for the activation of cytochrome oxidases, fumarate reductases and catalases; other functions have been identified that might explain the essentiality of heme in some species, as reviewed in (Gruss et al. 2012). For *Escherichia coli*, it has been shown that even though mutants not able to produce heme can grow anaerobically, they cannot do so in the presence of oxygen, as the expression of fermentative enzymes is limited to anaerobic growth conditions by the activity of redox response regulators (Rompf et al. 1998).

3.4.3.5 Cobalamins

Adenosylcobalamin, methylcobalamin and adocobalamin are important in isomerization reactions, methylations and dehalogenations. For *E. coli* and related species, they are only strictly essential in specific environments where glycerol, propanediol and/or ethanolamine are important sources of carbon or nitrogen and energy (Fowler et al. 2010). Other cases where essentiality was demonstrated are methanogenic archaea (Martens et al. 2002) and *Rhodocyclus purpureus* (Pfennig 1978). A comparative genomics study identified the absence of cobalamin biosynthetic genes and regulatory elements in most obligate pathogenic bacteria and in *Aquifex aeolicus* (Rodionov et al. 2003).

3.4.3.6 Lipoic Acid

Also called lipoate, it is essential for several key enzyme complexes in oxidative and one carbon metabolism, including pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Spalding & Prigge 2010). It is therefore not strictly essential in facultative anaerobes, anaerobic organisms and the special case of the microaerophilic *H. pylori* (Spalding & Prigge 2010).

3.4.3.7 UDP-Glc-Nac

UDP-n-acetyl-d-glucosamine is a universal donor in the transfer of N-acetylglucosamine residues, essential for the synthesis of the cell wall in prokaryotes (Namboori & Graham 2008). Currently it is accepted that only Mycoplasma does not require this cofactor, as it does not produce cell wall (Du et al. 2000).

3.4.3.8 Polyamines

The role of polyamines in prokaryotic metabolism (the most common in Bacteria and Archaea being putrescine and spermidine) is ubiquitous, as reviewed in (Schneider & Wendisch 2011). The analysis done here included them as organic cofactors mainly due to their classification in GSMs, which have included them broadly (49%). However, polyamines act more as stabilizers and signaling molecules and are not usually considered as cofactors (Shah & Swiatlo 2008) (for this reason, as with ACP, no essential enzymes are matched as depending on them). It has been reported that polyamines are essential for normal growth (Shah & Swiatlo 2008), even though their essentiality is not prevalent, with reported dispensability in *E. coli* (Hafner et al. 1979), *Yersinia pestis* (Patel et al. 2006) and several other species (Bitoni & Mccann 1987).

3.4.4 Other Details on Modeling Biomass Compositions

Biomass objective functions were formulated in different manners in the field of metabolic modeling: direct biosynthesis from precursor metabolites (Varma & Palsson 1993a,b); biosynthesis from building blocks (Feist et al. 2007, Varma et al.

1993) or biosynthesis from macromolecules (Liao et al. 2011), using lumped reactions for each (Villadsen et al. 2011). Also, there is no consensus on how each component should be included in BOFs. For example, Coenzyme A, an important cofactor in lipid metabolism, is represented in isolation in the solute pool in some cases, charged with lipids in some, and is even excluded in others. These different ways of formulating BOFs, together with nomenclature inconsistencies that have been addressed elsewhere (Bernard et al. 2014, Kumar et al. 2012, Sauls & Buescher 2014), hinder comparative studies involving manually curated GSMs.

There are few exceptions in which the introduction of an essential component in the BOF might bring additional questions unaddressed in the context of GSMs. This is the case of the acyl carrier protein (ACP) and potentially other protein-based components. ACP is included in the ModelSEED universal template for biomass composition and is considered to be essential in most organisms. However, its inclusion in the BOF implies the introduction of a biosynthetic pathway in the model, which might lead to inconsistencies, as no other protein has a dedicated pathway in GSMs. An alternative would be to include an artificial transport reaction, which implies adding information to the model that has no correspondence in reality.

Another situation with even higher biological relevance is that of RNA. Some models already include tRNA and the essential reactions and genes that charge each individual tRNA molecule with its respective amino acid. There is, however, a generalized absence of mature rRNA in GSMs, which pool also needs to be maintained stable, by duplicating with each cell division, involving metabolic transformations (Deutscher 2009).

The biomass composition of a cell can change with different growth conditions within the same strain (Blazewicz et al. 2013, Cotner et al. 2006, Pramanik & Keasling 1998, Vu et al. 2012). Setting a standardized average core biomass composition is only the starting point for increasingly better, more predictive genome-scale metabolic models.

3.5 Methods

3.5.1 Collection and Comparison of Detailed BOFs in GSMs

Manually-curated GSMs of prokaryotes were searched for in four major online databases: BiGG (Schellenberger et al. 2010), MetRxn (Bernard et al. 2014), BioModels (Chelliah et al. 2015), GSMNDB (Systems Biology and Metabolic Engineering Research Group at the Tianjin University 2014) and in an updated list of GSMs as per Palsson group website (Systems Biology Research Group at the University of California San Diego 2014). The biomass composition was, whenever possible, retrieved directly from the model file; if the model was not available or not accessible, the composition, along with the metadata, was taken from the publication (Supplementary Table 3.1). For the cases where several important macromolecules or the solute pool were represented in lumped reactions, the composition from the individual lumped reactions was deconstructed. For nomenclature standardization, an initial list with all the metabolites from BOFs of GSMs built with BiGG nomenclature was created. Each individual component of all remaining BOFs was matched against that list, with the help of mappings of ModelSEED (Henry et al. 2010b). The non-matching metabolites were checked manually for matches with alternative names. Several species-specific tagged metabolites were discarded, although if they could be matched as generalist lipids (e.g. phosphoethanolamine) or peptidoglycan the tag would be removed or the id would be substituted by the more general id. For yet non-matching metabolites, a new entity and id was created in the list (Supplementary Tables 3.2, 3.3 and 3.4).

The ModelSEED template for universal biomass components was obtained from the original publication (Henry et al. 2010a).

3.5.2 Cluster Analysis

Hierarchical clustering was performed using ‘pvclust’ R package (Suzuki & Shimodaira 2006) with binary distance as a dissimilarity metric and Ward 1 method as the linkage criterion. For accessing uncertainty, approximately unbiased p-values were calculated via multiscale bootstrap resampling. All statistical analyses were performed using R statistical software version 3.1.

3.5.3 BOF Swap

Five different GSMs were chosen by sampling high and low phylogenetic dissimilarity pairs in order to assess the impact of BOFs in predictions of essentiality (**Figure 3.2**a-b; Supplementary Table 3.1 for corresponding phyla). When adding a new BOF to a model, the model was verified to contain all new metabolites added, and if not, those were removed from the BOF (Supplementary Table 3.7). It was also checked that the wild-type network was viable with all the existing import drains set for simulation of nutrient import (20 mmol/gDW/h). Often some metabolites were not added, either for not being represented in the model at all, or for being end-points of blocked pathways in the network. The same media conditions were used for simulations before and after all swaps. The swaps likely alter the interpretation (units) of biomass in the BOF, which however does not affect the Boolean results of feasibility of biomass production.

3.5.4 Simulations of Reaction/Gene Deletion Phenotypes

Simulations of maximum growth rates for single-deletions of reactions and genes were performed using Flux Balance Analysis (FBA) (Savinell & Palsson 1992, Varma & Palsson 1993a). For the study of the impact of BOF swap in essentiality predictions, the flux through the BOF was calculated and mapped directly for each reaction deletion in each model. For the validation of results for *K. pneumoniae* with experimental data, individual gene knockouts were generated for all model genes and the flux through the BOF was accessed and mapped to each gene. All modeling procedures were implemented in C++ and solved using IBM ILOG CPLEX solver.

3.5.5 Mapping *In Silico* Essential Genes with Large-Scale Experimental Essential Datasets

Searches in DEG (Luo et al. 2014) were performed manually for each of the 52 new essential genes of iYL1228 (see Results section). Matching was done by searching for the corresponding gene annotation and, independently, with BLASTP in DEG with an E-value threshold of 10e-6.

3.5.6 Data Extraction and Integration

All enzyme-cofactor association data for prokaryotes was extracted using the Python SOAP access methods for BRENDA (Chang et al. 2015). Biosynthetic genes for each cofactor or class of cofactors identified in the cross-integration of DEG and BRENDA were extracted manually from Metacyc (Caspi et al. 2014). For the mapping of gene names in DEG with BRENDA and Metacyc, bioDBNet (Mudunuri et al. 2009) and KEGG (Kanehisa et al. 2014) were used.

3.5.7 Modification of iNJ661v

All changes described in the Results section were performed manually on the original SBML file for iNJ661v. To simulate Middlebrook media as used in the genome-scale experimental assay for validation of the predictions (Sassetti et al. 2003), new transporters for biotin and pyridoxine were added). The upper bounds of all the respective uptakes of the constituents were set to 20 mmol/gDW/h, with the exception of albumin, zinc, catalase and oleic acid (not modeled).

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CHAPTER 4

Essential and Ancestral Metabolic Functions in Prokaryotes

A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.

—MARIE CURIE, as quoted in *Madame Curie: A Biography* (1937), by Eve Curie Labouisse

In this chapter the essential reactions in several different metabolic networks of prokaryotic cells were analyzed to study the composition of early and minimal complex cellular systems. The main hypothesis is that essential and highly conserved metabolic functions are shaping constituents of theoretical minimal metabolic networks and were also present already in ancestral cells. Results from large-scale simulations of 15 manually curated genome-scale metabolic networks were integrated with 36 large-scale gene essentiality assays encompassing a wide variety of species and phyla of bacteria and archaea. Ancient metabolic genes were estimated from an analysis of conservation with 79 manually selected representative genomes from all the branches of the prokaryotic tree of life. The results indicate the tRNA charging module as an isolated winner in centrality and ancestry, pointing to an early information processing system supplied by ATP dependent transport systems in a rich primordial environment. The high conservation and essentiality of cofactor biosynthesis genes points to an early depletion that selected for cells that were autonomous for the production of these crucial catalysts. On a large-scale, highly essential genes tend to be highly conserved as opposed to non-essential genes which may be highly conserved or not.

The information presented in this Chapter is being prepared for submission to a peer reviewed journal:

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4.1 Introduction

4.1.1 Cellular Complexity and Genome-Scale Essentiality

Prokaryotes are the simplest contemporary life forms known, and nevertheless are characterized by an immense complexity. The debate on the requirement of such complexity for life and its breadth in the primordial life forms has been around for years (Kauffman 1995, Rasmussen et al. 2008, Schuster 1996), and was furthermore expanded and detailed since the advent of systems biology (Kim & Caetano-Anollés 2011, Oltvai & Barabási 2002, Peretó 2012). The study of essential genetic alleles has been crucial for detangling this complexity, relating some proteins with cell viability in specific conditions (Skouloubris et al. 1998) and others with cell viability in apparently all conditions (Fayet et al. 1989, Wu et al. 1999). Genome-wide essentiality studies based on collections of targeted mutants or random mutagenesis techniques have been conducted for a number of species, aiming mainly at antibiotic design or industrially relevant targets (see Chapter 2, Section 2.2.1 and Table 2.3). These data have been integrated in databases such as OGEE (Chen et al. 2012) and DEG (Luo et al. 2014) but their functional analysis is still incipient. Early work based on genome-scale essentiality for four bacterial species included the interesting finding that essentiality drives gene strand bias (Rocha & Danchin 2003). Later, a review was published with a critical analysis of this type of large essentiality datasets, in which the authors also conducted a preliminary analysis which integrated 6 genome-scale assays corresponding to 4 different species (Gerdes et al. 2006). Functional differences were highlighted, as the smaller number of essential genes in flavin synthesis in *B. subtilis,* a species known to have an active riboflavin salvage capability. The authors of the DEG database have also recently conducted a couple of integrative analysis on large-scale essentiality data. The first concluded that there are less essential genes inside than outside genomic islands, and some of those are related with virulence (Zhang et al. 2015). The second study (Luo et al. 2015) added to a previous finding based only on *E. coli* essentiality data where it was proposed that essential genes are more evolutionarily conserved than non-essential genes (Jordan et al. 2002). Luo and others used the same type of analysis, based on synonymous and non-synonymous substitution rates, to corroborate this finding (Luo et al. 2015). The authors also suggest that the most evolutionarily conserved COG categories of essential genes are: Carbohydrate transport and metabolism; Coenzyme transport and metabolism; Transcription; Translation ribosomal structure and biogenesis; Lipid transport and metabolism, and Replication, recombination and repair.

4.1.2 Genome-Scale Metabolic Models and the Core and Ancestral Metabolism

Genome-scale metabolic models (GSMs) are curated large repositories of metabolic data for individual species that expand possibilities of functional analysis of cellular physiology. More than improving or suggesting new functional annotations by reconstructing whole pathways (Overbeek et al. 2014), GSMs can be used for calculations of metabolic fluxes that permit the prediction of, among others, lethal phenotypes (Edwards & Palsson 2000) (see Chapter 1). Multi-species analysis of this type of phenotype predictions with different manually curated models has been scarce (Oberhardt et al. 2009), in part impaired by the poor knowledge basis for other species than the usual model organisms, but also by the deficient use of standards in building such models.

Comparative genomics is commonly used to find core essential genes for several species, and at the same time, given that it is based on the evolutionary key notion of orthology, to infer the group of genes present in common ancestors of the species analyzed (Koonin 2003). Assuming evolutionary parsimony, it is expected that genes present in a set of species have been vertically inherited from a common ancestor. Horizontal Gene Transfer (HGT) might have played a role even in ancient times before the divergence of the three main domains (Fournier et al. 2015). Nevertheless, when a gene is present in all or most species of a phylogenetic tree, the most parsimonious scenario is that HGT was not the cause of all, or at least the majority of the conservation. In the case of functional comparisons as in comparisons of metabolic reactions, the problems with sequence data and HGT are surpassed.

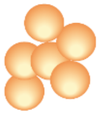
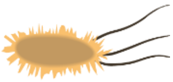
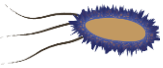
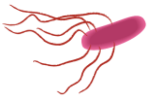
In this study, 36 experimental genome-scale essentiality assays were integrated with simulations of 15 genome-scale metabolic models and the screening of full genome sequences of 79 prokaryotic species in order to find core essential and ancestral functions in prokaryotic biology. It is expected that this knowledge on the minimal metabolic functions of prokaryotic cells can not only help uncovering the fundamental complexity of cellular systems but also, by building up on the concept of orthogonalization of metabolic modules (Mampel et al. 2013), here analyzed in the form of metabolic subsystems, improve future engineering approaches that use this type of organisms.

4.2 Methods

4.2.1 Genome-Scale Metabolic Models Used in Essentiality Predictions

For all essentiality predictions performed in this study, 15 genome-scale metabolic models were chosen based on curation, validation, and comparability of the nomenclature of metabolites and reactions. These comprise 7 prokaryotic phyla, including one archaea. Ten of these include more than 20% of the total number of the species ORFs. **Table 4.1** summarizes the details on these models including species name, strain, a small illustration, model ID, statistics and references.

Table 4.1 – Details on the models and corresponding species used in the *in silico* essentiality studies performed in this chapter.

Phylum	Species	Illustration	Model ID	Reactions	Metabolites	% ORFs	Reference
Firmicutes	*Bacillus subtilis*		iYO844	1020	988	21%	(Oh et al. 2007)
	Clostridium beijerinckii NCIMB 8052		iCB925	938	881	18%	(Milne et al. 2011)
	Staphylococcus aureus N315		iSB619	641	571	24%	(Becker & Palsson 2005)
Proteobacteria	*Escherichia coli K12*		iAF1260	2077	1039	29%	(Feist et al. 2007)
	Escherichia coli W (ATCC9637)		iCA1273	2477	1111	27%	(Archer et al. 2011)
	Helicobacter pylori 16695		iIT341	476	485	21%	(Thiele et al. 2005)
	Klebsiella pneumoniae MGH 78578		iYL1228	1970	1658	24%	(Liao et al. 2011)
	Pseudomonas putida KT2440		iNJ746	950	911	14%	(Nogales et al. 2008)
	Salmonella typhimurium LT2		STM_v1.0	2201	1119	28%	(Thiele et al. 2011)
	Shewanella oneidensis MR-1		iSO783	774	634	15%	(Pinchuk et al. 2010)
Actinobacteria	*Mycobacterium tuberculosis H37Rv*		iNJ661	939	828	15%	(Jamshidi & Palsson 2007)
Chloroflexi	*Dehalococcoides ethenogenes*		iAI549	518	549	27%	(Islam et al. 2010)
Cyanobacteria	*Synechocystis sp. PCC6803*		iJN678	863	795	21%	(Nogales et al. 2012)
Thermotogales	*Thermotoga maritima MSB8*		(None)	562	503	25%	(Zhang et al. 2009)
Euryarchaeota	*Methanosarcina barkeri str. Fusaro*		iAF692	476	485	14%	(Feist et al. 2006)

4.2.2 Parsing Genome-Scale Metabolic Models

All models were collected in SBML format from which they were then converted to bioopt, a format part of the BioMet Toolbox (Cvijovic et al. 2010). All the models were then parsed to model an environmental condition corresponding to rich media: all original exchange reactions in the model were set to a maximum uptake limit of -20 mmol gDW^{-1} h^{-1} to allow for the import of all transported metabolites (including oxygen, whenever it was possible).

4.2.3 Single Knockout of Metabolic Reactions

Flux Balance Analysis (FBA) was used to predict the essentiality of each metabolic reaction in all models (see Chapter 1 for details on the simulation methods). A threshold of 10% of the flux through the biomass reaction compared to the wild type was set as the limit to define an essential metabolic reaction. All modeling procedures were implemented in C++ and solved using IBM ILOG CPLEX solver. The Optflux platform (Rocha et al. 2010) was used occasionally to confirm and benchmark results.

4.2.4 Standardizing the Nomenclature of Essential Metabolic Reactions

The comparison of the reactions of the 15 GSMs used required resolving some nomenclature inconsistencies in the models. This included mostly the standardization of suffixes used in reaction IDs, including unnecessary or redundant indications of reversibility, species names allocated to reactions and other redundant tags. Irrelevant and irregular characters such as dashes were filtered out of all the nomenclature (see Supplementary Table 4.1).

4.2.5 Experimental Data and Subsystem Mapping

Large-scale experimental data on gene essentiality were collected from two databases, OGEE (Chen et al. 2012) and DEG (Luo et al. 2014). The content of the databases was compared and DEG was chosen for the analysis performed in this chapter as it is considerably larger, including wider and clearer annotation metadata for 36 prokaryotic datasets (**Table 4.2**). Genes were mapped to the subsystems present in the latest *Escherichia coli* genome-scale metabolic model (Orth et al. 2011). All essential reactions obtained after GSMs analysis were also mapped according to this updated list of subsystems.

Table 4.2 – Large-scale essentiality assays used in this study and respective original reference of publication. The corresponding annotated data was obtained from the DEG database (Luo et al. 2014).

Species name	Reference
Acinetobacter baylyi ADP1	(de Berardinis et al. 2008)
Bacillus subtilis 168	(Kobayashi et al. 2003)
Bacteroides fragilis 638R	(Veeranagouda et al. 2014)
Bacteroides thetaiotaomicron VPI-5482	(Goodman et al. 2009)
Burkholderia pseudomallei K96243	(Moule et al. 2014)
Burkholderia thailandensis E264	(Baugh et al. 2013)
Campylobacter jejuni subsp. jejuni NCTC 11168 = ATCC 700819	(Metris et al. 2011)
Caulobacter crescentus	(Christen et al. 2011)
Escherichia coli MG1655 I	(Gerdes et al. 2003)
Escherichia coli MG1655 II	(Baba et al. 2006)
Francisella novicida U112	(Gallagher et al. 2007)
Haemophilus influenzae Rd KW20	(Akerley et al. 2002)
Helicobacter pylori 26695	(Salama et al. 2004)
Methanococcus maripaludis S2	(Sarmiento et al. 2013)
Mycobacterium tuberculosis H37Rv	(Sassetti et al. 2003)
Mycobacterium tuberculosis H37Rv II	(Griffin et al. 2011)
Mycobacterium tuberculosis H37Rv III	(Zhang et al. 2012)
Mycoplasma genitalium G37	(Glass et al. 2006)
Mycoplasma pulmonis UAB CTIP	(French et al. 2008)
Porphyromonas gingivalis ATCC 33277	(Klein et al. 2012)
Pseudomonas aeruginosa PAO1	(Gallagher et al. 2011)
Pseudomonas aeruginosa UCBPP-PA14	(Liberati et al. 2006)
Salmonella enterica serovar Typhi	(Langridge et al. 2009)
Salmonella enterica serovar Typhi Ty2	(Barquist et al. 2013)
Salmonella enterica serovar Typhimurium SL1344	(Barquist et al. 2013)
Salmonella enterica subsp. enterica serovar Typhimurium str. 14028S	(Khatiwara et al. 2012)
Salmonella typhimurium LT2	(Knuth et al. 2004)
Shewanella oneidensis MR-1	(Deutschbauer et al. 2011)
Sphingomonas wittichii RW1	(Roggo et al. 2013)
Staphylococcus aureus N315	(Ji et al. 2001)
Staphylococcus aureus NCTC 8325	(Chaudhuri et al. 2009)
Streptococcus pneumoniae	(Thanassi et al. 2002)
Streptococcus pyogenes MGAS5448	(Le Breton et al. 2015)

Table 4.2 – Large-scale essentiality assays used in this study and respective original reference of publication (continued)

Streptococcus pyogenes NZ131	(Le Breton et al. 2015)
Streptococcus sanguinis	(Xu et al. 2011)
Vibrio cholerae N16961	(Cameron et al. 2008)

4.2.6 Analysis of Genetic Conservation

To analyze the conservation and infer ancestry of all the metabolic genes annotated in metabolic subsystems of GSMs, a local protein blast was performed against representative genomes of all the 35 prokaryotic phyla with at least one fully sequenced quality genome in the NCBI genome database (accession date: June 2015). For this task, translated genomes were selected and downloaded for all 53 unique species of prokaryotes for which there is a GSM (Supplementary Table 3.1); to these, 26 representative genomes for phyla not modeled with GSMs were added. This totaled in 79 translated genomes representing the fully sequenced phyla in the prokaryotic tree of life (see Supplementary Figure 4.1). The metabolic genes of *E. coli* K12 were used as queries. The threshold e-value considered was 1e-4 as used elsewhere in blasts against single genomes (Rahman et al. 2014, Seringhaus et al. 2006). All the procedures were implemented using the Biopython package (Cock et al. 2009).

4.2.7 Numerical and Statistical Analysis of Essentiality and Conservation

For assessing the conservation of essential reactions and essential genes in each metabolic subsystem, the weighted sum of essentiality was calculated, as the value of **W,** for each subsystem **m**, as:

$$W_m = \sum_{i=1}^{t} n_i . i$$

$\boldsymbol{n_i}$ being the number of reactions or genes essential in **i** models or datasets, where **t** is the total of models or datasets, 15 and 36 respectively.

The average experimental essentiality for each metabolic subsystem $\bar{E}_m$ was calculated as the average of the number of essential genes in that subsystem E_m for all experimental datasets:

$$\bar{E}_m = \frac{\sum_{i=1}^{t} E_m}{t}$$

Average non-essentiality was calculated in the same manner. Average conservation for metabolic subsystems was calculated as the average of the number of genomes where each gene in that subsystem was conserved.

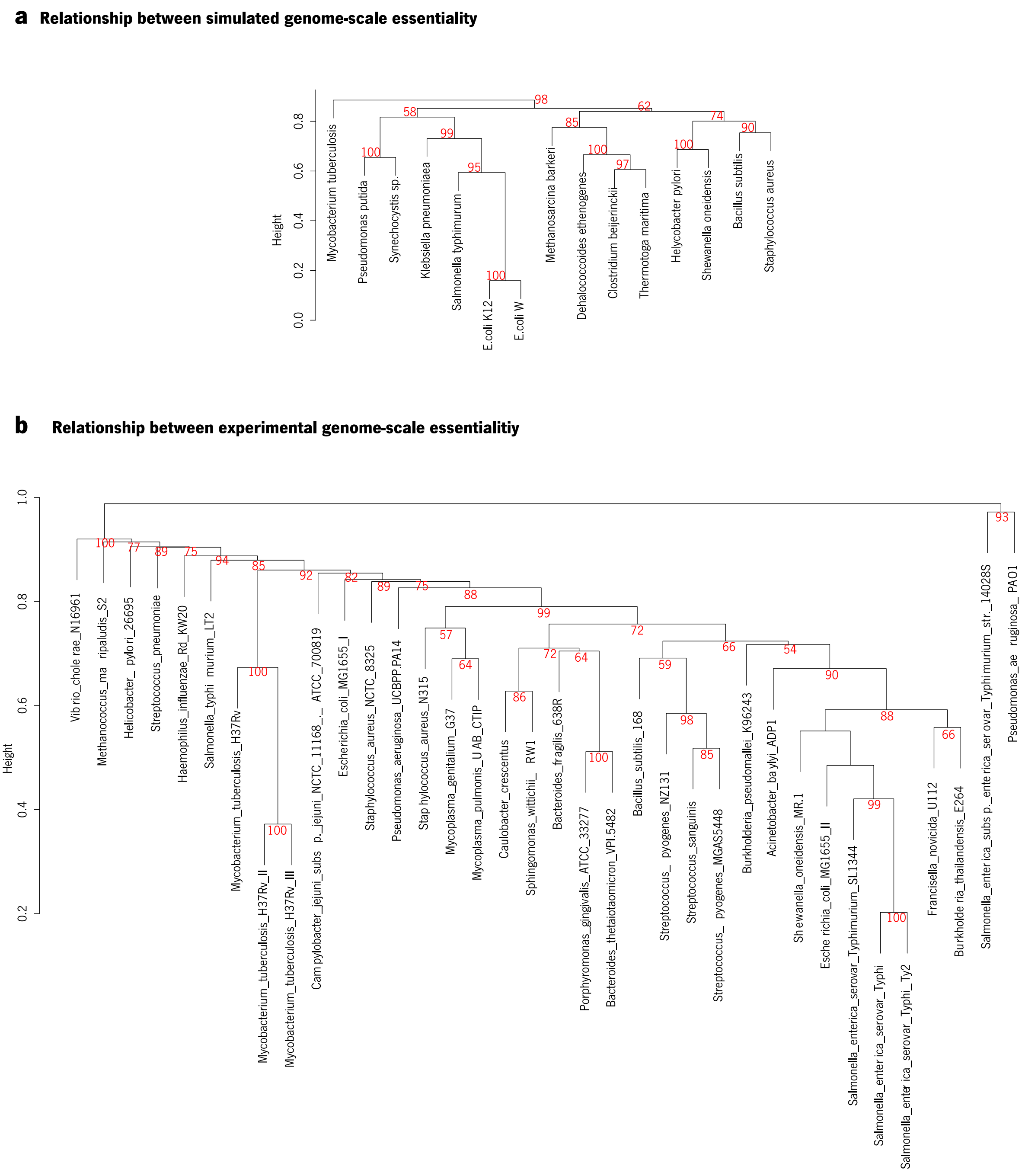
All statistical analyses and calculations of polynomial regressions were performed using R statistical software version 3.1. Hierarchical clustering was performed using the 'pvclust' R package (Suzuki & Shimodaira 2006) with binary distance as the dissimilarity metric and Ward 1 method as the linkage criterion. Pvclust was also used for assessing uncertainty by calculating approximately unbiased p-values via multiscale bootstrap resampling.

4.3 Results

4.3.1 Patterns of Essentiality Are Validated by Phylogenies

To analyze the validity of the essentiality results on a large scale the different models were clustered based on single-reaction essentiality predictions and the different datasets available on DEG (Luo et al. 2014) were clustered based on the content of essential genes. **Figure 4.1** shows both clusters. Given that both the simulations and the majority of the experiments were performed in rich media conditions (see Methods) common essentiality patterns are expected to reflect similarities among the networks. In the case of the simulated essentiality, strongly supported clusters (more than 75% of 1000 bootstrap replicas) are phylogenetically consistent at the level of the phylum, with the exception of the models of *C. beijerinckii* and *P. putida*. *H. pylori* and *S. oneidensis* show up in the same cluster, but not together with the rest of the Proteobacteria (although these higher-level clusters are not statistically supported). The lower number of available exchange reactions in *H. pylori* and *S. oneidensis* models and in *P. putida* (74, 95 and 89 respectively) compared with other Proteobacteria models (*K. pneumoniae, E. coli* K12, *S. typhimurium* and *E. coli W* with 289, 299, 305 and 310 respectively) might justify these results, as less exchanges cause more reactions in the network to be essential. *C. beijerinckii's* model is also very restricted with regards to exchange reactions, with only 19.

Regarding the experimental data, fewer clusters are statistically supported, although there is a pattern of clustering of some taxonomically related species. One well-supported phylogenetic cluster is that of several gamma and beta-proteobacteria including *Acinetobacter baylyi*, dataset II of *E. coli K12*, three Salmonellas, one *Shewanella* and one *Francisella*. Others are the cluster of Tenericutes (both Mycoplasmas), the one of Bacteroidetes, the cluster with all three datasets of *M. tuberculosis* and the cluster of the alpha-proteobacteria, *Sphingomonas* and *Caulobacter*. One interesting outlier is the highly supported cluster including *Pseudomonas aeruginosa PAO1* and *Salmonella enterica subsp. Enterica serovar Typhimurium str. 14028S*, datasets of essential genes that are significantly smaller than the others, as they are not saturated genome-wide gene-essentiality screens (as identified and discussed in Chapter 3 of this thesis). Surprisingly, Firmicutes are spread all across the tree and both *E.coli* sets are very distant from each other. Both the original studies were checked, and although they were performed under rich media conditions, one yielded 609 essential genes (Gerdes et al. 2003) and the other yielded only 296 (Baba et al. 2006). This difference is due to the use of different technologies to perform the large-scale assays, the first being random mutagenesis and the screening of mixed populations, and the second the screening of libraries of targeted mutants, as reviewed in (Gerdes et al. 2006).



a Relationship between simulated genome-scale essentiality

b Relationship between experimental genome-scale essentialitiy

Figure 4.1 – Relationships between simulated (a) and experimental (b) genome-scale essentialities of prokaryotes. Clusters show approximately unbiased p-values in red (percentage) calculated by multiscale bootstrap re-sampling with 1000 replicas (see Methods for details).

4.3.2 Cofactor Metabolism, Cell Wall and Lipids: Most Essential Subsystems in Metabolic Networks

For an initial analysis of the simulations of single-reaction knockouts, all the essential reactions calculated for the 15 GSMs were mapped to the corresponding metabolic subsystem (see Methods; Supplementary Table 4.1). The total number of essential reactions varies significantly between subsystems, as the total number of reactions in those subsystems in the models, as shown in **Figure 4.2**. Both totals are independent in the majority of the subsystems (p-value smaller than 0.05 in a Fisher exact test). The subsystems of cofactor and prosthetic group biosynthesis, cell envelope biosynthesis, membrane lipid metabolism and glycerophospholipid metabolism are isolated with more than double the amount of essential reactions than the following most essential subsystem, Transport.

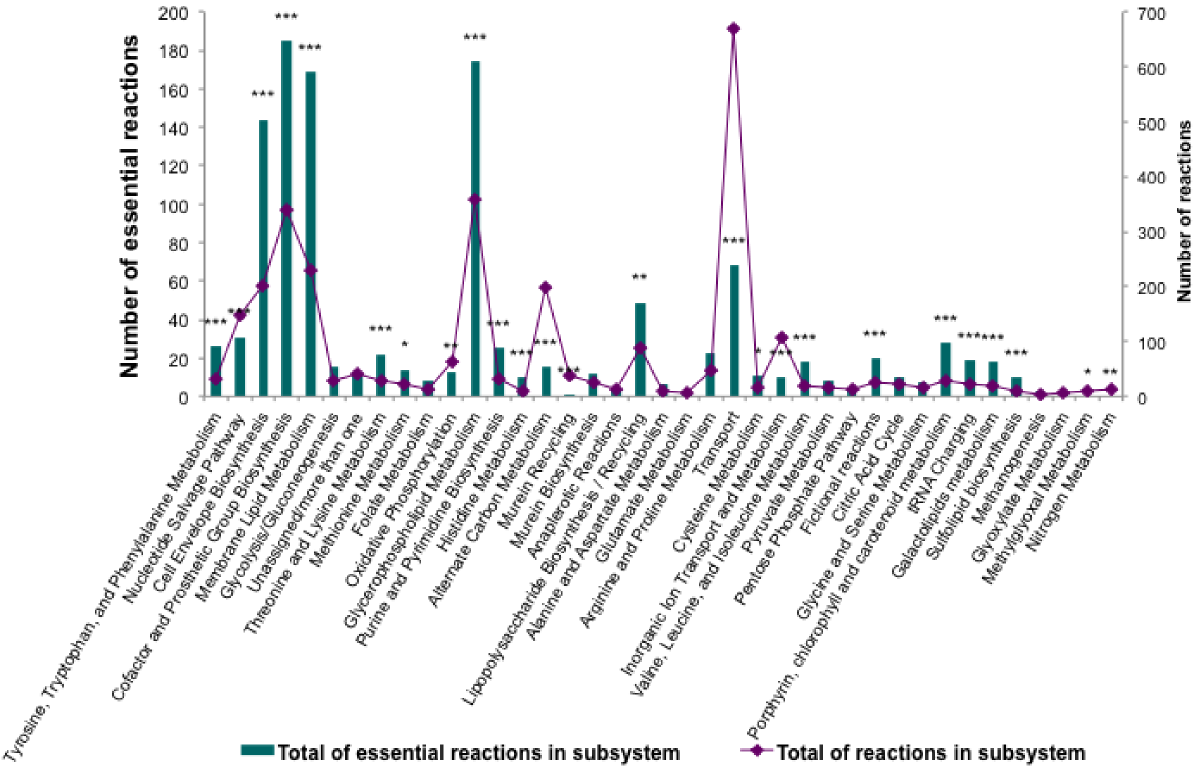


Figure 4.2 – Total number of essential reactions for biomass production calculated for fifteen genome-scale metabolic models compared with the total number of reactions in those models for each metabolic subsystems. Single, double and triple asterisks indicate p-values smaller than 0.05, 0.01 and 0.0001, respectively, after a Fisher's exact test for count data.

Different models show different proportions of essential reactions for each metabolic subsystem. For the majority of the models, the most essential subsystem is that of cofactor and prosthetic group biosynthesis. Forty seven point eight percent of the essential reactions in the simulations with the GSM of *E. coli* K12 were related with this subsystem (**Figure 4.3**). Although in rich media, this model does not contain all the transport reactions for cofactors that can be uptaken (e.g. riboflavin) making the corresponding non-essential biosynthetic steps to be predicted as essential (riboflavin synthase). However, several of these essential reactions were confirmed to be essential steps in the biosynthesis of the active forms of cofactors that cannot be uptaken (e.g. dihydrofolate synthase and dihydrofolate reductase for the biosynthesis of tetrahydrofolate and derivatives and NAD kinase for obtaining NADP).

For *M. tuberculosis, D. ethenogenes, S. typhimurium and K. pneumoniae* the most represented subsystems were those related with lipid metabolism (30.5 and 25.1% essential reactions, respectively). Discrepancies regarding results for each individual model are not only related with the metabolic network but are also dependent on the formulations of the biomass equation and environmental conditions. In the next section the focus lies on common or conserved essential reactions among models, which diminishes biases caused by individual models, nevertheless these are explored in greater detail. This discussion was also deepened in Chapter 3 of this thesis and will be further expanded in section 4.4 of this Chapter.

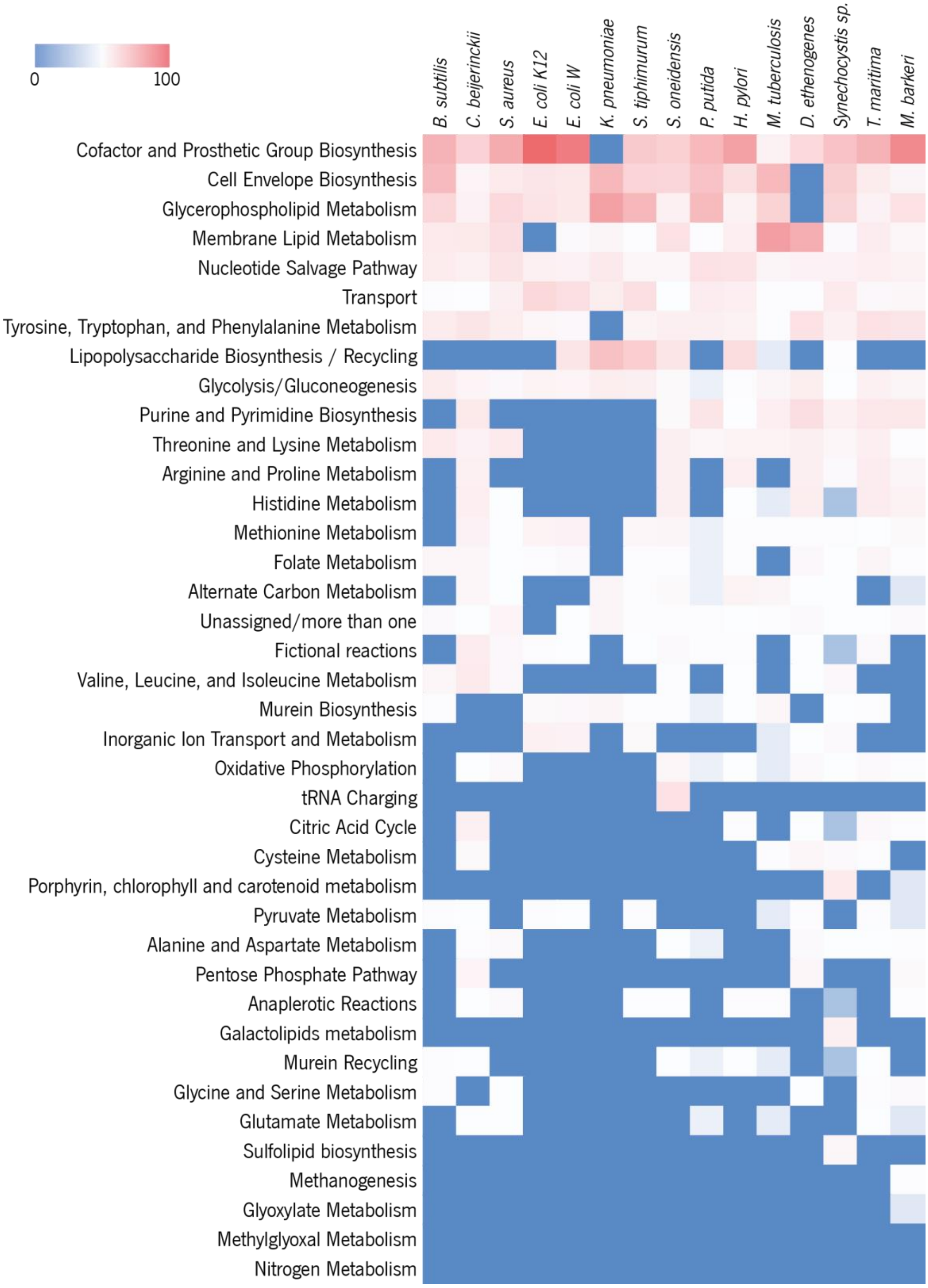


Figure 4.3 – Percentage of essential reactions for biomass production of each of 15 genome-scale metabolic models corresponding to each metabolic subsystem. The colour bar represents the normalized percentage of essential genes for each subsystem compared to the total number of essential genes for that model.

To further explore which of these essential reactions in each subsystem were essential for more than one model, the conservation of essentiality across models was analyzed for each metabolic subsystem (**Figure 4.4**). Strikingly, not one essential reaction was essential for all the models analyzed. However, three reactions related with aromatic amino acids metabolism (tyrosine, tryptophan and phenylalanine) were essential in 14 out of the 15 models simulated. Eight out of the 15 models can directly uptake all three aromatic amino acids and other two can uptake two of them, with just five models completely relying on their *de novo* anabolic pathways to obtain them. The model where these reactions are not essential is *K. pneumoniae,* which can directly uptake all three amino acids. The notable difference between this model and the others is that it lacks cofactors and prosthetic groups in its biomass equation. These three reactions correspond to the three last steps in the synthesis of chorismate, which is part of the shikimate pathway, which connects central metabolism with aromatic amino acid metabolism. However, this pathway is also the route taken to synthesize several other compounds in the cell, including quinones and folates (Coggins et al. 2003). The latter are present in the biomass equations of 13 of the models used, except *K. pneumoniae* and *M. tuberculosis*. The highly essential reactions annotated to belong to the cofactor and prosthetic group biosynthesis subsystem are also related with biosynthesis of folates and the phosphorylation of NAD to produce NADP. Two reactions involved in the salvage pathways of nucleotides were also essential for 14 of the models – the biosynthesis of gdp and dttp. Three reactions essential in 13 models are related with the biosynthesis of cell wall components and just not essential in *B. subtilis* and *D. ethenogenes*. Acetyl-CoA carboxylase, related with membrane lipid metabolism, is essential in 12 of the 15 models. One reaction not assigned to any subsystem, the HCO_3 equilibration reaction, was essential in 11 of all 15 models.

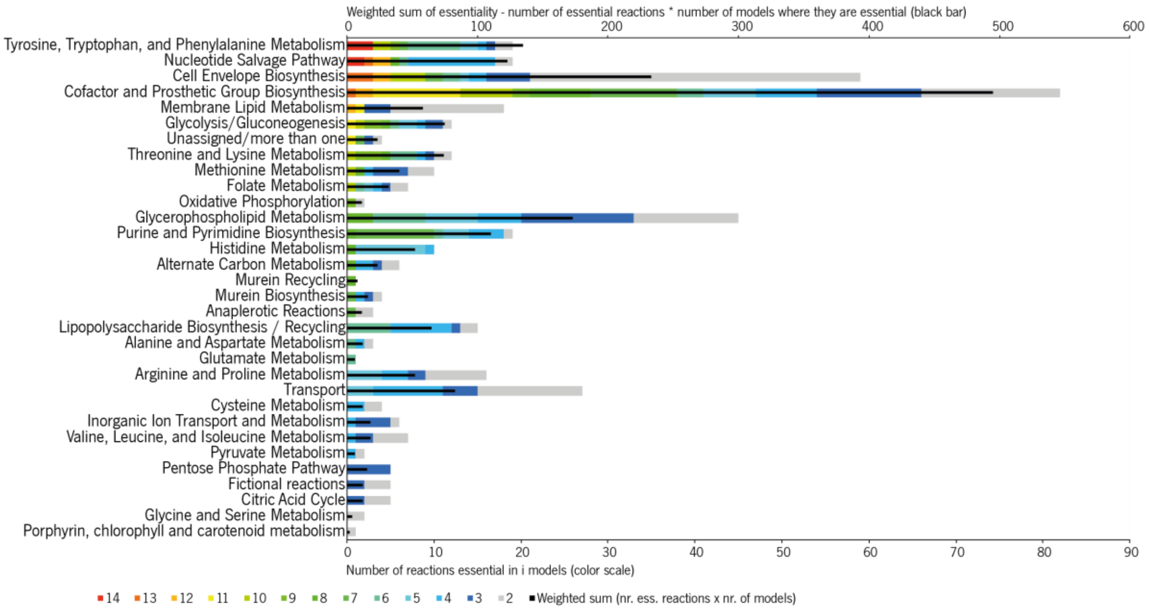


Figure 4.4 – Conservation of essentiality of metabolic subsystems in 15 genome-scale metabolic models. Red indicates highest conservation (reactions that are essential for biomass production in 14 GSMs) and grey the least (essential in only two GSMs). Black bar: weighted sum of essential reactions given the number of models in which they are essential.

4.3.3 Experimental Data Corroborates and Elaborates on the Patterns of Essentiality Given by GSMs

To validate the predictions of essentiality of metabolic modules given by the results obtained with GSMs, each gene in DEG was annotated according to its function. COG annotations were obtained from DEG and functional categories are shown in **Figure 4.5**. Strikingly, a quarter of the prokaryotic essential genes in the database are either of unknown function or were attributed a general function prediction. 44% of the genes correspond to metabolic functions. COG metabolic functional categories are much less detailed than those used in the annotation of metabolic models in GSMs. Both the “Energy production and Conversion” and “Amino acid transport and metabolism” functional categories encompass several of those that are detailed with GSMs. The transport category is one isolated in GSMs, but distributed by each category of major biomolecules (amino acids, coenzymes, carbohydrates) in the COG system. Some misleading COG annotations were also found. One case is thiO, a gene that is essential for the biosynthesis of thiamine diphosphate (Settembre et al. 2003) (an important organic cofactor) which is annotated in category E (Amino acid transport and metabolism). Another case is that of csd, a cysteine desulfurase, essential in 7 datasets that is involved in the formation of Fe-S clusters (Loiseau et al. 2005), cofactors crucial in several redox reactions, also annotated in category E. For these reasons, the DEG database was annotated to the subsystem categories used in GSMs (**Figure 4.6**). This new annotation comprised more annotations (1363 metabolic genes annotated in total compared with a total of 906 unique metabolic COGs). This is also a highly curated dataset that could be directly compared to the modeling results and included some genes annotated in the "General function prediction only" COG category.

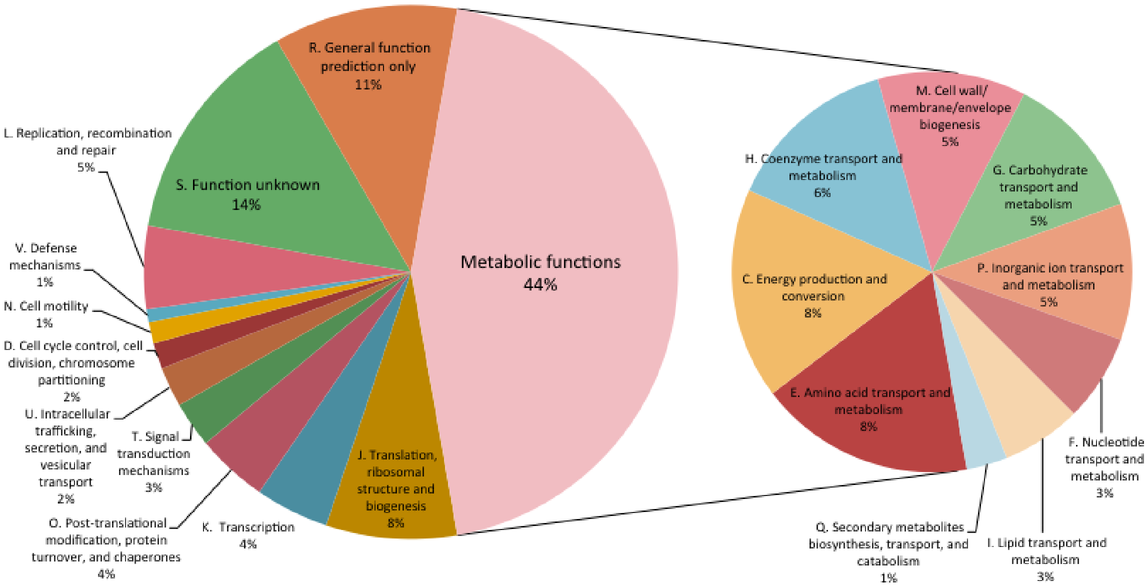


Figure 4.5 – COG functional categories and their prevalence for prokaryotic essential genes in DEG.

In the new annotation of experimentally essential metabolic genes with the subsystems used in GSMs, genes related with cofactor metabolism comprise the majority of the annotated functions (**Figure 4.6**). The category of tRNA charging appears much more evidently as the second highest representative in experimental data, in contrast with the low result in the simulations of GSMs. This occurs due to this category being modeled in only one GSM (*S. oneidensis,* **Figure 4.3**). Cell envelope biosynthesis genes follow as the third most essential functional module, in accordance with the modeling results. To overview the relationship between modeling and experimental results, both weighted sums for each set of results (**Figure 4.4** and **Figure 4.6**) were correlated. **Figure 4.7** shows the high correlation obtained between the weighted essentiality for each subsystem when excluding the tRNA charging subsystem. It is expected that if this subsystem is included in the 14 remaining models it will be highly essential, as the biomass function would include all 20 tRNAs charged with the corresponding amino acids.

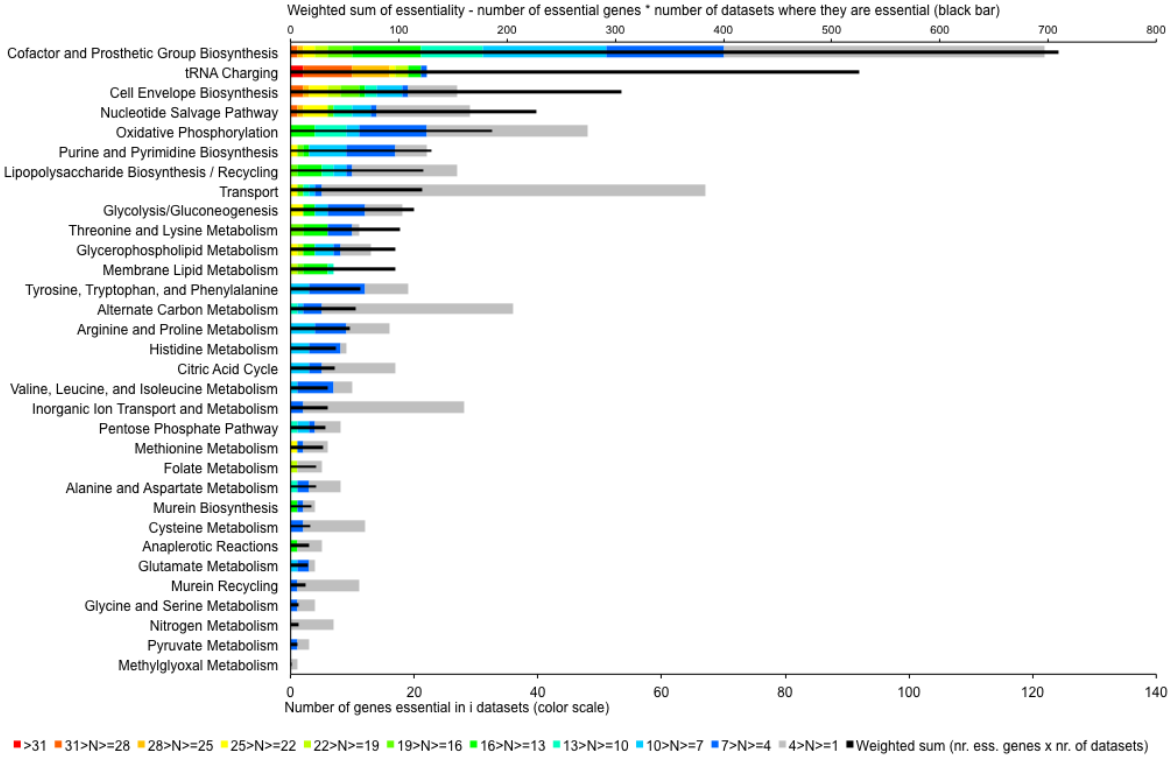


Figure 4.6 – Conservation of essentiality of metabolic subsystems in 36 large-scale gene essentiality datasets. Red indicates highest conservation (genes essential for growth in more than 31 experiments) and grey the least (essential in less than 4 experiments). Black bar: weighted sum of essential genes given the number of datasets in which they are essential.

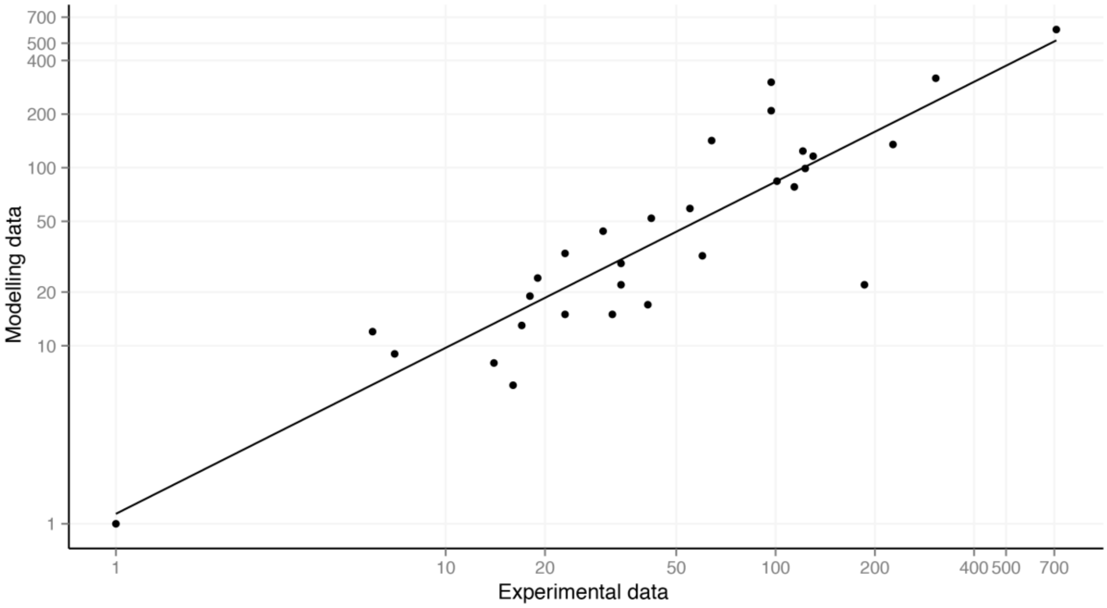


Figure 4.7 – Correlation between modelling and experimental genome-scale essentiality data at metabolic subsystem level (r^2 is 0.804, Pearson correlation coefficient of 0.896 with p-value 6.28e-14). Both axis are represented in log scale and correspond to the weighted sum of essentiality for each type of data.

4.3.4 tRNA Charging, Transport, Oxidative Phosphorylation and Cofactor Metabolism: The Core Conserved Metabolism

Based on the premises of evolutionary parsimony and orthology (Koonin 2003), this work proceeded to the analysis at a large scale of the conservation of metabolic genes in the prokaryotic tree of life to infer potential ancestral metabolic functions. 79 genomes were assayed representing all the known prokaryotic phyla with a fully sequenced genome (see Methods for details). A phylogenetic tree with these 79 species is available in Supplementary Figure 4.1. All of the annotated metabolic genes of *E. coli* K12 were used as queries to search the set of genomes for conserved metabolic genes and respective functions. The results on conservation of metabolic genes are summarized in **Figure 4.8**.

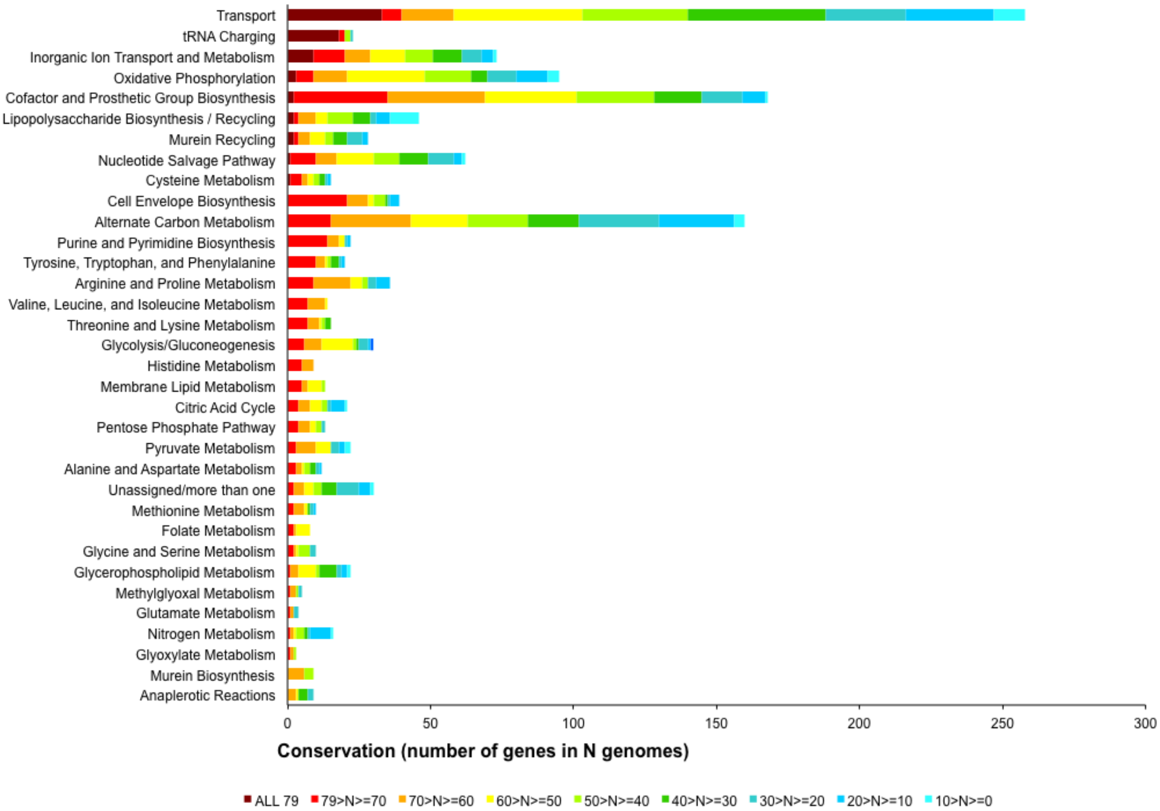


Figure 4.8 – Conservation of metabolic subsystems in genomes of all prokaryotic phyla with at least one fully sequenced genome. Dark red indicates highest conservation (genes that are conserved in all 79 genomes accessed) and light blue the least (present in less than 10 genomes).

The metabolic subsystem with more prevalent genes in more genomes is Transport, followed by the universal tRNA charging genes (aminoacyl-tRNA synthetases). It should be noted though that all the 33 transport genes conserved in all 79 genomes correspond to ABC transporters (**Table 4.3**). The ATP-binding domain in these genes is ubiquitous across all domains of life, and therefore it is not clear if all the hits correspond to the same transported metabolites annotated for *E. coli*. Three genes involved in oxidative phosphorylation were also conserved in all genomes analysed: atpA, atpD (ATP synthase subunit alpha and beta, respectively) and trxA (thioredoxin). In the subsystem of cofactors and prosthetic group biosynthesis, glutX and sufC were also conserved in all genomes analysed. It should be noted though that glutX corresponds actually to a tRNA charging protein, a glutamyl-tRNA synthetase involved in the biosynthesis of heme, that should have a double annotation; sufC is an atypical cytoplasmic ABC/ATPase required for the assembly of iron-sulphur clusters (Nachin 2003).

Table 4.3 – Ubiquitous transporter genes in prokaryotic genomes. Essentiality is given as the number of datasets in DEG in which each gene is essential. The description is that of the corresponding annotated ORF in the genome of *E. coli* K12.

Gene name	Description (*E. coli* K12)	Essentiality
alsA	D-allose ABC transporter ATPase	0
araG	L-arabinose ABC transporter ATPase	1
artP	arginine ABC transporter ATPase	1
btuD	vitamin B12 ABC transporter ATPase	0
ccmA	heme export ABC transporter ATPase	2
cydC	glutathione/cysteine ABC transporter export permease/ATPase	7
cydD	glutathione/cysteine ABC transporter export permease/ATPase	3
ddpD	D,D-dipeptide ABC transporter ATPase	0
ddpF	D,D-dipeptide ABC transporter ATPase	0
dppD	dipeptide/heme ABC transporter ATPase	3
dppF	dipeptide/heme ABC transporter ATPas	1
fhuC	iron(3+)-hydroxamate import ABC transporter ATPase	0
glnQ	glutamine transporter subunit	0
gltL	glutamate/aspartate ABC transporter ATPase	1
gsiA	glutathione ABC transporter ATPase	0
hisP	histidine ABC transporter ATPase	0
livF	branched-chain amino acid ABC transporter ATPase	0
livG	branched-chain amino acid ABC transporter ATPase	0
malK	maltose ABC transportor ATPase	0
metN	DL-methionine transporter subunit	0
mglA	methyl-galactoside ABC transporter ATPase	1
potA	spermidine/putrescine ABC transporter ATPase	3
potG	putrescine ABC transporter ATPase	0
proV	glycine betaine/proline ABC transporter periplasmic binding protein	0
rbsA	D-ribose ABC transporter ATPase	0
ssuB	aliphatic sulfonate ABC transporter ATPase	0
tauB	taurine ABC transporter ATPase	0
thiQ	thiamine/thiamine pyrophosphate ABC transporter ATPase	1
ugpC	sn-glycerol-3-phosphate ABC transporter ATPase	2
xylG	D-xylose ABC transporter dual domain ATPase	0
ydcT	putative ABC transporter ATPase	1
yehX	putative ABC transporter ATPase	0
ytfR	putative sugar ABC transporter ATPase	0

Although the vast majority of genes found conserved in all the genomes analysed correspond to ABC ubiquitous domains, the high conservation (between 70 and 79 genomes) of other genes is still prominent. In the case of cofactor and prosthetic group biosynthesis genes, there are 33 highly conserved genes that are crucial in the biosynthesis of core cofactors in prokaryotic species described in Chapter 3 (**Table 4.4**).

Table 4.4 – Highly conserved cofactor biosynthesis genes in prokaryotic genomes. Essentiality is given as the number of datasets in DEG in which each gene is essential. The description is that of the corresponding annotated ORF in the genome of *E. coli* K12. Biosynthesized cofactors were manually retrieved from the detailed information available in the Metacyc database (Caspi et al. 2014).

Gene Name	Conservation	Description	Cofactor	Essentiality (DEG)
Gor	78	glutathione oxidoreductase	glutathione	2
sufS	78	cysteine desulfurase, stimulated by SufE; selenocysteine lyase, PLP-dependent	FeS clusters	2
iscS	77	cysteine desulfurase (tRNA sulfurtransferase), PLP-dependent	FeS clusters	10
entA	77	2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase	siderophores	0
ispB	76	octaprenyl diphosphate synthase	quinones	12
ispA	76	geranyltranstransferase	quinones	9
ispU	76	undecaprenyl pyrophosphate synthase	quinones	2
Dxs	75	1-deoxyxylulose-5-phosphate synthase, thiamine triphosphate-binding, FAD-requiring	thiamine; isoprenoids;	16
glyA	75	serine hydroxymethyltransferase	Folates	14
hemL	75	glutamate-1-semialdehyde aminotransferase (aminomutase)	porphyrins	14
ubiE	75	bifunctional 2-octaprenyl-6-methoxy-1,4-benzoquinone methylase/ S-adenosylmethionine:2-DMK methyltransferase	quinones	13
ribD	75	fused diaminohydroxyphosphoribosylaminopyrimidine deaminase and 5-amino-6-(5-phosphoribosylamino) uracil reductase	riboflavin	8
bioA	75	7,8-diaminopelargonic acid synthase, PLP-dependent	Biotin	3
nadK	75	NAD kinase	nad/nadp	0
pdxB	74	erythronate-4-phosphate dehydrogenase	pyridoxal-5-p	2
pabA	74	aminodeoxychorismate synthase, subunit II	Folates	0

ribE	73	riboflavin synthase beta chain	riboflavin	7

Table 4.4 – Highly conserved cofactor biosynthesis genes in prokaryotic genomes (continued)

ribB	73	3,4-dihydroxy-2-butanone-4-phosphate synthase	riboflavin	5
bioC	73	malonyl-ACP O-methyltransferase, SAM-dependent	biotin	4
epd	73	D-erythrose 4-phosphate dehydrogenase	pyridoxal-5-p	1
ribF	72	bifunctional riboflavin kinase/FAD synthetase	flavins	15
entC	72	isochorismate synthase 1	terpenoids	1
menF	72	isochorismate synthase 2	menaquinones	1
ligA	71	DNA ligase, NAD(+)-dependent	?	23
coaE	71	dephospho-CoA kinase	CoA	20
folC	71	bifunctional folylpolyglutamate synthase/ dihydrofolate synthase	Folates	9
entE	71	2,3-dihydroxybenzoate-AMP ligase component of enterobactin synthase multienzyme complex	siderophores	0
coaD	70	pantetheine-phosphate adenylyltransferase	CoA	22
folP	70	7,8-dihydropteroate synthase	folates	7
menE	70	O-succinylbenzoate-CoA ligase	menaquinones	6
spoT	70	bifunctional (p)ppGpp synthetase II/ guanosine-3',5'-bis pyrophosphate 3'-pyrophosphohydrolase	GTP	5
ribC	70	riboflavin synthase, alpha subunit	riboflavin	3
entF	70	enterobactin synthase multienzyme complex component, ATP-dependent	siderophores	0

4.3.5 Common Essential Genes Are Rarer and Prone to Be Highly Conserved, Contrarily to Common Non-Essential Genes

On a first overlook there is no direct correlation between essentiality and conservation at the individual gene level, as indicated by the number of DEG datasets where the highly conserved cofactor biosynthesis genes are essential (**Table 4.4**). The same is even more evident in the case of the highly conserved ABC domains in transporters (**Table 4.3**), with the majority (20/33) not being essential in any dataset in DEG. This substantiates the fact that highly conserved genes are not necessarily highly essential. For Membrane Lipid Metabolism, though, the correlation is positive and significant (pearson coefficient 0.95, p-value 1.07e-06). There is also a good fit by a 3rd degree polynomial function to the relationship between average essentiality of each subsystem with its average conservation (**Figure 4.9**a, adjusted R-squared of 0.7359 and p-value 1.041e-09) in contrast to the absence of any significant fit for the relationship between average non-essentiality for each subsystem and conservation (**Figure 4.9**b). It is evident though that this correlation is completely dependent on the tRNA charging subsystem, that is isolated as the most conserved and most essential subsystem.

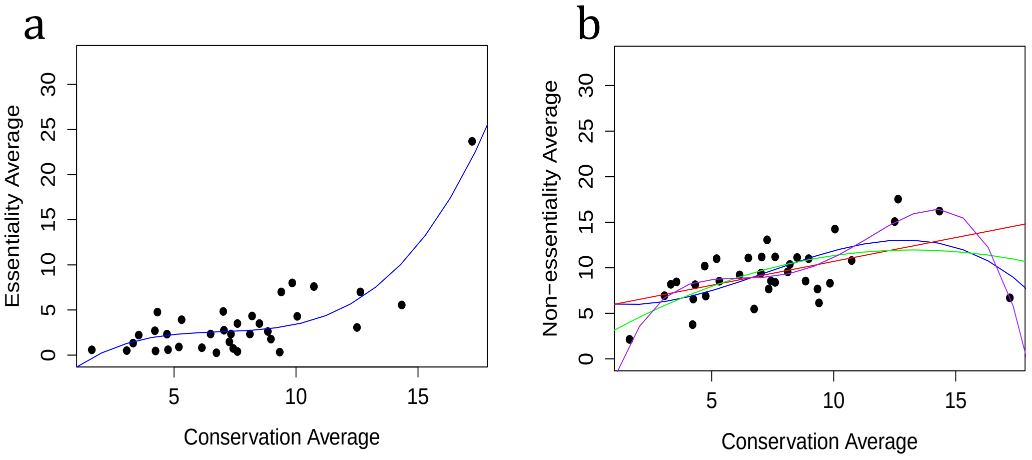


Figure 4.9 – Average essentiality vs. average conservation (a) and average non-essentiality vs. average conservation (b) for metabolic subsystems of prokaryotes with corresponding fitting models (see section 4.2.7 for details). In red, 1st degree polynomial regression model; green – second degree; blue – 3rd degree; purple – 4th degree.

To access the unbiased relationship between essentiality and conservation at the individual gene level, the data for non-essential genes in DEG was integrated in the analysis. For this purpose, the number of times a gene was found non-essential in an experimental assay was added as a negative value to the number of times that gene was found essential, totaling in the sum of essentiality shown in **Figure 4.10**. The vast majority of metabolic genes lie on the left area of the plot. However, on the right side of the plot, where the genes with a positive sum of essentiality lie, the clear majority of genes are highly conserved. There are some interesting outliers as glyS, essential in 21 datasets in DEG but conserved in only 48 of the 79 genomes assayed. This corresponds to one instance in which the monophyly rule is violated: *E. coli's* type is common for most bacteria, but another type is common to some other bacteria, archaea and eukarya (Mazauric et al. 1996, Woese et al. 2000).

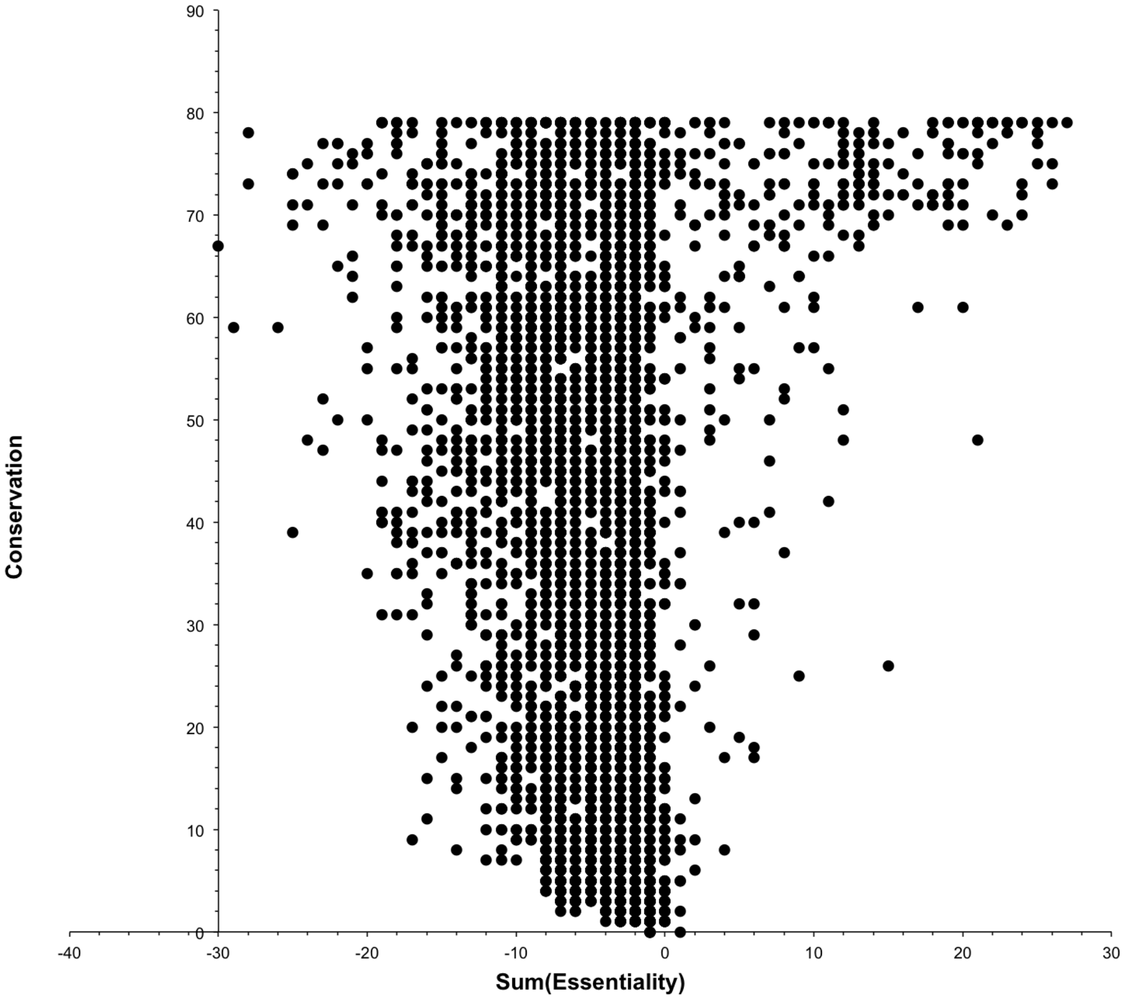


Figure 4.10 – Conservation (number of genomes where a gene is present) vs. sum of essentiality (number of times a gene is essential minus the time it is non-essential in datasets in DEG) for all metabolic genes annotated in this study.

4.4 Discussion

The integration done here was the first of the kind for a wide variety of phyla of the bacteria and archaea domains, encompassing experimental phenotypic data, results of large-scale computational simulations and sequence data. The experimental genome-scale essentiality data reveals that approximately 25% of prokaryotic essential genes encode for unknown or general functions (categories S and R in **Figure 4.5**), which is a strong warning on the need for experimental studies on the phenotype of these essential proteins for prokaryotic physiology. While those are not available, computational models can be valuable tools aiding in the task of decoding prokaryotic metabolism.

Although GSMs are limited by the quality of the genome annotations, the biomass equation and environmental formulations, the integration performed here tried to reduce the impact of these limitations. First of all, the choice of the models was based on a large survey of high-quality manually curated models (Supplementary Table 3.1) for which 15 balanced, validated, comparable models were chosen, that at the same time included wide phylogenetic diversity (**Table 4.1**). Secondly, the analysis filtered out the unique essential reactions that might represent specific errors related with individual models, to find core and common features to most of them. Also, as recognized by other authors, the predictive power of comparative analysis can be significantly enhanced by using it within the functional context of pathways and subsystems (Gerdes et al. 2006). The prediction with GSMs of which metabolic subsystems have genes that are more commonly essential in multiple species was accurate (**Figure 4.7**). The exception of the experimentally demonstrated highly essential tRNA-charging functionality that was not reflected in the simulations is due to the hindrance of just one model including this subsystem (Pinchuk et al. 2010).

The problem of the unstandardized biomass composition, evidenced by the GSM of *K. pneumoniae* not predicting any essential reaction involved in cofactor and prosthetic group biosynthesis (**Figure 4.3**) was explored in Chapter 3 of this thesis. Due to the incompleteness of the networks, it was not possible to complete the equations with the missing cofactors without an impractical manual editing and curation of most models. However, considering the results obtained here, this incompleteness did not impair the prediction of an overwhelming majority of essential reactions related with this subsystem. Interestingly, reactions annotated in the metabolic subsystem of tryptophan, tyrosine and phenylalanine biosynthesis were detected as commonly essential in metabolic networks but not in the experimental data. It was found that these reactions are involved in the shikimate pathway and have a broader functionality that is essential not only for the *de novo* synthesis of aromatic amino acids but also the biosynthesis of folates, which are essential in one carbon metabolism (Chapter 3). The active folates are core prokaryotic cofactors present in most biomass equations (13 out of 15) and can't be directly uptaken (see section 4.3.2; Chapter 3 of this thesis). It could be expected that essential vitamins could be uptaken directly in rich media by most prokaryotes, but that is not the case, as it was confirmed by the results of experimental essentiality (**Figure 4.6**). A closer look at the individual genes shows that other essential cofactors cannot be uptaken in their active forms and therefore some enzymes essential for their biosynthesis are essential for cell viability even in rich media (eg. nadE for NAD; coaD and coaE for coenzyme A; hemC for heme; dxr for isoprenoids).

The results of the comparison of modeling with essentiality results can help raising specific hypothesis and directions for more detailed investigation. One example is that of chorismate synthase. In the rich media essentiality datasets studied, although this enzyme has been shown to be essential in some cases, it is non-essential in the majority. However, in minimal media the knock-out of this gene in *E. coli* impairs growth (Joyce et al. 2006). It has been shown that when provided with p-aminobenzoic acid (PABA), para-hydroxybenzoic acid (PHBA) or a combination of a precursor from PABA with a non-biological catalyst, the growth of *E. coli* aroC mutant in M9 minimal medium could be rescued (Lee et al. 2013). Transporters for these compounds or others that might compensate for the lethal phenotype in rich media remain to be integrated in the genome-scale metabolic models and further explored.

Still regarding the results of simulations with GSMs, the subsystems of membrane lipid metabolism and cofactor and prosthetic group biosynthesis display a similar total of essential reactions (169 and 184, respectively), but a very different weighted sum of essentiality (**Figure 4.4**, W of 209 and 597; see section 4.2.7 for details). This indicates that although both subsystems are crucial for cell viability, the latter uses the same metabolic tools since early evolution, while the former diversified early in a wider variety of entities and functionalities. This was confirmed by the results of the analysis of conservation of the sequences of metabolic genes in prokaryotic genomes (**Figure 4.8**). The experimental data reflects these results (**Figure 4.6**), except for the total of essential genes in lipid metabolism, which is highly likely to occur because of the use of *E.coli* to annotate the metabolic genes, which excludes the lipid genes of Gram positive bacteria.

The analysis of conservation of metabolic genes here was the first using a manually curated annotation system for metabolic pathways and subsystems, with the latest and largest genome-scale metabolic model of a prokaryote to date (Orth et al. 2011). Regarding inferences on ancestry, it is important to note that genes encoding for functions that were lost throughout the evolution of prokaryotes might have been present in the last common ancestor of prokaryotes and will not be identified with this analysis. However, the genes identified here as present in all genomes of all representative phyla are most likely genes present in the last common ancestor (Koonin 2003).

Overall, the results of high conservation of the tRNA charging system, Transport and Oxidative Phosphorylation point to a last common ancestor metabolic network of the prokaryotes where most of the nutrients were uptaken with nonspecific transporters at the expense of ATP and in which tRNA charging were already present. This hypothesis might help bridge the gap in the debate of metabolism or replication first (Pross 2004), by establishing a connection of simpler information processing systems in the hypothetical RNA world with the metabolic reactions that provided the required energy. The results also suggest that the catalytic power of cofactors and prosthetic groups was a coin highly sought for in early prebiotic systems. It is highly likely that genes encoding for enzymes aiding in cofactor biosynthesis were selected for early in primordial evolution, as was suggested elsewhere for the origin of anabolic pathways in prebiotic systems (Fani & Fondi 2009).

This work also expanded considerably on previous related studies regarding the relationship between gene conservation and essentiality in width and depth. Jordan and coauthors used only *E. coli* essentiality data that was mapped to *H. pylori* and *N. meningitidis* to show that essential genes are more evolutionary conserved that non-essential (Jordan et al. 2002). Luo and coauthors used 23 experimentally essential assays to corroborate that finding (Luo et al. 2015). Both studies used the ratio of non-synonymous substitutions to synonymous substitutions in the genomes to estimate conservation (Ka/Ks). Here, 36 experimentally essential datasets were used, that included one Archaea (**Table 4.2**). The conservation was analyzed just by looking at the presence of each gene in 79 genomes that were manually selected to represent all the phyla with one fully-sequenced genome in the prokaryotic tree of life. Because each gene is essential in some datasets in DEG, non-essential in others and not assayed in yet others, instead of analyzing essential genes separately from non-essential as in the two aforementioned studies, an alternative method was used: a measure of essentiality for each gene (sum of essentiality) that takes into account the datasets where it shows up as essential and those where it is non-essential (**Figure 4.10**). The results show that genes with a positive sum of essentiality (more datasets showing essential that non-essential) are much scarcer than those with a negative sum, however it is much more likely that they are highly conserved. For genes with a negative sum of essentiality, there is no tendency for high or low conservation, with a uniform distribution of these genes for all the values of conservation. Here, the results expanded also both previously mentioned studies by integrating functional assessment of the data. The function of highly conserved metabolic genes was explored, with the conclusion that with the exception of tRNA charging subsystem, the majority of highly conserved genes related with transport and cofactor biosynthesis are not highly essential (**Figure 4.10**, **Table 4.3** and **Table 4.4**). This confirms a well-known remarkable redundancy in metabolic networks (Freilich et al. 2010) that is reflected in the resilience and robustness of life forms, which was responsible for life's endurance for the billions of years that it has existed on Earth. Most of this redundancy is not only based on known alternative metabolic routes but on promiscuous, general enzymatic activities, a large amount of which is still poorly understood (evident in the percentage of genes with general function prediction only in **Figure 4.5**) and that might be even more prevalent than previously thought, although in many cases unpredictable (Patrick et al. 2007).

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CHAPTER 5

Generating Minimal Metabolic Networks with a Curated Universe of Prokaryotic Reactions

[...] *from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.*

—CHARLES DARWIN, *On the Origin of Species* (1859)

In this chapter the outputs from previous chapters are integrated in a strategy to generate minimal metabolic networks for growth based on highly curated data. The universally essential cofactors from Chapter 3 were integrated in a universal prokaryotic biomass equation. Together with a highly curated universe of prokaryotic metabolic reactions updated from Chapter 4, this biomass composition was used to predict minimal metabolic networks viable in different growth media: a complete medium simulated with all 496 exchanges in the universe of reactions and abstractions of LB medium and M9 minimal medium with 67 and 20 components respectively. One thousand variations of the minimal networks were generated for each medium. The minimal networks obtained were analyzed regarding size, metabolic subsystems, maximum growth rates, ATP and cofactor requirements. The results are consistent with the sizes of minimal metabolic networks in previous works, indicating to a core metabolism of ~250 metabolic reactions. The results indicate no significant differences when using the complete or the LB medium. Transport, cofactor and prosthetic groups, nucleotide and energy metabolism make up the core of the networks in rich media. A significant increase in the number of reactions relating to cofactor and prosthetic groups and nucleotide and amino acid metabolism occurs when generating the networks in the minimal medium. Several outliers in growth capacity and ATP and NAD(P) requirements indicate future routes of investigation. The method and data provided may allow for further exploratory studies of prokaryotic metabolism.

The information presented in this Chapter is being prepared for submission to a peer reviewed journal:

Xavier JC, Machado D, Patil KR, Rocha I. Prediction of Minimal Metabolic Networks With Diverse Manually Curated Data (in preparation).

5.1 Introduction

How many molecular components are necessary to sustain a living cell? This bold and yet nebulous question has been gathering attention from the scientific community especially since the advent of genomics, with a great focus on essential genes that are part of hypothetical minimal genomes (Gil et al. 2002, 2004; Hutchison et al. 1999, Itaya 1995, Mushegian & Koonin 1996). The motivations are several and overlap partially, in the fundamental drive to uncover the core essential requirements of life, the investigation of the origin of life, the contemporary search for new antibiotic targets and the industrial requirements of highly modular but at the same time versatile chassis cells (see Chapter 2 of this thesis).

The use of metabolic networks as an alternative approach to the question of cellular minimization has been substantially less explored than pure genomics, albeit it conveys a whole additional layer of information – that of functionality – and it can be explored quantitatively with graph theory and other mathematical tools (Gabaldón et al. 2007, Ravasz et al. 2002). Manually curated genome-scale metabolic models (GSMs) are the prime representative of this approach and have been widely used in predicting essential and non-essential reactions and metabolites of specific species (Imieliński et al. 2005, Kim et al. 2010, 2011; Suthers et al. 2009) but also in comparative studies to find core metabolic functions (Alam et al. 2011, Almaas et al. 2005). Moreover, these models can serve as databases of curated reactions to predict smaller, viable minimal metabolic networks.

Escherichia coli, as a model organism, has been extensively used as a starting point to achieve small viable networks with different approaches. An early model was employed in a pioneer study with mixed-integer linear programming (MILP) that concluded on minimal networks with 224 reactions on a glucose-only medium and 122 in a rich medium (Burgard et al. 2001). The GSM iJR904 (Reed et al. 2003) was used in a topological method of random addition and deletion of reactions to achieve minimal reaction sets having between 140 and 232 reactions (Jiang et al. 2010). Another study used the same metabolic model, variable biomass compositions and experimental assays to infer minimal metabolic networks for three different substrates (Taymaz-Nikerel et al. 2010). The authors removed all unnecessary transport reactions, dead-end reactions and zero-flux reactions and reached a minimal model for glucose with 276 reactions. Other authors used *E. coli* and a graph-theory approach combined with mixed integer linear programming to achieve a minimal network; however, the model used represented only the central carbon metabolism of this organism (Jonnalagadda & Srinivasan 2014). Central carbon metabolism was also the starting point of a study intending to predict a minimal *E. coli* cell for efficient production of ethanol that reduced the functional space of the network from over 15,000 pathway possibilities to 6 pathway options that support cell function (Trinh et al. 2008). Another quite ingenious study predicted the reductive evolution of the endosymbiotic bacteria *Buchnera aphidicola* and *Wigglesworthia glossinidia* with 80% accuracy by simulating the successive loss of genes of *E.coli'*s network (Pál et al. 2006).

In the present work, a large universe of prokaryotic metabolic reactions with re-annotated and curated metabolic subsystems was built and used in a subsequent generation of minimal metabolic networks. Three different environmental conditions were employed together with a manually curated biomass function representing the universal metabolic requirements of prokaryotes common to both bacteria and archaea. The main goal was to find core metabolic functions for minimal prokaryotic metabolisms, expanding previous work with *E. coli* only, as here the universe of reactions encompasses 15 GSMs representing several different phyla of bacteria and one archaea. The aim was to analyze the differences between networks generated in different growth conditions, in terms of size, growth capacity, cofactor requirements, but also functional composition concerning the most represented metabolic subsystems.

5.2 Methods

5.2.1 Construction of a Universe of Diverse Prokaryotic Metabolic Reactions

A curated universe of metabolic reactions encompassing a wide variety of prokaryotic phyla was built by integrating all models used in Chapter 4 (see Chapter 4, Table 4.1), with the exception of the model of *Escherichia coli* K12, iAF1260 (Feist et al. 2007), instead of which the newer and more complete model, iJO1366 (Orth et al. 2011) was used. All models were imported in the original SBML format and tested for feasibility in biomass production using default flux bounds. When the same reaction was present in one model as reversible and irreversible in another, the reversible version was kept. When different reactions were present in different models with the same ids, new unique ids were created. All exchange reaction fluxes were set to a limit of -10 mmol gDW^{-1}h^{-1} for the lower bound, representing the consumption of the metabolite, and 1000 mmol gDW^{-1}h^{-1} for the upper bound representing the excretion. All other reaction bounds were cleaned, except for irreversible reactions where the lower bound was fixed to zero. The universe obtained in this manner was tested for feasibility with all biomass reactions from the individual models. All blocked reactions and dead-end metabolites were computed using FVA and removed from the universe. All artificial metabolite sinks were also removed (with the exception of R_DM_4CRSOL, R_DM_5DRIB, R_DM_AMOB, R_DM_MTHTHF).

5.2.2 Growth Media

Three growth media compositions were tested for the design of minimal metabolic networks, all simulated at an exchange rate of 10 mmol gdW-1h-1 for each component. The first composition was an extremely rich medium where all the 496 exchanges available in the 15 models were allowed to carry flux in the simulations, representing a theoretical situation where there are no nutritional limitations, that is also an approximation of a possible ancestral rich prebiotic environment (Martin et al. 2008). The second condition was a common undefined laboratory rich growth medium, Lysogeny Broth (LB) (Bertani 1951), for which an abstraction was obtained from the ModelSEED culture media repository (ArgonneLBMedium, (Henry et al. 2010)); the trace elements molybdenum and nickel were added (as required by the imposed composition of the core biomass) resulting in a total of 67 medium components. The third and final growth medium used was the defined minimal medium M9, which composition was obtained from (Joyce et al. 2006) and adapted with the addition of the trace elements molybdenum, nickel, zinc, manganese, copper, cobalt and iron 2 and 3 resulting in a total of 20 available components. **Table 5.1** shows the composition of both LB and M9 media used.

Table 5.1 – Growth media used in the generation and simulation of minimal metabolic networks. The abbreviations shown are those used for each reaction identifier in the universe of reactions.

Component	M9	LB	Reaction ID in the Universe
NH_4	X		EX_nh4_e
Fe^{2+}	X	X	EX_fe2_e
Fe^{3+}	X	X	EX_fe3_e
Cobalt	X	X	EX_cobalt2_e;EX_cobalt3_e
Cu^{2+}	X	X	EX_cu2_e
Mn^{2+}	X	X	EX_mn2_e
Mobd	X	X	EX_mobd_e
Ni^{2+}	X	X	EX_ni2_e
Zn^{2+}	X	X	EX_zn2_e
Na^{+}	X	X	EX_na1_e
K^{+}	X	X	EX_k_e
Cl^{-}	X	X	EX_cl_e
Mg^{2+}	X	X	EX_mg2_e
Ca^{2+}	X	X	EX_ca2_e
HPO_4	X	X	EX_pi_e;R_EX_h_e
SO_4	X	X	EX_so4_e
Glucose	X	X	EX_glc_e
Water	X	X	EX_h2o_e
O_2	X	X	EX_o2_e
Adenosine		X	EX_adn_e
AMP		X	EX_amp_e
Arsenate		X	EX_aso3_e
Cd^{2+}		X	EX_cd2_e
Chromate		X	EX_cro4_e
CMP		X	EX_cmp_e
Deoxyadenosine		X	EX_dad-2_e
Deoxycytidine		X	EX_dcyt_e
Folate		X	EX_fol_e
Glycine		X	EX_gly_e
GMP		X	EX_gmp_e
Guanosine		X	EX_gsn_e

Table 5.1 – Growth media used in the generation and simulation of minimal metabolic networks (continued)

H_2S	**X**	**EX_h2s_e**
Heme	X	EX_pheme_e
Hg^{2+}	X	EX_hg2_e
Hypoxanthine	X	EX_hxan_e
Inosine	X	EX_ins_e
L-Alanine	X	EX_ala-L_e
L-Arginine	X	EX_arg-L_e
L-Aspartate	X	EX_asp-L_e
L-Cystine	X	EX_cyst_e;EX_cys_L_e
L-Glutamate	X	EX_glu-L_e
L-Histidine	X	EX_his-L_e
L-Isoleucine	X	EX_ile-L_e
L-Leucine	X	EX_leu-L_e
L-Lysine	X	EX_lys-L_e
L-Methionine	X	EX_met-L_e
L-Phenylalanine	X	EX_phe-L_e
L-Proline	X	EX_pro-L_e
L-Serine	X	EX_ser-L_e
L-Threonine	X	EX_thr-L_e
L-Tryptophan	X	EX_trp-L_e
L-Tyrosine	X	EX_tyr-L_e
L-Valine	X	EX_val-L_e
Lipoate	X	EX_lipoate_e
Niacin	X	EX_nac_e
PAN	X	EX_pnto-R_e
Pyridoxal	X	EX_pydx_e
Riboflavin	X	EX_ribflv_e
Thiamine	X	EX_thm_e
Thymidine	X	EX_thymd_e
UMP	X	EX_ump_e
Uracil	X	EX_ura_e
Uridine	X	EX_uri_e
Vitamin B12	X	EX_adocbl_e;EX_cbl1_e

5.2.3 Universal Biomass

The universal biomass equation used in all simulations was adapted from the core biomass equation of *E. coli*'s model iJO1366, R_Ec_biomass_iJO1366_core_53p95M (Orth et al. 2011). A manual curation was done based on Chapter 3 of this thesis and other publications (Chopra et al. 2010, Mendum et al. 2011, Orth & Palsson 2012, Paliy & Gunasekera 2007). All amino acids and building blocks of RNA and DNA were maintained, for which all coefficients were kept as in the original equation (**Table 5.2**). Alterations were done to the pool of organic cofactors: bis-molybdopterin guanine dinucleotide, biotin, 2-Octaprenyl-6-hydroxyphenol, Undecaprenyl-diphosphate, tetrahydrofolate, protoheme and siroheme were removed and riboflavin was substituted directly by the active cofactor flavin mononucleotide. The cytoplasmic lipid species were substituted by a common precursor to both archaea and bacteria, dihydroxyacetone phosphate (Koga 2011), for which a new coefficient was recalculated from the stoichiometry of the substituted lipids. Murein and lipopolysaccharide were removed for those are not universal components in the biomass of prokaryotes, the latter being specific of Gram negative bacteria and the former of bacteria with cell wall (exceptions in prokaryotes being Mollicutes and archaea).

Table 5.2 – Biomass composition adapted from the core biomass equation of *E. coli*'s model iJO1366 used in the generation of minimal metabolic networks to simulate the core universal components in prokaryotes. Column "Alteration" indicates substitutions and exclusions from the original. If an original metabolite was substituted, that is indicated with a new metabolite name and a corresponding new calculated coefficient; if the original metabolite was excluded for not being universal in prokaryotes, that is indicated with the tag "Removed".

Macromolecule /Class	Metabolite ID	Coefficient (mmol/gDW)	Alteration	New coefficient
		REAGENTS		
Protein	ala-L[c]	0,513689		
	arg-L[c]	0,295792		
	asn-L[c]	0,241055		
	asp-L[c]	0,241055		
	cys-L[c]	0,091580		
	gln-L[c]	0,263160		
	glu-L[c]	0,263160		
	gly[c]	0,612638		
	his-L[c]	0,094738		
	ile-L[c]	0,290529		
	leu-L[c]	0,450531		
	lys-L[c]	0,343161		
	met-L[c]	0,153686		
	phe-L[c]	0,185265		
	pro-L[c]	0,221055		
	ser-L[c]	0,215792		
	thr-L[c]	0,253687		
	trp-L[c]	0,056843		
	tyr-L[c]	0,137896		
	val-L[c]	0,423162		
DNA	datp[c]	0,026166		
	dctp[c]	0,027017		
	dgtp[c]	0,027017		
	dttp[c]	0,026166		
RNA	ctp[c]	0,133508		
	gtp[c]	0,215096		
	utp[c]	0,144104		
LIPID	pe160[c]	0,017868	M_dhap_c	0,072022
	pe161[c]	0,021060		
	pe160[p]	0,045946	Removed	
	pe161[p]	0,054154	Removed	

Table 5.2 – Biomass composition adapted from the core biomass equation of *E. coli*'s model iJO1366 used in the generation of minimal metabolic networks to simulate the core universal components in prokaryotes (continued)

Inorganic Ions	2fe2s[c]	0,000026		
	4fe4s[c]	0,000260		
	ca2[c]	0,005205		
	cl[c]	0,005205		
	cobalt2[c]	0,000025		
	cu2[c]	0,000709		
	fe2[c]	0,006715		
	fe3[c]	0,007808		
	k[c]	0,195193		
	mg2[c]	0,008675		
	mn2[c]	0,000691		
	mobd[c]	0,000007		
	nh4[c]	0,013013		
	ni2[c]	0,000323		
	so4[c]	0,004338		
	zn2[c]	0,000341		
Organic Cofactors	10fthf[c]	0,000223		
	amet[c]	0,000223		
	coa[c]	0,000576		
	fad[c]	0,000223		
	mlthf[c]	0,000223		
	nad[c]	0,001831		
	nadp[c]	0,000447		
	pydx5p[c]	0,000223		
	ribflv[c]	0,000223	FMN	0,000223
	thmpp[c]	0,000223		
	bmocogdp[c]	0,000122	Removed	
	2ohph[c]	0,000223	Removed	
	btn[c]	0,000002	Removed	
	pheme[c]	0,000223	Removed	
	sheme[c]	0,000223	Removed	
	thf[c]	0,000223	Removed	
	udcpdp[c]	0,000055	Removed	
Murein	murein5px4p[p]	0,013894	Removed	
LPS	kdo2lipid4[e]	0,019456	Removed	
Growth Associated Maintenance	atp[c]	54,124831		
	h2o[c]	48,601527		

Table 5.2 – Biomass composition adapted from the core biomass equation of *E. coli*'s model iJO1366 used in the generation of minimal metabolic networks to simulate the core universal components in prokaryotes (continued)

PRODUCTS	
adp[c]	53,950000
h[c]	53,950000
pi[c]	53,945662
ppi[c]	0,7739030

5.2.4 Curation of the Reaction Universe and Generation of Feasible Minimal Metabolic Networks

Minimal feasible metabolic networks were generated based on the universe of metabolic reactions with a linear programming approach, by minimizing the number of reactions in a model that would still allow for a positive flux through the universal biomass reaction. The minimization was performed by individually scaling the absolute flux of each reaction (v) by a random weighting factor (w) drawn from a uniform distribution U(0,1):

$$\begin{aligned}
&\min \sum_{i=1}^{n} w_i |v_i| \\
&\text{s.t} \\
&\quad S \cdot v = 0 \\
&\quad lb \leq v \leq ub \\
&\quad v_{\text{biomass}} \geq 1 \\
&\quad w_i \sim U(0,1)\ \forall i
\end{aligned}$$

Where S corresponds to the stoichiometric matrix and lb and ub to the lower and upper bounds of each individual reaction, respectively. This sampling procedure generates an ensemble of alternative flux distributions with minimal support vectors.

One thousand alternative feasible minimal networks were initially built for the theoretical complete medium and the 47 reactions present in more than 70% of the networks were manually checked. Six reactions were excluded based on this analysis. Those were two reactions involved in the direct transport of Coenzyme A in *M. tuberculosis*, since this cofactor has no known direct transport and there is evidence of essentiality of its biosynthetic enzymes in *M. tuberculosis* (Chapter 3 of this thesis; Kumar et al. 2007; Ambady et al. 2012). The other four reactions excluded were reactions of transport of AMP, GMP, dTMP and CMP that are symports with hydrogen, originally present only in the *B. subtilis* network. After this curation, a new set of 1000 feasible minimal metabolic networks was generated for each environmental condition: complete, LB and M9 medium.

5.2.5 Model Analysis

Each set of 1000 minimal metabolic networks was analyzed for the frequency of individual reactions in the networks, network size, maximum biomass flux, ATP, NADH and NADPH requirements. Simulations were performed with parsimonious Flux Balance Analysis (pFBA) implemented with Gurobi optimizer 6.0. Cofactor requirements were calculated as the turnover of each metabolite normalized by the maximum biomass flux.

5.3 Results and Discussion

5.3.1 Universe of Prokaryotic Metabolic Reactions

The curated universe resulting of the integration of the 15 prokaryotic genome-scale metabolic models is composed of 5768 metabolic reactions. These were checked against each model metadata on metabolic subsystems and pathways, resulting on 233 subsystem names that were manually integrated into a final set of 23 highly curated metabolic subsystems (**Figure 5.1**). A small set of 512 reactions - 8.88% of the universe - is composed of fictional reactions used to allow for simulations (exchanges, sinks and lumping reactions). However, 496 of those are exchange reactions that represent the individual components of the media available for simulations. A large portion of the universe (25.7%) corresponds to transport reactions in the different models, which allow for the passage of components between the external compartment loaded by exchange reactions and the cytoplasm or periplasm. These reactions include different alternatives for the transport with different symports, antiports and energetic requirements. Given that six models used in the construction of the universe include a periplasm compartment and the other nine do not, several of the transport reactions are duplicated and the real size of this subsystem in the universe is smaller. Following in size is the subsystem of Lipid metabolism with 13.3% of the reactions in the universe and Cofactor and Prosthetic Group metabolism with 10.1%. The universe is rich and diverse with 381 reactions allowing for the metabolism of alternative carbon sources and 131 reactions related with secondary metabolism and other miscellaneous functions.

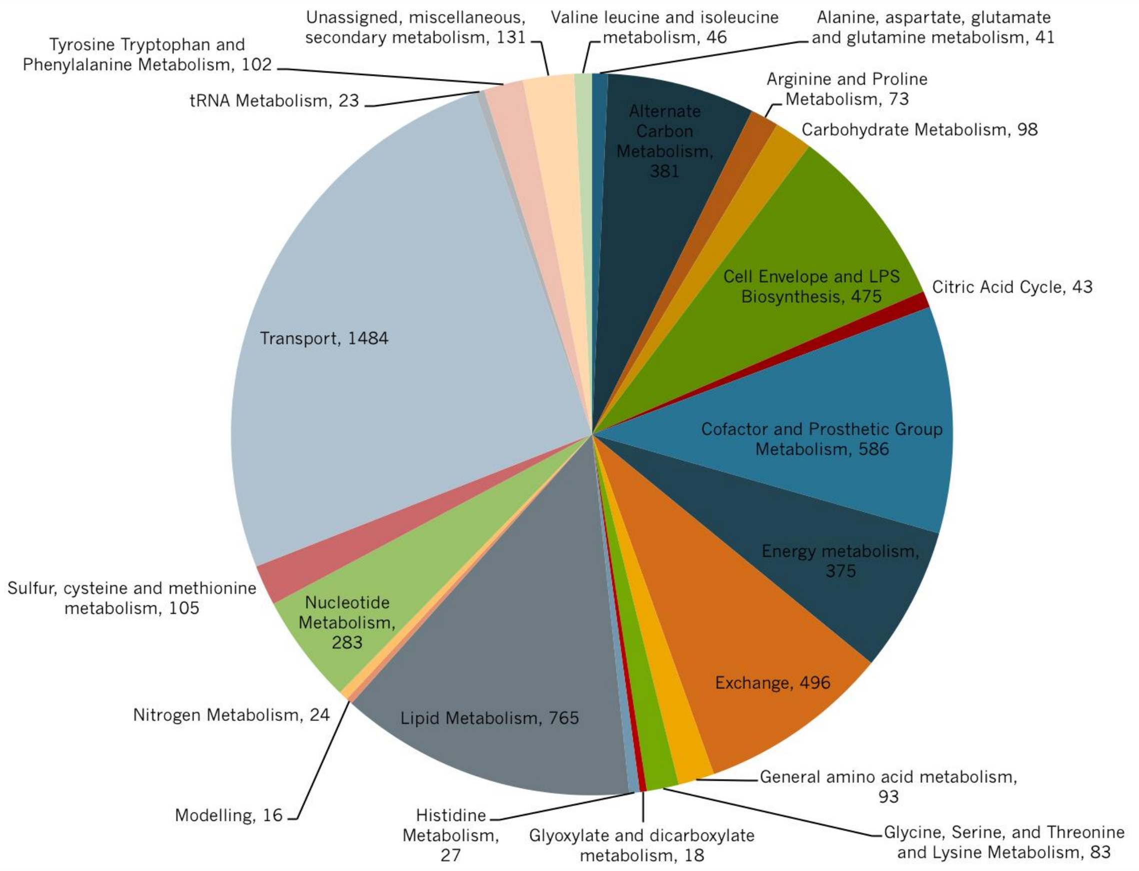

Figure 5.1 – Metabolic subsystems in the integrated universe of prokaryotic metabolic reactions. Aggregated and curated subsystem names are shown together with the number of respective individual reactions in the subsystems.

5.3.2 Network Sizes

Minimal networks generated for different growth media differed regarding the final network size, i.e. the minimal total number of reactions necessary for growth (**Figure 5.2**). Surprisingly, there is no significant difference between the size of the networks generated in a complete medium compared to the LB medium, which in fact yielded an average network size smaller – 217 - than the former - 219. For M9 minimal medium, however, the difference is significant with an average network size of 274 reactions. Other authors obtained similar sizes for viable minimal networks of 224 reactions in a glucose-only based medium (Burgard et al. 2001). The biomass requirements in that study were based on an early *E. coli* model with the same composition used in iJR904 (See Supplementary Table 3.1 and 3.2 for composition) with no inorganic cofactors and less organic cofactors than those used here, on another side it included some lipids and cell wall components. Another study using the same biomass composition and a defined minimal medium similar to M9 concluded on 276 reactions (Taymaz-Nikerel et al. 2010), which is highly similar to what was obtained here.

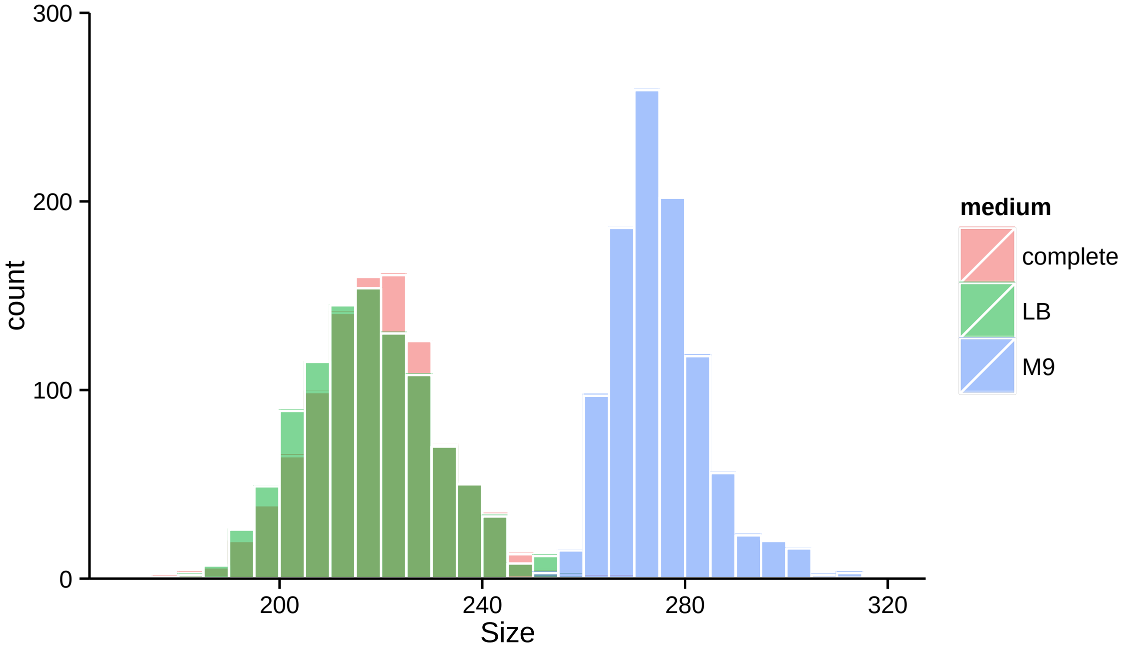

Figure 5.2 – Distribution of network sizes for each set of 1000 minimal networks generated in different media conditions. Red - complete theoretical medium; green - LB medium and blue - M9 medium.

5.3.3 Metabolic Subsystems in the Minimal Networks

The smallest networks generated for the complete, LB and M9 media had 178, 182 and 249 reactions respectively and the composition of each was analyzed regarding the representation of each curated metabolic subsystem (**Figure 5.3**). Even though the biomass reaction used for the generation and simulation of the networks was a universal reaction with only amino and nucleic acids, core universal cofactors and one lipid precursor, there is a good representation of all metabolic subsystems in the generated networks. Only reactions belonging to the subsystems of tRNA charging and glyoxylate metabolism are never represented in these three networks. The results for both rich media conditions are similar, with a vast majority of reactions being transports, followed by cofactor and prosthetic group, and nucleotide and energy metabolism reactions. Nevertheless, even in complete medium, the networks generated do not uptake directly all amino acids. One example is in the complete medium where the network uses two reactions in the subsystem of Arginine and Proline Metabolism to generate the latter amino acid. Only approximately 30% of the networks generated in this medium included a direct uptake of proline, which requires two reactions as well (one exchange to generate the proline in the medium and one transport to the cytoplasm). The existence of a vast alternative of metabolic routes allows for the use of less common reactions for the biosynthesis of universal biomass components. One example is the use of one reaction of Alternate Carbon metabolism in complete medium (a methionyl aminopeptidase originally from *Shewanella oneidensis*) that allows for the production of aspartate in one single step after the import of a dipeptide from the growth medium. Also, both networks fixate nitrogen with a nitrogenase from *D. ethenogenes*, which generates 16 ATP molecules for each nitrogen molecule generated. This is the best alternative in terms of ATP generation with the least number of reactions possible.

The network generated in M9 minimal medium has a considerably smaller number of active exchange and transport reactions which translates in a vast increase of the number of reactions in other subsystems, mainly pertaining to cofactors, nucleotides, energy and amino acid reactions. Reactions associated with aromatic amino acids and valine, leucine and isoleucine appear only in this network.

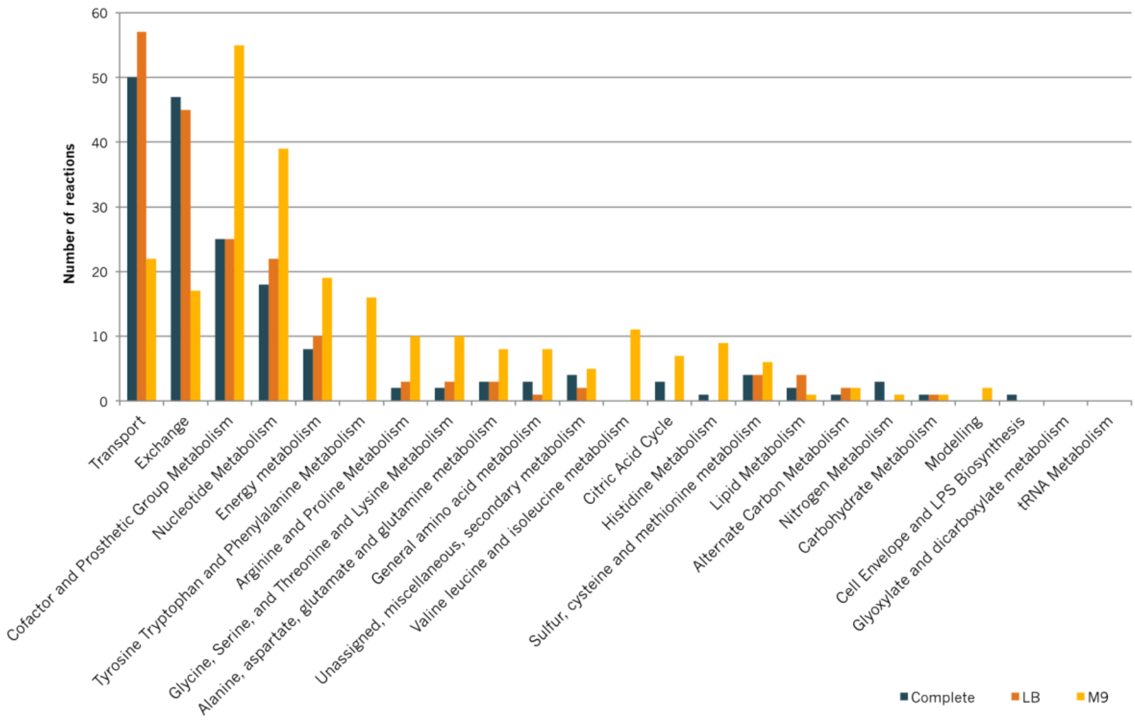

Figure 5.3 – Number of reactions in the different metabolic subsystems for the smallest minimal networks generated with complete, LB and M9 medium and a universal prokaryotic biomass reaction.

5.3.4 Growth Rates

The distribution of maximum growth rates for the sets of networks generated for different media was quite scattered, with maximum growth rates ranging from 1 to 22.19 h^{-1} among the 3000 networks generated (**Figure 5.4**). Given the non-normalized biomass equation (where some compounds were removed and the total mass doesn't sum to one gram) and the absence of expensive compounds as membrane and cell wall components, the values of growth rates reach theoretical values that are much higher than real growth rates. However, these are still comparable among networks, given that all networks were generated using this same biomass composition.

The average growth was 4.83 for M9 medium and again unexpectedly larger for the LB medium than for the complete medium, with 6.24 for the former and 5.3 for the latter. There are several outliers, especially in the cases of both rich media, which is confirmed by high standard deviations (3.44 for M9, 3.89 for LB and 3.45 for complete medium). The upper limit of growth in M9 medium is fixed at 11.65.

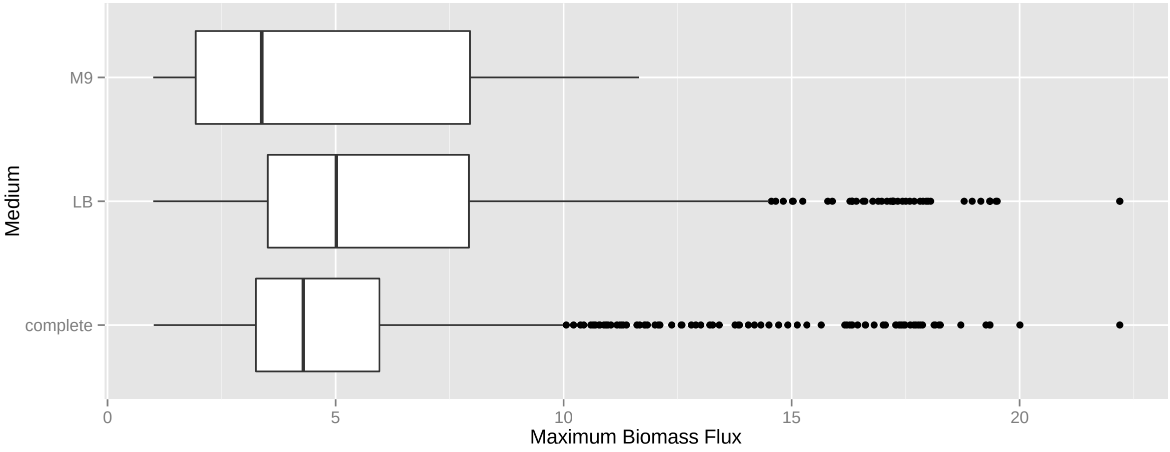

Figure 5.4 – Maximum flux through the biomass objective function for each set of 1000 minimal networks generated in different media conditions.

5.3.5 Cofactor Requirements

As in the case of growth rates, the distribution of ATP, NADH and NADPH requirements for the different networks shows a considerable number of distant outliers (**Figure 5.5**). In general, there is a much higher requirement for ATP (between 55.5 and 292.8), followed by NADH (minimum 0 and maximum 117.6) and NADPH (between 0 and 93.8). Interestingly, both extreme values of NADPH requirement occur in the networks generated in complete medium. However, the average requirements are lower for both rich media in the three cases, with a marginal difference between the complete medium and the LB medium, with the former producing smaller average requirements. The higher average requirements in M9 medium are justified for its single carbon source, glucose.

It is notable that in complete medium there are networks where NADH and NADPH are not required for biomass production. Both these cofactors appear in the universal biomass composition utilized in the simulations in their oxidized form. A closer look at these networks reveals that there is no network where both turnovers for the redox cofactors are zero: either it is zero for NADH or NADPH. Looking into the three networks where the NADH turnover is zero with more detail, it is notable that the only exchange reaction that all three utilize that is not present in the simulation of LB medium is the transport of nicotinamide mononucleotide, a direct precursor of NAD. This precursor is converted to NAD, which is directly routed to biomass and converted to NADP by a direct phosphorylation. NADP is then converted to NADPH, which is then utilized for its reductive power in the network. NADH is therefore absent and unnecessary in these networks. The zero turnover for NADPH in other 5 networks remains to be explained.

When comparing the maximum growth with the ATP requirements normalized by growth for all networks, the results are very similar again for both the complete and LB media, with a high variation of maximum growth but quite fixed ATP requirements for the vast majority of the networks, while in the case of M9 medium the ATP requirement varies more at lower maximum growth rates (**Figure 5.6**). This result indicates that the ATP requirements of the network are not dependent on the maximum biomass production. Interestingly, there is a gap between approximately 110 and 170 for ATP requirements, the latter being the point where the significant outlier networks start to lie with small maximum growth rates.

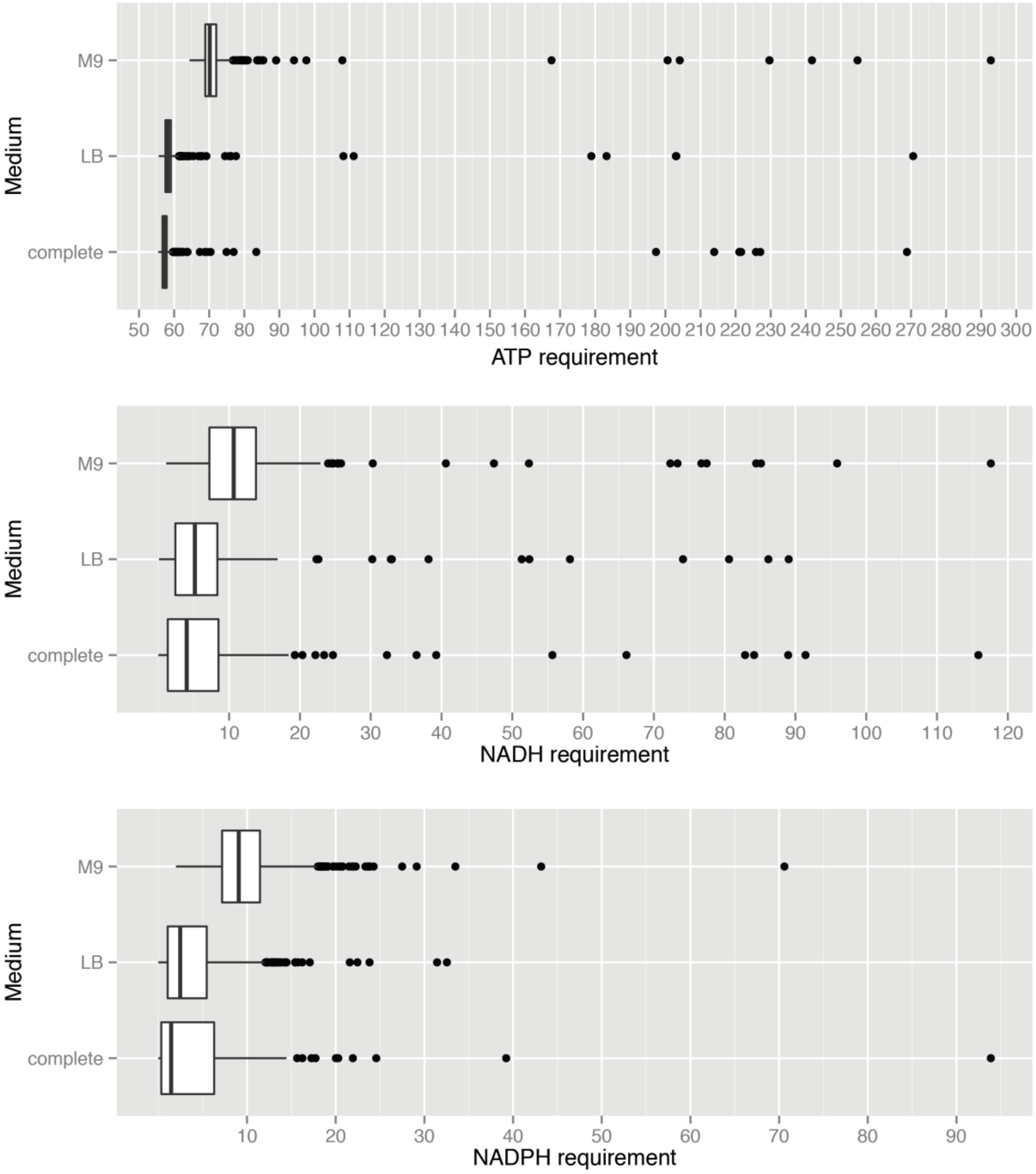

Figure 5.5 – Distribution of the value of cofactor requirements for the 3000 networks generated in three different media conditions (complete, LB and M9). Requirements were calculated as the turnover of each metabolite normalized by growth.

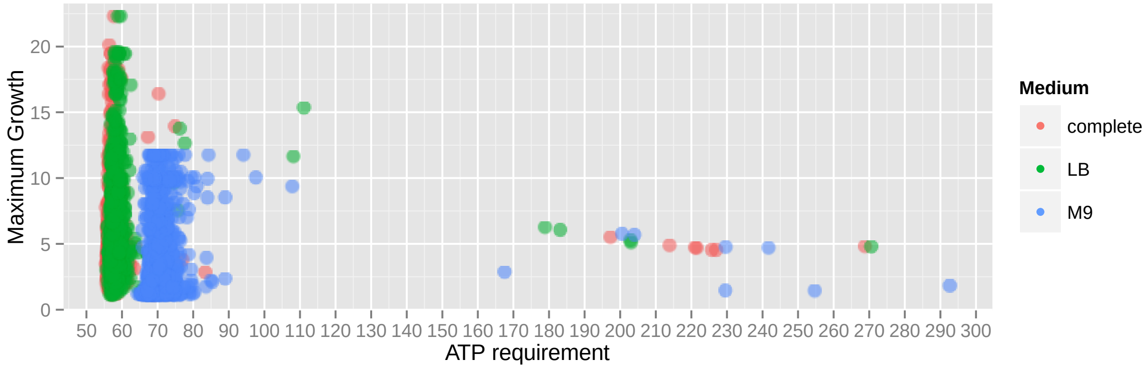

Figure 5.6 – Maximum growth and ATP requirements for the 3000 minimal networks generated for the three growth media conditions. Red – complete medium, green – LB medium and blue – M9 medium.

5.4 Conclusions

The present work derived a universe of 5768 curated metabolic reactions depicting a wide variety of metabolic capacities of different bacteria and archaea in an unprecedented manner, based on which, using a curated biomass reaction representative of universal compounds in prokaryotes, 1000 minimal and viable metabolic networks were generated for different growth media conditions. Average network sizes of 219, 217 and 274 reactions for a complete medium, an abstraction of LB medium and M9 minimal medium, respectively are highly similar to the results obtained by other authors that concluded on viable networks of 224 (Burgard et al. 2001) and 276 (Taymaz-Nikerel et al. 2010) reactions and to other theoretical minimal genome sizes with 206 (Gabaldón et al. 2007, Gil et al. 2004) and 256 genes (Mushegian & Koonin 1996).

The minimal networks obtained for the abstraction of LB media with 67 components are surprisingly similar in size, metabolic content, cofactor requirements and maximum growth rates to those obtained with a complete media with 496 available components. This result indicates that the optimal conditions for growth, among those tested, are represented in the LB medium and further additional components are unnecessary to generate the smaller networks. It is however evident from the results with M9 medium that the removal of some specific components will affect drastically the sizes and capabilities of the minimal networks. A closer look at the composition of LB medium compared with M9 and the subsystem distribution in the different networks indicates that the components that are highly essential for generating smaller networks are amino acids and vitamins and cofactors. An iterative study with individual randomly generated growth media might shed a light on individual nutrients which have the highest impact on the generated networks.

The universe of reactions and the minimal network generation method provided here allow for future estimation of minimal networks with different biomass requirements and growth media. Interesting studies may include the minimal network requirements for the production of different compounds of interest and growth on different metabolic modes, including autotrophy, methanogenesis and nitrogen fixation. Although the universal biomass equation used here excluded lipids and cell wall components, necessary reactions for their production are available in the universe.

It is admitted in the current status of prokaryotic systems biology that there is probably no single minimal genome or metabolic network, due to the extremely high redundancy of prokaryotic networks (Koonin 2003)(see Chapter 2). This work not only confirms this postulate by generating thousands of alternative hypothetical minimal metabolic networks for a complete medium, but also allows for future explorations of the trade-off between network capacities and the environment. It is expected that this exploration can lead to a better understanding of the core metabolism of prokaryotes, but also in the design of viable, optimized and modular chassis cells for different biotechnological processes.

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CHAPTER 6

Conclusions and Perspectives on Future Research

We can only see a short distance ahead, but we can see plenty there that needs to be done.

— Alan Turing, *Computing Machinery and Intelligence* (1950)

In this final chapter the reader can find the main conclusions achieved by the research described in this thesis. Some selected perspectives on future research according to these conclusions and to the hypotheses raised throughout this work are also advanced.

6.1 General Conclusions

The research conducted in this thesis had the overall objective of studying minimal and essential metabolic functions within prokaryotic species. In order to achieve this general objective, specific research aims were defined in Chapter 1 of this thesis. Answering to those aims, the main conclusions obtained in each chapter are discussed here.

In Chapter 2, an extensive review of the broad field of minimal and simpler cells was performed. Several entangled concepts were uncovered and systematically described (Table 2.1) together with the traditional and emergent systems biology approaches to the field (Figure. 2.1). It became evident that the traditional analytical, top-down approach has been prominent with large-scale identifications of essential genes with a special emphasis on minimal genomes (Table 2.3). A fundamental difference between two main goals in this field was exposed: the minimization of cellular components that has been prominent in the traditional analytical approach to the field, versus the simplification of cellular complexity that is more patent in integrative approaches that include mathematical modeling.

In Chapter 3, the biomass objective function used in the modeling of metabolic networks at genome-scale was analyzed. The main goal was to identify core components that are essential for all prokaryotic species for further prediction of core metabolic reactions in minimal networks. This goal was achieved and surpassed with several side conclusions. A comparison of all the available prokaryotic genome-scale metabolic models (GSMs) at the time of this work (Supplementary Table 3.1) revealed a large heterogeneity in the definition and formalization of the biomass composition in these models (Figure 3.1). With sequential simulations of 5 GSMs with interchangeable biomass equations, it was shown that the biomass composition can impact drastically the predictions of essential reactions for growth (Figure 3.2). A set of universally essential organic cofactors for prokaryotic species was uncovered (Figure 3.3 and Figure 3.5): nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), S-adenosyl-methionine (SAM), flavin adenine dinucleotide (FAD), pyridoxal 5-phosphate (P5P), coenzyme A (COA), thiamin diphosphate

(THMPP) and flavin mononucleotide (FMN) plus one class of cofactors, which was identified as one-carbon carriers (tetrahydrofolates for bacteria and tetrahydromethanopterins for most archaea). A set of highly essential but not universal cofactors was also identified and discussed. The universal cofactors allowed for a revision of essentiality predictions in *Klebsiella pneumoniae* and the prediction of a biosynthetic pathway absent in the model of *M. tuberculosis* that was later found to have been confirmed experimentally by Dick and co-authors (Dick et al. 2010). Moreover, in the same study, the authors validated the prediction done in this work of the essentiality of vitamin B6 for the survival of *M. tuberculosis*.

In Chapter 4 fifteen comparable and validated GSMs were simulated in rich media abstractions, predicting the metabolic subsystems of cofactor and prosthetic group biosynthesis, cell envelope biosynthesis and membrane lipid and glycerophospholipid metabolism to have the highest number of essential reactions in all models (Fig. 4.2). The reactions that were essential in more models belonged to the metabolic subsystems of aromatic amino acid metabolism, nucleotide salvage pathway, cell envelope biosynthesis and cofactor and prosthetic group metabolism (Fig. 4.4). These results were confirmed by experimental data, except for tRNA metabolism, which was shown to be highly essential experimentally but that is not included in most models (Figure 4.6). Three reactions essential in 14 out of the 15 metabolic models related with the shikimate pathway and annotated in the aromatic amino-acid metabolism were shown to be essential for the biosynthesis of folates, and therefore require a re-annotation. More specifically, the essentiality of chorismate synthase in models but not in the experimental data led to the hypothesis that a transporter for chorismate or another metabolite further down in the biosynthesis of folates from chorismate is missing in the metabolic models. This hypothesis was confirmed in the literature, with the experimental rescuing of a mutant for chorismate synthase in minimal medium with p-aminobenzoic acid (Lee et al. 2013). Still in this chapter, the results of essentiality were compared at a large-scale with results of ancestry inferred from a BLAST of *E. coli* genes against a manually-selected set of species representing all the phyla with one fully-sequenced quality genome in the tree of life. The comparison revealed that genes with a positive sum of essentiality (more datasets showing essential that non-essential) are much scarcer than those with a negative sum. However, it is much more likely that those are highly conserved, and therefore, likely to be ancestral. In the case of genes with a negative sum of essentiality, there is no tendency for high or low conservation. A functional mapping to metabolic subsystems revealed that the genes more likely to be ancestral are those in the tRNA charging subsystem, Transport and Oxidative Phosphorylation.

Finally, in Chapter 5, the results obtained in the previous chapters were integrated by devising of a method to generate minimal metabolic networks based on highly curated data. A large universe of 5768 prokaryotic reactions was built and re-annotated employing the 15 GSMs used in Chapter 4 (with the exception of the model of *E. coli* for which a newer version was used (Orth et al. 2011)) and using a new set of 23 curated metabolic subsystems (Figure 5.1), revealing a wide variety of metabolic capacities of different bacteria and archaea in an unprecedented manner. Three media conditions were tested (one theoretical complete medium and two abstracted real media compositions, LB and M9) with a newly curated core biomass reaction that included the universal cofactors identified in Chapter 3. The sizes of minimal metabolic networks were consistent with previous works, indicating a core metabolism of ~250 reactions (Burgard et al. 2001, Gabaldón et al. 2007, Gil et al. 2004, Mushegian & Koonin 1996, Taymaz-Nikerel et al. 2010). No significant change was found between the characteristics of the networks when using the complete or LB medium, which leads to the conclusion that LB represents the optimal conditions for growth among those tested. This can be an indication of a minimal set of components for the design of economically viable rich media for chassis cells. Reactions involved in transport, cofactor and prosthetic groups metabolism, nucleotide metabolism and energy metabolism make up the core of the networks in rich media. The minimal medium generates networks with several more reactions relating to the biosynthesis of cofactors and prosthetic groups, nucleotides and amino acids. With the generation of thousands of alternative minimal networks for all growth conditions, the results of this work confirm the postulate of high metabolic redundancy in prokaryotic metabolism.

6.2 Perspectives on Future Research

In any scientific project, several new questions arise in each step of the process, and the current thesis is no exception. A long-winded discussion could follow on new enquiries that were side results of this work. A selected set of topics requiring further exploration is presented below.

- **The standardization of manually curated genome-scale metabolic models.** Several efforts have been taken by the community towards this goal (Bernard et al. 2014, Henry et al. 2010, King et al. 2015, Kumar et al. 2012, Sauls & Buescher 2014). However, the current state of the art still portrays a heterogeneity that is large and impairs the comparison and integration of results of GSMs. Very recently, an interesting and important debate on the adoption of standards by the community was raised (Chindelevitch et al. 2015, Ebrahim et al. 2015). It is expected that these standards will indeed be applied, which would greatly facilitate the emergence of a comparative systems biology based on GSMs that can be used to answer fundamental biological questions as the ones that were the main goals of this thesis.
- **The use of more complete models and the inference of not only minimal networks but minimal virtual cells.** Different models of minimal cells were described in Chapter 2, section 2.5.5 that represent more than only the metabolic functions of cells. It should be highlighted also the new ME models published recently that account for metabolism and gene expression in *Thermotoga maritima* and *E. coli* (Lerman et al. 2012, O'Brien et al. 2013). Although these are still not representative of a large-diversity of prokaryotic phyla and species, they reveal features of regulation that seem to be central and essential to prokaryotes. It remains to be found which of these regulation features are core features for life.
- **The study of ancestral metabolic networks.** Here, the subject of ancestral metabolism was very briefly touched upon. The definition of a highly curated ancestral environment based on solid geochemical science could help constrain a metabolic model to infer an ancestral metabolism with the resources provided in this thesis.

- **The further exploration of the generation of minimal metabolic networks.** In Chapter 5 of this thesis, an algorithm was devised to generate minimal metabolic networks based on a highly curated and diverse universe of metabolic reactions. It was interesting to see the usage of different reactions to produce a core set of biomass components given different growth media, which included for example reactions of the archaea in some cases, and nitrogen fixation from *D. ethenogenes* in most of the networks generated in rich media. However, much remained to be explored. More complete biomass equations, for example including a full set of essential lipids should be investigated. Special conditional cofactor requirements can be analyzed for specific purposes with the resources provided in Chapter 3. Furthermore, a panoply of possibilities are open related with the exploration of different growth media and nutritional requirements for different biomass objectives. Randomized large-scale simulations can be easily developed. Another direction of investigation is the design of specific minimal networks for the production of specific metabolites of interest coupled with maximum or fixed growth.

6.3 Supplementary Material

All the Supplementary Material mentioned in this thesis is freely available for download in http://darwin.di.uminho.pt/jcxavier/ and within the CD containing the digital version of this document.

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