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Producing high-value chemicals in *Escherichia coli* through synthetic biology and metabolic engineering

Helena Shomar Monges

PRODUCING HIGH-VALUE CHEMICALS IN *ESCHERICHIA COLI* THROUGH SYNTHETIC BIOLOGY AND METABOLIC ENGINEERING

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by

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With unconditional love and admiration
Remember that life is not a journey
There's no set destination that we should hurry to reach
Life is a musical
The whole point is to play it and dance it together

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INTRODUCTION

For millennia, humans have used microbes to produce industrial products through fermentation processes. In recent years, the application of engineering principles to microbiology have dramatically expanded our ability to develop novel microbial cell factories for the production of a wide variety of commercial products: from food and commodity chemicals, to biofuels and fine chemicals such as pharmaceuticals, fragrances, cosmetics or dyes. In this chapter, we present a historical overview of major scientific and industrial milestones that have led to the advent of metabolic engineering and synthetic biology. We describe how these fields have considerably advanced industrial microbiology and hold great potential to enable a sustainable bioeconomy. We introduce the main principles of these modern biotechnologies to engineer microbial cell factories, which served as framework for the work presented in this thesis. Finally, we discuss notable challenges in the field addressed by our research.

1

1.1 INTRODUCTION: A HISTORY OF INDUSTRIAL MICROBIOLOGY

Microorganisms belong to an extremely wide variety of taxonomic phyla, ranging from Bacteria and Archaea, to Fungi and microscopic plants. Most microbes on Earth consist of unicellular organisms, and it has been estimated that about 99% of them have not yet been characterized[1].

Throughout history, microorganisms have been widely exploited by humans to develop key processes and technologies that were essential to their survival, culture and expansion[2] (**Figure 1.1**). In fact, our knowledge and study of microorganisms have been closely related to their industrial use. Long before the 17th century when they were first visualized and discovered by Antonie van Leeuwenhoek in Delft, microbes had been employed to produce food and beverages via a process called 'fermentation'. For millennia, fermentation processes were based on poorly understood methods of manipulation and transformation of food that were acquired through experimentation. Fermentations were extensively used to produce a diversity of products such as wine, beer, bread, cheese, vinegar or pickled foods. These microbial processes effectively allowed humans to preserve food for longer, contributed to enhance their quality of life and greatly defined cultural habits. It was not until the second half of the 19th century that Louis Pasteur, while working with wine manufacturers, demonstrated that fermentation processes resulted from the biological activity of specific microbes. Soon after, Pasteur and Robert Koch considerably accelerated the field of microbiology by discovering that microbes provoke food spoilage, and that their proliferation in the body of animals and humans is the cause of many diseases. Such discoveries established the 'germ theory of disease' and the practice of pasteurization (application of heat to kill pathogenic bacteria), which allowed controlling the spread of infectious diseases. These discoveries considerably helped in the development effective therapies, and transformed the food industry. Further biochemical studies demonstrated that specific proteins (called 'enzymes') catalyze chemical reactions inside living cells, and that isolated enzymes can carry out fermentation processes in the absence of living microbes.

Towards the end of the 19th century, the impressive progress in the field of microbiology further advanced industrial applications, and fermentations became highly controlled and scalable processes. By the beginning of the 20th century microbiology applications quickly expanded into other industry sectors, marked by the development of new apparatuses such as bioreactors (engineered vessels in which living microorganisms are cultivated in a controlled manner to produce a product or biomass), which allowed scaling-up new bioprocesses for commercial purposes[3]. Microbial processes for the large-scale production of valuable chemicals from starch and sugars rapidly emerged, notably during World War I to manufacture glycerol, acetone and butanol[2]. Other relevant fermentation processes were developed to meet the

demand for commercial biomolecules such as vitamin C, citric acid, enzymes and a variety of organic acids.

A new era of industrial microbiology started with the discovery of penicillin by Alexander Fleming in 1928, which became the first antimicrobial agent widely produced to treat bacterial infections[4]. Tremendous efforts to advance the large-scale production of penicillin, from the very inefficient *Penicillum* strain originally isolated by Fleming, launched the tight connection between genetic manipulation and industrial microbiology. Despite poor understanding of the underlying genetic and biochemical mechanisms involved in penicillin production, a combination of strain selection, mutagenesis (notably through X-ray treatment) and optimization of culture conditions, led to fermentation improvements that allowed saving the lives of thousands of soldiers during World War II. Following the success of penicillin, strain improvement technologies and mutagenesis became effective tools to develop large-scale fermentation processes for the commercial production natural or semi-synthetic antibiotics. This marked the advent of the antibiotic 'wonder drugs' that subsequently transformed modern medicine. Mutagenesis and strain selection remained for decades one of the most efficient technologies to improve microbial fermentation processes, and to generate new biomolecules with novel properties[5].

The discovery of the molecular structure of DNA in 1953, followed by the establishment of the central dogma of molecular biology[6] and the molecular regulation of genetic expression[7], significantly improved our understanding of the link between molecular blocks and cellular metabolism. These discoveries implied that generally all the biochemical traits of a given organism are encoded within its genome. Consequently, this suggested that technologies to exchange functional DNA blocks could be employed to deliberately transfer biochemical traits between organisms. During the second half of the 20th century, the development of new methods to manipulate DNA led to the expansion of recombinant DNA technologies, which enabled the transfer of genetic information between different organisms. Molecular cloning techniques were thus developed to assemble and introduce recombinant DNA into microbial hosts, mainly by constructing replicating plasmid vectors. Such technologies considerably expanded the repertoire of natural products commercially manufactured with fermentation. Indeed microbes could be harnessed for the first time as host organisms for the large-scale production of molecules originally produced by organisms from different species (e.g. heterologous production). The first industrial success enabled by DNA technology was the commercial production of human insulin using recombinant bacteria in 1982[2]. From that point, numerous novel recombinant bioprocesses were developed for industrial exploitation, and in particular for the production of pharmaceuticals[8].

From the 1990s, significant progress in the field of biotechnology progressively transformed biology into an engineering discipline. Increasing amounts of data generated by high-throughput techniques to measure and

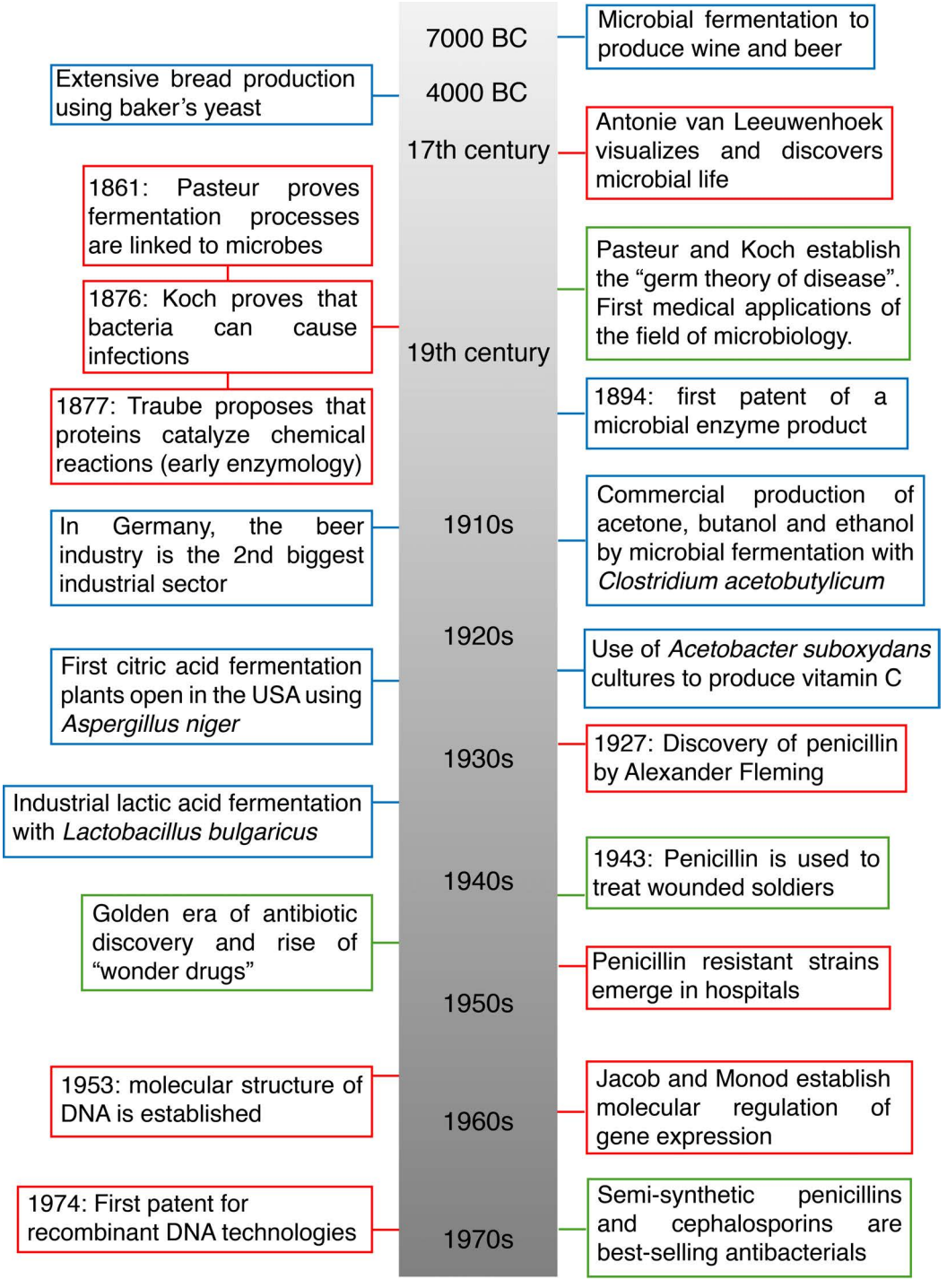
characterize molecular networks – of DNA (genomics), RNA (transcriptomics), proteins (proteomics) or metabolites (metabolomics) – analyzed with the help of classical engineering and computational tools, enabled to develop quantitative models to describe and predict biological systems[9] (e.g. systems biology). This interdisciplinary and holistic approach was successfully combined with recombinant technologies to modulate and optimize metabolic pathways for the large-scale production of valuable chemicals: the field of 'metabolic engineering' was born[10]. The tools of metabolic engineering employ a systemic view of biochemical pathways that improves our understanding of their functioning and limitations. This information can be used to develop efficient strategies to redirect metabolic fluxes toward the production of desired chemicals. Such strategies can be implemented using combinatorial methods that control the state of the metabolism, including: tuning the expression of pathway components, improving the efficiency and specificity of enzymes, or balancing metabolic networks to increase product yield. This field quickly expanded and has have been successfully applied to develop commercially viable fermentation processes[11].

The interdisciplinary field of synthetic biology, which emerged at the beginning of the 20th century, provided new tools to improve the model-driven design and construction of biological systems. This field introduced a bottom-up engineering approach to biology, which complements the top-down approach of systems biology. Indeed, synthetic biology applies design principles borrowed from classical engineering disciplines to biological systems. Engineers introduced abstraction and standardization principles to decompose cells, seen as 'biological machines', into modular 'parts'. They also demonstrated that these parts could be successfully combined to construct decoupled functional and regulatory modules with desired behaviors[12]. By analogy with the manner electrical engineers construct electrical circuits, synthetic biologists aim to develop genetic 'circuits' to carry out tightly controlled functions, which can be described with mathematical models. While the complexity, context-dependency and unpredictability of biological systems has challenged the founding concepts of synthetic biology, the field has considerably expanded our ability to engineer biological systems with industrial applications[10,13]. The development and spread of the enabling technologies of synthetic biology generated interdisciplinary tools that have been directly applied to the development of microbial cell factories[12], including: (i) increasingly accurate and inexpensive DNA synthesis and sequencing; (ii) tools for rapid DNA assembly and genetic manipulation; (iii) high-throughput analytics to characterize the behavior of natural and engineered biological systems; (iv) high-level bioinformatics and modeling tools to study complex systems; (v) scalable automation of laboratory protocols. Altogether, the tools and methods of synthetic biology have greatly boosted our ability to revise/extend the catalytic and/or regulatory networks of living organisms.

In addition to DNA synthesis and traditional molecular cloning, the emergence of robust genome editing tools, in particular CRISPR/Cas9, have accelerated our capacity to modify DNA sequences. Indeed, these tools can be used to perform targeted modifications within the genome of almost any organism with unprecedented precision and efficiency. By advancing genetic tractability of a wide range of organisms, CRISPR/Cas9 has greatly expanded the opportunities for engineering biological systems

As DNA technologies are becoming incredibly cheap, and the convergence between biotechnology and engineering keeps improving, numerous industry sectors are increasingly interested in the potential of biology to become on of the most promising manufacturing technologies of the future[14]. Indeed, not only it is becoming possible to fully exploit the tremendous diversity of natural metabolic pathways, but modern biotechnologies have also the unprecedented ability to design *de novo* synthetic pathways to produce compounds of interest[15]. Microbial biosynthesis also offers an unprecedented scalability to the production certain natural products that were unavailable for industrial exploitation. The recent commercialization of products made from biosynthetic spider silk is a striking example of it. Indeed, a unique scalable and cost effective fermentation process was developed using engineered yeast to produce modified spider silk proteins to manufacture textiles with particular properties[16]. Consequently, several companies are increasingly employing recombinant microbial biosynthesis to generate previously untapped proteins for the sustainable production of new textiles and materials.

The escalating progress in synthetic biology and metabolic engineering has the potential to enable the intelligent design of novel microbial cell factories to manufacture valuable industrial products from renewable materials. Because of the growing potential of these technologies to transform the commercial market, global industrial companies and emerging dedicated business ecosystems have launched or expanded R&D programs to accelerate biomanufacturing technologies[17]. Over the past decade, significant developments of the enabling technologies in synthetic biology (i.e. DNA synthesis and sequencing, automation, high-throughput analytics, bioinformatics), have broadened the range of enabled products (i.e. pharmaceuticals, chemicals, materials, biofuels) that can be commercially produced through microbial fermentation. Pioneering companies in the field of biomanufacturing include specialized companies such as Gingko Bioworks, Zymergen, Amyris and Evolva, as well as established global groups such as DuPont, Intrexon or Royal DSM. Overall, the synthetic biology market is estimated to reach $38.7 billion by 2020, with an annual growth rate of 46.4% between 2014 and 2020 (according to an Allied Market Research report).


7000 BC
Microbial fermentation to produce wine and beer
Extensive bread production using baker's yeast
4000 BC
17th century
Antonie van Leeuwenhoek visualizes and discovers microbial life
1861: Pasteur proves fermentation processes are linked to microbes
Pasteur and Koch establish the "germ theory of disease". First medical applications of the field of microbiology.
1876: Koch proves that bacteria can cause infections
19th century
1877: Traube proposes that proteins catalyze chemical reactions (early enzymology)
1894: first patent of a microbial enzyme product
In Germany, the beer industry is the 2nd biggest industrial sector
1910s
Commercial production of acetone, butanol and ethanol by microbial fermentation with Clostridium acetobutylicum
1920s
First citric acid fermentation plants open in the USA using Aspergillus niger
Use of Acetobacter suboxydans cultures to produce vitamin C
1930s
1927: Discovery of penicillin by Alexander Fleming
Industrial lactic acid fermentation with Lactobacillus bulgaricus
1940s
1943: Penicillin is used to treat wounded soldiers
Golden era of antibiotic discovery and rise of "wonder drugs"
Penicillin resistant strains emerge in hospitals
1950s
1953: molecular structure of DNA is established
1960s
Jacob and Monod establish molecular regulation of gene expression
1974: First patent for recombinant DNA technologies
1970s
Semi-synthetic penicillins and cephalosporins are best-selling antibacterials

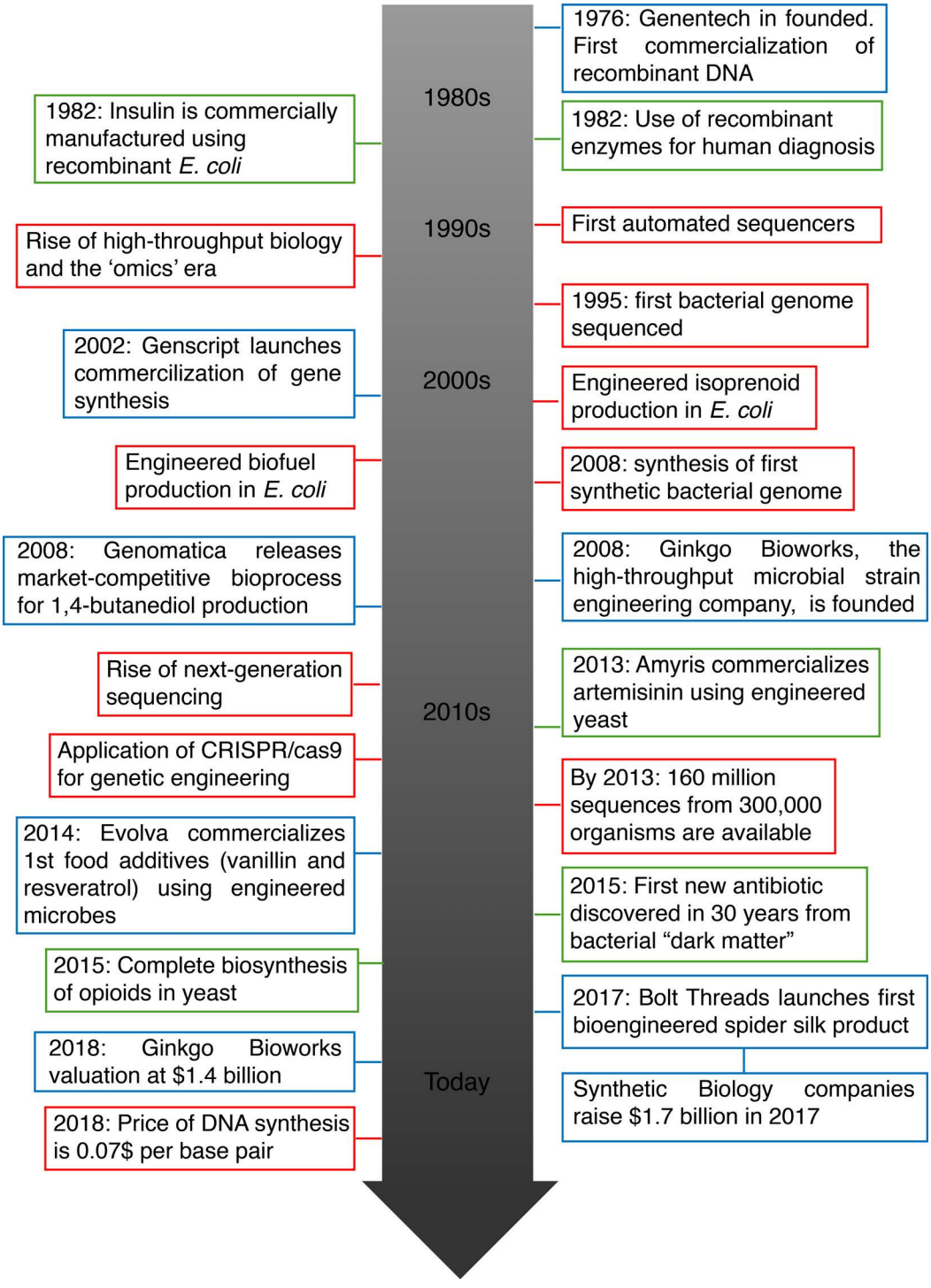


Figure 1.1 Timeline – A history of industrial microbiology. Key to coloured boxes: scientific or technical milestones (red); industrial milestones (blue); therapeutic applications (green). The information used to build this timeline was obtained from ref. [2] and www.synbiobeta.com

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1.2 THE PROMISE OF A BIOECONOMY ENABLED BY ENGINEERED MICROBES

Microbial cell factories developed by synthetic biology and metabolic engineering have the capacity to produce chemicals of social and economical value from renewable feedstocks[18]. Within the context of the increasing population, current environmental crisis and climate change awareness, engineered cell factories can be harnessed to develop sustainable processes for manufacturing a wide range of products and chemicals for human exploitation[19].

Microbial bioprocesses represent an attractive and sustainable alternative to current industrial production methods, which mainly rely on chemical synthesis or extraction from native producers. Advanced biomanufacturing technologies would not only provide sustainable economic benefits (by reducing the monetary cost of production of useful chemicals), but also offer social and environmental benefits. For instance, compared to chemical methods, these technologies would contribute to reducing the waste and use of toxic/hazardous intermediates, diminishing the generation of greenhouse gases and other polluting by-products, or reducing energy costs (i.e. use of milder conditions)[18]. Increasing the industrial use of microbial bioprocesses has the potential to enable an economy and energy sector independent of petroleum and other fossil fuels. Hence, the combination of these factors could greatly contribute to developing a sustainable society.

Recent examples of engineered microbial processes that transform cheap and renewable carbon sources into industrially relevant compounds[19] include: (i) commodity chemicals (such as solvents, detergents, polymers); (ii) fuels (i.e. ethanol, biodiesel) and energy (i.e. hydrogen gas); (iii) food and food additives (amino acids, flavors and vitamins); (iv) materials (i.e. biomimetic materials, textiles); (v) proteins; (vi) fine and specialty chemicals such as pharmaceuticals, fragrances, cosmetics, dyes or agrochemicals. Moreover, engineered microbes can be used to develop bioremediation strategies that degrade environmental pollutants, or even transform them into high-value chemicals. While most of these bioprocesses still remain laboratory-scale demonstrations, increasing academic and industrial research efforts are gradually translating them into industrial-scale processes[20]. Economically viable bioprocesses to produce useful chemicals have been successfully implemented by scaling-up fermentations using engineered microbial strains (through bioprocess engineering). Novel bioprocesses for the industrial production of a variety of products have marked the early success of metabolic engineering[13,17,21] (Table 1.1).

In addition to replacing current manufacturing processes, heterologous microbial biosynthesis likely offers the only viable platform to produce certain natural products at industrial scales. Indeed, many relevant compounds cannot be viably manufactured through chemical synthesis, due to their chemical

structure and/or complexity. In addition, native organisms often produce these compounds at undetectable/low levels, which are insufficient to develop viable extraction or fermentation methods[22,23]. Moreover, microbial production platforms can be used to generate new compound/protein variants with useful properties and functions[13], which can be used to produce novel products, such as pharmaceuticals, biofuels or materials.

Table 1.1 Examples of chemicals commercially manufactured with engineered microbes

Chemical	Microbial cell factory	Application	Company
1,5-pentanediamine	*Escherichia coli*	High polymers	Cathay Industrial Biotech
1,4-Butanediol	*Escherichia coli*	Solvent, building block for plastic and fibers	Genomatica
Polyhydroxyalkanoates	*Escherichia coli*	Polyesters	Metabolix
1,3-Propanediol	*Escherichia coli*	Building block for materials, cosmetics, food products	Dupont and Tate&Lyle joint venture
Artemisinic acid	*Saccharomyces cerevisiae*	Anti-malarial drug	Sanofi Aventis (process developed by Amyris)
Isobutanol	*Saccharomyces cerevisiae*	Advanced biofuel	Gevo, Butamax
Farnesene	*Saccharomyces cerevisiae*	Hydrocarbon building block	Amyris
Squalene	*Saccharomyces cerevisiae*	Cosmetics and pharmaceuticals	Amyris
Vanillin	*Saccharomyces cerevisiae*	Food additive	Evolva
Butanol	*Clostridium acetobutylicum*	Chemical building block	Green Biologics
7-ADCA	*Penicillium chrysogenum*	Antibiotics	DSM

For instance, heterologous microbial biosynthesis could greatly benefit the fight against antimicrobial resistance, which is considered one of the greatest public health challenges of the 21st century[24]. Indeed, the rising spread of antibiotic resistant infections increasingly threatens the efficiency and costs of current therapeutic options. This could eventually compromise the effective use of antibiotics in modern medicine. Thus, in addition to preserving the effectiveness of our current arsenal, we must urgently develop new antibiotics and deploy them rapidly to the clinic[25]. Recent technologies have uncovered new antibiotics that, if harnessed, might help alleviate this crisis[26]. However, most of these new antibiotic compounds are far too complex for

economical chemical synthesis, and are naturally produced by unculturable and/or genetically intractable microbes incompatible with industrial fermentation[23,27].Therefore these discoveries may not prove beneficial without sustainable processes to produce the antibiotics on an industrial scale. Developing new heterologous microbial platforms for antibiotic production may be an efficient solution for harnessing the clinical potential of these molecules and their commercialization[28,29].

The microbial production of isoprenoids is another striking example of the contribution of biomanufacturing to building a sustainable bioeconomy. Isoprenoids represent one of the largest families of natural compounds (over 50,000 molecules) with an incredible number of practical uses, and of great commercial value[30]: from high-value compounds such as many pharmaceuticals, fragrances and flavors, to commodity chemicals such as solvents, rubber or advanced biofuels. Traditionally, isoprenoids have been extracted from their natural producers, usually plants, which grow slowly and often accumulate useful compounds at very low concentrations[31]. Consequently, isoprenoid extraction often requires expensive and laborious methods, which have greatly limited their commercialization and industrial use. Metabolic engineering and synthetic biology have proven to be effective in generating microbial cell factories for the large-scale production of isoprenoids for a variety of sectors[31–33]. However, current bioprocesses remain in their infancy to fully enable an economically viable delivery of isoprenoids to the market.

In order to build and sustain the promise of a bioeconomy for the 21st century, metabolic engineering is under pressure to continue to provide large-scale, sustainable and cost-competitive bioprocesses that meet global needs. Here we focus on the development of microbial strains to accelerate the biological production of 2 different families of high-value compounds of prominent biotechnological relevance: antibiotics and isoprenoids. We will describe the current status and some of the main principles of metabolic engineering, which served as a framework for the work presented in this thesis. Then we will discuss prevailing obstacles to engineering microbial cell factories for industrial manufacturing, with an emphasis on the challenges tackled by our research.

1.3 PRINCIPLES OF METABOLIC ENGINEERING FOR THE MICROBIAL PRODUCTION OF VALUABLE COMPOUNDS

In order to engineer microbes to produce specific compounds of interest, it is necessary to understand the function of the molecular networks of the cell, their respective interactions, and how their modification can alter metabolic reactions (**Figure 1.2**).

All the hereditary genetic information that defines the structure and composition of any living organism is encoded in the universal macromolecule DNA (deoxyribonucleic acid)[34]. DNA can be used to produce other macromolecules that determine the phenotype and behavior of the cell: RNA (ribonucleic acid) and proteins (chains of amino acids). The 'central dogma of molecular biology' (Figure 1.3) establishes that genetic information flows into proteins via two processes: first a fragment of DNA is amplified and transcribed into an RNA molecule (transcription), which is subsequently translated into amino acid sequences that form one or several proteins (translation). Within organisms, proteins perform the majority of functions required for their survival and growth, including catalyzing chemical reactions, ensuring structural support, transporting molecules inside/outside the cell, or composing communication and signaling networks.

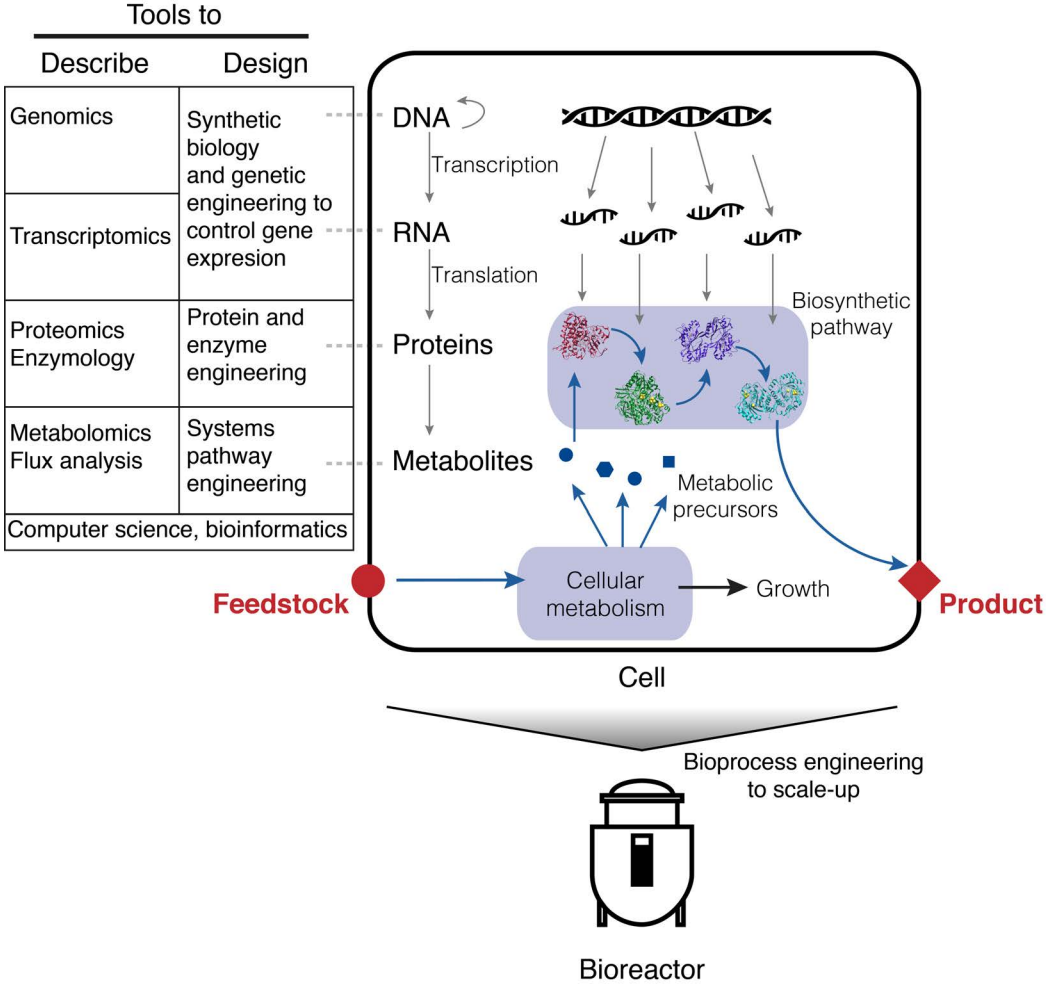

Figure 1.2 – Tools and principles for engineering biosynthetic pathways for the production of desired products

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Protein catalysts, referred to as enzymes, are responsible for most metabolic reactions carried out by living organisms. Metabolic pathways produce specific biochemical products from substrate compounds (known as metabolites) through series of linked biochemical reactions catalyzed by enzymes[34]. According to the flow of information in biological systems defined by the 'central dogma', it is assumed that most natural metabolic pathways are encoded and stored in the genome of the organisms that perform them.

Metabolic engineering optimizes the biological production of valuable chemicals by modifying, tuning and combining natural and/or *de novo* metabolic pathways. This often requires the coordinated manipulation of multiple molecular networks within the cell (DNA, RNA, proteins and/or metabolites)[10]. To tackle this complexity, the field employs a holistic and interdisciplinary approach to characterize the networks involved in the biosynthesis of molecules of interest, with the purpose of increasing their production[17]. Hence, metabolic engineering efforts often implicate the dynamic and precise control of complex biosynthetic pathways, by coordinating the expression of multiple genes and tuning the concentration of several metabolites.

1.3.1 TYPICAL WORKFLOW FOR ENGINEERING MICROBIAL CELL FACTORIES

Here we will detail the typical workflow used in metabolic engineering to generate microbial strains for the production of desired chemicals (**Figure 1.3**). In order to illustrate the basic principles of the field, we will briefly explain how they were directly applied to engineer the proof-of-principle strains presented in this thesis. In this section, we focus on our endeavor to engineer metabolic pathways for the production of relevant antibiotics within a microbial host.

Identification of a biosynthetic pathway for the production of a compound of interest

The first step in the process of engineering microbial cell factories for the production of a compound of interest commonly involves the identification of existing biochemical pathways that synthetize it. Indeed, one must search for enzymes that could be used to produce a specific chemical from given metabolic substrates within the cell. Over the past decades biochemical studies have allowed identifying and characterizing a tremendous amount of genes encoding biosynthetic pathways and enzymes from organisms across all kingdoms of life. Thanks to these studies, in combination with DNA sequencing technologies, many pathways that synthetize industrially relevant compounds have been identified[35], and can be found in online databases and scientific publications. Effective computational tools to search databases to obtain potential pathways for the production of specific chemicals have been developed to accelerate this process[13]. In addition, recent metagenomics techniques have the ability to uncover previously unknown pathways, including from unculturable organisms. The ever-growing power of DNA technologies

and bioinformatics are accelerating the discovery and annotation of natural biosynthetic pathways, hence expanding the genetic and biochemical repertoire that can be used for engineering cell factories[36,37].

Moreover, these mining efforts have been harnessed to engineer *de novo* biosynthetic routes to synthetize target compounds for which natural synthesis pathways have not been found. Diverse synthetic biology tools have been developed to (re)design chimeric pathways that combine enzymes from diverse organisms and natural routes[18]. Reverse-engineering approaches with computational tools can be employed to design *de novo* biosynthetic pathways by combining biocatalysts and regulatory components from different organisms[15,35]. For instance, metabolic engineering of artemisinin production in *E. coli* and yeast required the construction chimeric pathways composed of enzymes from different plants and microorganisms[35].

In the context of our work to implement heterologous antibiotic production, the biosynthetic clusters containing the enzymes involved in the synthesis of the desired compounds had already been identified in natural producers. While in some cases, the individual biochemical steps and enzyme mechanisms had not yet been fully characterized, these clusters served as the starting point for engineering heterologous production.

Selecting a suitable microbial chassis

Once a biosynthetic pathway (or several candidate pathways) has been selected for metabolic engineering, a crucial step in designing microbial cell factories is the selection of a suitable microbial 'chassis' strain. Indeed, the organism that will be used to implement a given biosynthetic pathway has to be compatible with the intended application. Some of the criteria to take into consideration when selecting a suitable production chassis involve[38,39]: (i) the functional compatibility with the components of the biosynthetic pathway (ii) the existence of genetic and molecular biology tools for their modification; (iii) extensive biological knowledge and available characterization tools; (iv) the native metabolic environment and molecular components; (v) growth conditions and nutritional requirements; (vi) tolerance to the desired product and/or pathway intermediates.

The choice of the microbial chassis will therefore determine most of the downstream bioprocess design considerations for optimizing production. Microbial chassis can be selected from a collection of established organisms in the field, such as *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae*, or emerging chassis organisms that include *Pichia pastoris*, as well as *Synechocystis, Geobacillus,* and *Streptomyces* species[38,40]. The advantages and disadvantages associated with a specific strain need to be carefully analyzed depending on the desired application. While *E. coli* is one of the most popular microbial chassis due to the high abundance engineering tools and its extensive characterization, alternative strains must be considered. For instance, if the bioprocess requires proteins glycosylation, *S. cerevisiae* might

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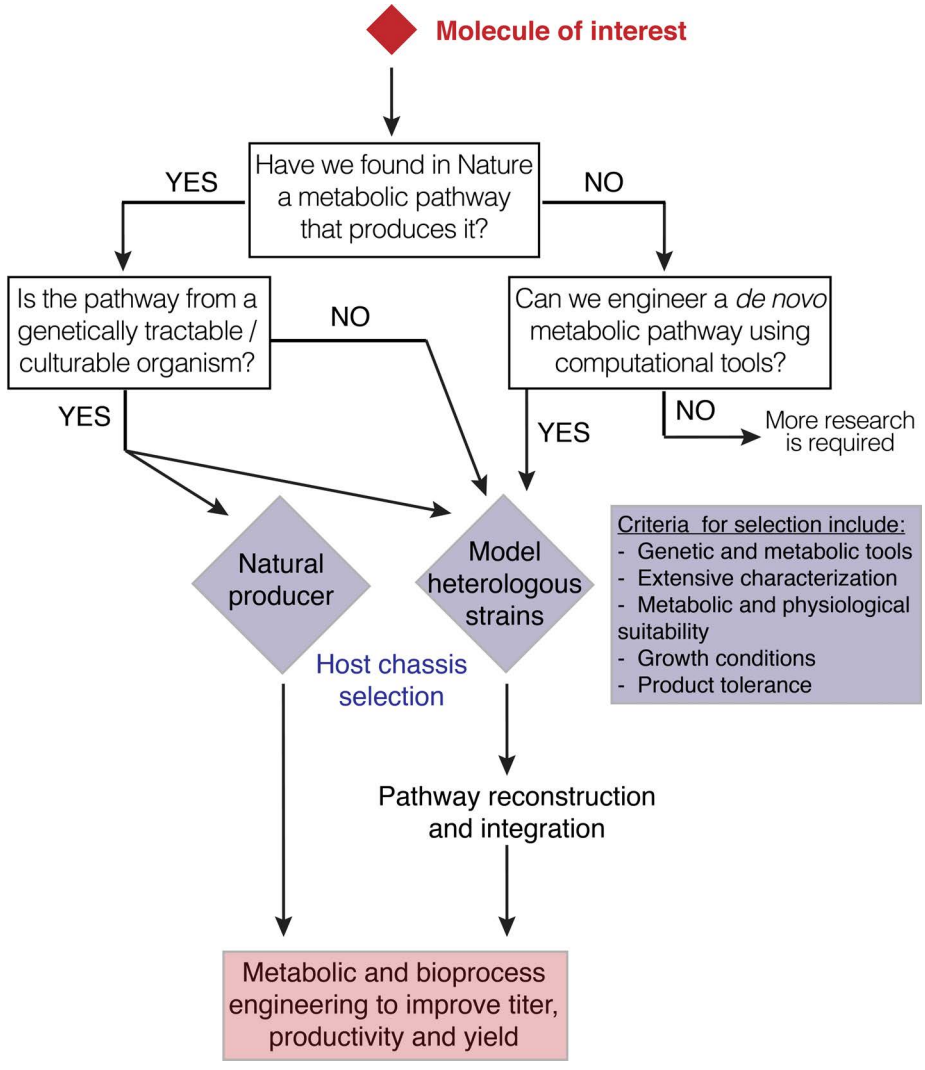

Figure 1.3 – Typical workflow for engineering microbial cell factories

be a preferred chassis; if high tolerance towards certain chemicals and solvents is necessary, *Pseudomonas putida* strains can be used instead. Consequently, considerable synthetic biology efforts have been focused on domesticating an increasing amount of organisms as suitable chassis for developing a wide range of bioprocesses[38].

In order to accelerate the deployment of novel antibiotics, we decided to transplant antibiotic producing pathways within *E. coli*. Indeed, with the choice of this chassis we aimed to capitalize on the genetic tractability, fast growing time, and long experience with *E. coli* as a production host. This would allow us to bypass the need for developing engineering tools for natural producers. Moreover, using *E. coli* as a host allows to rapidly prototype, test and optimize

modified biosynthesis pathways required for engineering large-scale production. Another advantage to the use of *E. coli* chassis involves decreasing bioprocessing costs by greatly reducing fermentation times (which can take up to several days with alternative hosts). Nevertheless, an universal obstacle to using a bacterial chassis for antibiotic production is rather obvious: the toxicity of the antibiotic products to the host[41]. Indeed, the production of potent antibiotics at high levels would likely result in in cell death and subsequently hinder production. Therefore, additional efforts to improve the tolerance of the chassis to the antibiotic product were implemented and described in this work.

Microbial strain construction

The tools of synthetic biology allow designing and constructing the genetic and regulatory programs required for implementing engineered biosynthetic pathways within a selected chassis. To this purpose, standardized functional blocks of DNA, known as 'Biobricks' (**Figure 1.4**), are typically assembled using a 'Lego-like' combinatorial approach[12] to design genetic circuits that encode desired predictable properties and behaviors. The community has focused much effort on improving the design, construction and characterization on biological parts, in order to advance the synthetic biology toolbox. Each part from this toolbox is characterized by a descriptive function, and conceptually abstracted from its complexity within natural biological systems[38]. For instance, 'promoter' regulatory parts are used and classified based on their features to control transcription rates; ribosome binding sites (RBS) with different strengths are used to control translation rates of downstream genes. The use of these parts to design biological pathways and circuits has greatly improved the predictability and reduced the context dependency of engineered biological systems. An increasing number of parts are available for use in established and emerging microbial chassis. In parallel, a number of tools have been developed to design multi-part transcriptional circuits and to predict their performance *in silico*[12].

A combination of recombinant DNA technologies, DNA synthesis and gene editing tools are subsequently used to construct the rationally designed genetic circuits. Cheap, fast and scalable methods to assemble specific pieces of DNA have been successfully implemented[38] (i.e. Gibson assembly, BglBrick, Golden Gate assembly, …). In addition, the dramatic drop in the price of DNA synthesis has greatly accelerated the construction of genetic circuits. Moreover, the emergence of gene editing tools, such as TALEN, MAGE and CRISPR/Cas9, has expanded our capacity to modify existing genomes. Altogether, these technologies have drastically advanced our ability to manipulate metabolism and optimize the production of desired chemicals within a given chassis. The type of manipulation that synthetic biology enable include[13,28]: (i) introducing/removing/modifying genes involved in specific pathways; (ii) timing and controlling enzyme expression; (iii) regulating metabolic flux in response to metabolites or environmental conditions; (iv)

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engineering microbial consortia; (v) engineering spatial organization of pathway components.

As mentioned previously, we selected *E. coli* as a chassis for engineering antibiotic production. We used widespread synthetic biology tools to construct our proof-of-principle strains, transcriptional circuits, and the antibiotic synthesis pathway. Some of the steps that were undertaken to construct high antibiotic producing strains included: (i) using online computational tools to codon-optimize the genes from a foreign organism for expression in *E. coli;* (ii) synthesizing codon-optimized genes using a commercial DNA synthesis service; (iii) choosing characterized inducible promoters to control pathway expression, notably due to the toxicity of our antibiotic product; (iv) assembling DNA parts in the laboratory using established protocols and introducing them into standardized plasmids developed by the synthetic biology community (BglBrick vectors[42]) for expression in *E. coli*; (v) using DNA recombination tools to modify specific enzymes within the genome; (vi) designing post-transcriptional circuits to tune the degradation rates of a specific enzyme.

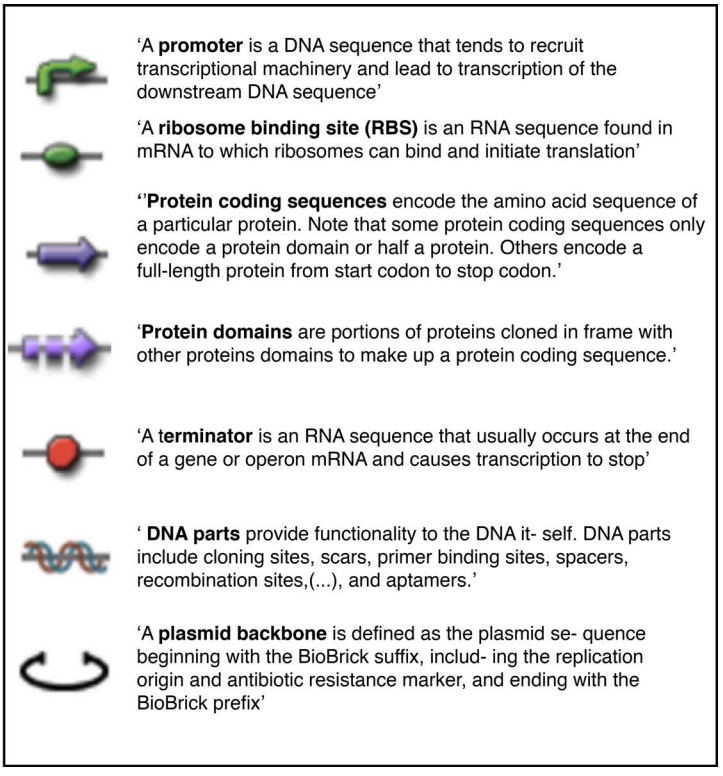

Figure 1.4 – Example of Biobricks™ with their respective schematic representation and definition found on the Registry of Biological Parts (http://parts.igem.org)

Testing the performance of constructed strains to optimize production

The performance of engineered microbial strains can be tested with the help of analytical tools that offer a global picture of cellular metabolism and cognate molecular networks. Transcriptomics, proteomics and metabolomics are therefore essential to metabolic engineering, as they offer a precise and systemic analysis of metabolic activities under specific physiological and environmental conditions. This information is critical for engineering and optimizing a given bioprocess. Indeed, the performance of engineered pathways remains unpredictable, regardless of the sophistication of the tools used to design them. Due to the context-dependency of biological parts and the intrinsic complexity of the host's metabolism, 'debugging' interventions are often required to increase production.

The characterization of metabolic fluxes, by combining '-omics' technologies with mathematical modeling, allows identifying critical components and parameters that determine pathway performance (by revealing pathway bottlenecks for example). Indeed, these metabolic flux profiles offer valuable insights regarding the optimal conditions and engineering approaches that could be implemented to increase product synthesis and improve yields[14]. Rational approaches to further optimize pathway performance include designing and/or tuning genetic circuits to engineer favorable metabolic states (i.e. balancing enzyme and metabolites levels), enhancing the properties and catalytic activities of pathway enzymes (via enzyme engineering or directed evolution), or improving peripheral pathways that alter production (such as cofactor regeneration or electron transfer pathways)[20].

In order to test the performance of our engineered antibiotic producing strains, we had to develop novel analytical methods to quantify pathway metabolites. We used these methods to compare the metabolic profiles of different strains and growth conditions, which allowed us to design additional approaches to increase production. For instance, we found that the availability of certain metabolic precursors was limiting, and therefore engineered strategies to increase their intracellular concentration. An available transcriptional biosensor tool developed by the synthetic biology community was used to estimate the levels of a precursor of interest and to test the effect of our genetic modifications. Subsequently, our analytical methods allowed us to identify a bottleneck enzyme in our engineered pathway, which could be a target for future protein engineering.

1.3.2 THE DESIGN-BUILD-TEST-LEARN CYCLE: IMPROVING THE PERFORMANCE OF ENGINEERED BIOSYSTEMS

The typical process for engineering microbial cell factories for the production of target molecules, which we described in the previous section, can be decomposed in interdependent modules. Like classical engineering disciplines, the field of metabolic engineering uses a Design-Build-Test-Learn (DBTL) cycle to decompose this process[18]. The steps required to translate laboratory-scale

fermentations performed with proof-of-principle strains into industrial-scale bioprocesses are realized through consecutive DBTL cycle iterations (**Figure 1.5**):

Design - This module involves the selection and/or rational design of all the suitable elements, across all scales of the process, to construct a bioprocess with the intended characteristics and applications. The selection of elements such as appropriate biosynthetic pathways, regulatory networks, enzymes (catalytic activities and biophysical properties), microbial chassis, and fermentation conditions are included in this module. Robust mathematical models and computational tools to engineer metabolism and its dynamic regulation are crucial for designing optimal genetic circuits. These tools are ‘‘open-ended’’ and constantly fueled by research and new knowledge about biological systems.

Build – Building microbial cell factories guided by the inputs from the Design module, involves the (re)construction and/or modification of selected biosynthetic pathways within the selected chassis. As previously described, this involves the use of synthetic biology tools to develop strains that contain engineered metabolic circuits.

Test – The performance of the engineered strains (constructed by Build) is tested to determine if the biological systems are appropriately carrying out the tailored functions defined by Design. This module analyses and integrates the data collected by measurement tools (such as transcriptomics, proteomics, and metabolomics) and modeling (systems biology, flux analysis) in order to identify elements that do not perform as initially expected.

Learn- Debugging, optimizing and scaling-up a bioprocess requires the analysis and integration of the collected data in order to improve the Design, Build and Test modules. Often, several rounds of the cycle are required in order to realize the desired performance of engineered microbial cell factories and develop highly-efficiency industrial bioprocesses. This module produces new knowledge that can be used to advance our understanding of biological systems and further accelerate the development of high-performing microbial cell factories.

Scientific and technological advances that support the predictability, throughput, speed, efficiency, scale, and cost of the tools required in each module of the Design-Build-Test-Learn cycle, continuously accelerate the prototyping of engineered microbes and improve our capacity to develop industrial biomanufacturing processes. For instance, synthetic biology tools and DNA synthesis have greatly accelerated the Build module by enabling the generation of large libraries of biological parts and strain variants. However, current analytical methods to evaluate their performance are often low-throughput, costly and/or restricted to a few proteins or metabolites[13]. Therefore, new high-throughput analytics and screening methods are required to perform routine analysis of such large part/strain libraries. Along with higher-

scale testing, more sophisticated data-analysis tools (i.e. machine learning) applied to metabolic engineering will be needed to integrate the increasing amounts of collected data.

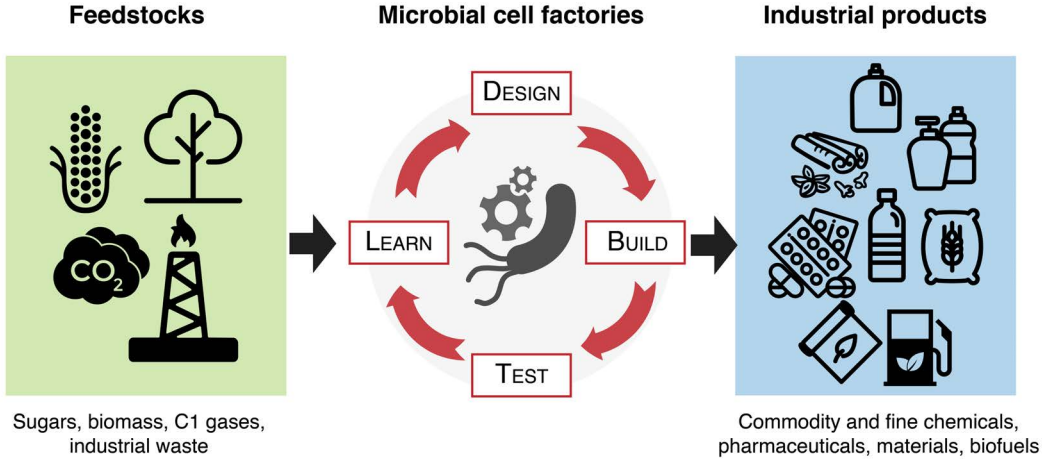

Figure 1.5 – The Design-Build-Test-Learn cycle of engineering microbial strains to produce industrial chemicals from renewable feedstocks

1.4 CURRENT CHALLENGES OF ENGINEERING NOVEL MICROBIAL CELL FACTORIES

Here we describe some challenges encountered in the field of metabolic engineering and synthetic biology, and which are addressed in this thesis. We focus in particular on relevant obstacles associated with the development of proof-of-principle strains for the laboratory-scale production of high-value chemicals.

1.4.1 MANY BIOSYNTHESIS PATHWAYS ARE NOT FULLY CHARACTERIZED

Advances in metagenomics, '-omics' analytical technologies, genetic engineering and biochemical assays have enabled the identification of a remarkable collection of biosynthetic gene clusters for the production of a wide-range of valuable compounds[43,44]. Many biosynthesis pathways encoded in these clusters could be harnessed to engineer microbial cell factories through synthetic biology and metabolic engineering. However, many complex pathways comprised of multiple enzymes have not been fully characterized. As a result, their specific enzymatic steps, pathway intermediates and conditions required to their optimal functioning (i.e. essential cofactors or reducing

partners) remain unknown. This concerns in particular pathways encoded in the genome of unculturable organisms and/or with poor genetic tractability.

The opportunities to optimize such pathways through metabolic engineering are limited, and further characterization studies are needed to fully exploit their potential. However, the complete elucidation of complex biosynthetic pathways can be a laborious and time-consuming task. This is notably the case for pathways that contain several multi-enzymatic steps, such as antibiotic synthesis pathways. For instance, while the fosfomycin biosynthetic gene cluster from *Streptomyces wedmorensis* was identified and sequenced during the 1990s[45], the synthesis pathway (comprised of six enzymes) was only entirely characterized in 2017[46]. Similarly, the complete characterization of the thienamycin biosynthesis pathway from *Streptomyces cattleya*, which involves at least 6 enzymatic steps, remains to be achieved[47].

Implementing new experimental and analytical assays to increase the throughput of pathway characterization would accelerate their application to the development of novel microbial cell factories[44]. Moreover, synthetic biology tools can be used to reconstruct relevant multi-gene pathways within heterologous hosts in order to uncover detailed mechanisms involved in product synthesis[48]. In this thesis, transferring an antibiotic pathway within *E. coli* allowed us to shed light on the potential function of two proteins encoded in the native biosynthetic cluster. We notably identified a reducing cofactor protein that boosted antibiotic biosynthesis upon overexpression.

1.4.2 MANY ENZYMES ARE INACTIVE WHEN EXPRESSED IN HETEROLOGOUS HOSTS

The context-dependency of biological parts has greatly limited the (re) construction of heterologous biological pathways within selected chassis[49]. In particular, many enzymes do not maintain activity when expressed within foreign hosts, hence frustrating the development of novel microbial cell factories. Moreover, the factors that determine the transferability of an enzyme across different microbial species are poorly understood. Therefore, the activity of a given enzyme within a foreign organism remains unpredictable. Consequently, finding enzymes that are functional within a selected chassis can be a time-consuming effort often based on blind trial-and-error. These difficulties can significantly increase with the complexity of the pathways of interest. At present, few synthetic biology tools to address this issue have been developed.

The activity of an enzyme can depend on multiple factors including its ability to fold, bind cofactors, and maintain catalytic activity within the cellular environment. Enzymes that are poorly transferrable often require post-translational modifications (PTMs) for their catalytic activation[50], which are mediated by a set of accessory proteins. Many enzymes involved in the synthesis of powerful antibiotics are known to require PTMs, and are notorious for displaying poor or no activity within common microbial chassis[47,51,52].

Therefore, in this thesis, we aimed to develop novel synthetic biology tools to understand and circumvent the barriers to the transferability of a specific family of enzymes involved in relevant antibiotic pathways. Our work demonstrates that inactive foreign enzymes can be recovered within *E. coli* by importing cognate PTM machineries from native organisms. The further development of modular tools for enzyme activation could untap the potential many relevant enzymes for metabolic engineering.

1.4.3 PRODUCT TOXICITY

The toxicity of the desired products or pathway intermediates to biological organisms hinders the production of certain chemicals via fermentation. Indeed, the high accumulation of these molecules inside the cell or the culture medium would result in poor metabolic performance, reduced cell viability or even cell death. As a result, developing bioprocesses to produce valuable compounds with toxic effects at titers compatible with industrial scales remains challenging. To address the issue of product toxicity, different biological engineering tools have been previously implemented including: (i) in situ product removal[53]; (ii) engineering the excretion/degradation/modification of the toxic product[54,55]; (iii) balancing gene expression to avoid accumulation of toxic intermediates[14]; (iv) protecting/removing cellular or metabolic targets within the host[54].

Toxicity has remained a notorious obstacle to the development of bioprocesses for the production of our compounds of interest: antibiotics[54], and certain isoprenoids[56] (i.e.monoterpenes). Because powerful antibiotics kill bacteria, the production of bactericidal and lysogenic antibiotic compounds within common bacterial hosts had not yet been demonstrated. In this thesis, novel strategies to overcome the toxic effect of potent antibiotics had been implemented to improve fermentation titers obtained with the *E. coli* chassis.

1.5 IN THIS THESIS

This thesis is an account of engineering efforts to design and develop novel microbial cell factories for the production of compounds of social and economic relevance. In particular our work is focused on engineering metabolic pathways for the production of powerful antibiotics and isoprenoid compounds, within the established microbial chassis *Escherichia coli*.

In **Chapter 2**, we explore how bacteria could be engineered to produce an antibiotic. We engineered *E. coli* to produce Car, a small antibiotic from the carbapenem family. Carbapenems are considered last-resort weapons against multi-drug resistant infections, and their production relies on costly chemical synthesis[57]. Metabolic engineering of the Car pathway in *E. coli* improved production rates of Car by 60-fold over our initial design. We further increase antibiotic productivity by engineering *E. coli* growth-arrested cells with improved

tolerance against carbapenem-induced lysis. The combination of our approaches surpasses reported titers obtained from natural carbapenem-producing strains. Our results demonstrate how *E. coli* can be harnessed as a heterologous platform for biosynthesis of bactericidal antibiotics.

Just as complex carbapenem pathways[47], many other industrially-relevant metabolic pathways contain biocatalysts that employ metal cofactors to perform remarkable biochemical reactions[32,51,58,59], referred to as metalloenzymes. The functional expression of metalloenzymes relies on post-translational modifications (PTMs) that often involve multi-protein maturation and/or electron transfer pathways. The dependence of metalloenzyme on PTMs often limits their functional expression and catalytic activity in heterologous organisms. This has greatly hindered their application to the development of novel microbial cell factories. **Chapter 3**, is a comprehensive literature review that highlights the biotechnological relevance of metalloenzymes in natural product biosynthesis, and discusses recent biological engineering strategies developed to enable/improve their activity in native and foreign bacterial hosts.

In **Chapter 4**, we describe our efforts to develop novel synthetic biology strategies to enable functional heterologous expression of a specific family of biosynthetic metalloenzymes: iron-sulfur (FeS) cluster enzymes. These enzymes hold a tremendous untapped potential for biomanufacturing. Indeed, many FeS enzymes are involved in the biosynthesis of a huge variety of valuable natural products, such as biofuels[60], fragrances and flavors[61], or pharmaceuticals (including carbapenems and other antibiotics)[47,62,63]. We demonstrate that inactive FeS enzymes can be recovered by co-expression of foreign FeS maturation and electron transfer proteins. We show that these proteins can act as efficient “plug adapters” to activate foreign FeS within *E. coli*. In addition, we explore the application of activated FeS enzymes for the biosynthesis of the biofuel precursor compound bisabolene, and the antibiotic fosfomycin.

Finally **Chapter 5**, provides concluding remarks regarding the work presented in this thesis and discusses perspectives on the future of industrial biomanufacturing technologies.

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2

METABOLIC ENGINEERING OF A CARBAPENEM ANTIBIOTIC BIOSYNTHESIS PATHWAY IN *ESCHERICHIA COLI*

Carbapenems, a family of β-lactam antibiotics, are among the most powerful bactericidal compounds in clinical use. However, as rational engineering of native carbapenem-producing microbes is not currently possible, the present carbapenem supply relies upon total chemical synthesis of artificial carbapenem derivatives. To enable access to the full diversity of natural carbapenems, we have engineered production of a simple carbapenem antibiotic within Escherichia coli. By increasing concentrations of precursor metabolites and identifying a reducing cofactor of a bottleneck enzyme, we improve productivity by 60-fold over the minimal pathway and surpass reported titers obtained from carbapenem-producing Streptomyces species. We stabilize E. coli metabolism against antibacterial effects of the carbapenem product by artificially inhibiting membrane synthesis, which further increases antibiotic productivity. As all naturally-occurring carbapenems known are derived from a common intermediate, our engineered strain provides a platform for biosynthesis of tailored carbapenem derivatives in a genetically-tractable and fast-growing species.

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2.1 INTRODUCTION

New antibiotics are critically needed in the fight against antimicrobial resistance. While metagenome sequences[1], difficult-to-culture organisms[2], and cryptic biosynthesis operons[3] have been successfully mined for novel antibiotics, such compounds are produced at titers far too low for large-scale production[2,4] or biochemical characterization[3]. Prior to the arrival of modern genetic engineering methods, the use of fermentation engineering and untargeted mutagenesis yielded strains capable of large-scale antibiotic production. Unfortunately these techniques do not guarantee higher titers[5–7] and cannot be applied to microbial species that cannot be cultivated[2]. Furthermore, improving production by rational metabolic engineering is intractably slow or not yet possible for most species[8]. Thus, without a platform for high-titer production, many antibiotics will remain beyond our reach and clinically irrelevant. Developing new platforms for antibiotic synthesis (i.e. heterologous hosts or novel production methods) is therefore as urgent as discovering new antibiotics and biosynthetic pathways.

The use of fast-growing, genetically-tractable species (such as *Escherichia coli*[8] and *Saccharomyces cerevisiae*[9]) as heterologous hosts for antibiotic biosynthesis could bridge discovery with production. The immense wealth of genetic tools available for such hosts enables rapid design and testing of engineered pathways. Improved pathways could be reintroduced into native antibiotic producers when possible[5], or large-scale production could be further developed using the heterologous host. Heterologous production is likely the only solution for harnessing biosynthesis pathways from uncultivable organisms such as sponge symbionts[10]. Heterologous expression could also enable the generation of novel antibiotic derivatives (via *in vivo* combinatorial biochemistry or enzyme engineering[11]). However, any attempt to engineer heterologous production, especially within susceptible bacterial species, must address the effects of the antibiotic on the host. This is especially true for bactericidal antibiotics, which cause cell death, disrupt metabolism, and arrest antibiotic synthesis. While bacteriostatic (growth-arresting) antibiotics such as erythromycin has been produced using *E. coli*[12], its suitability for production of small-molecule bactericidal antibiotics at high titers has not yet been systematically explored.

The carbapenems, a family of β-lactams, exemplify a class of bactericidal antibiotics that must be synthesized chemically due to difficulties improving production by natural microbial hosts. Carbapenems are relied upon as last-resort treatments against multi-drug resistant infections[13] and are particularly valuable due to their broad-spectrum activity and superior resistance against the vast majority of β-lactamase enzymes[14]. However, resistance to carbapenems is rapidly increasing due to the proliferation of genes encoding specific metallo-β-lactamases active against carbapenems[15]. Carbapenem resistance could be addressed using the more than 40 naturally

occurring known carbapenems if large-scale production of such compounds were possible. Unfortunately, carbapenem titers from microbial production are typically low (e.g., 1-4 mg/L thienamycin from wild-type *Streptomyces cattleya*)[16], and a lack of sophisticated genetic engineering tools has precluded extensive metabolic engineering of native hosts[8]. Engineering carbapenem production within heterologous hosts that are more genetically tractable could circumvent this obstacle and accelerate deployment of natural and novel carbapenems to the clinic.

Here we engineer heterologous biosynthesis of the carbapenem antibiotic (5*R*)-carbapen-2-em-3-carboxylic acid (known as Car) within *E. coli*. Despite our use of low-density shake-flask cultures, we surpass carbapenem titers reported using wild-type *Streptomyces* species cultivated in tank fermenters. As the biosynthesis pathways of all known carbapenems share early intermediates[17], our pathway provides a universal platform that can be further engineered for production of any carbapenem, including novel derivatives generated via *in vivo* combinatorial biochemistry. We additionally demonstrate how *E. coli* cells can be stabilized against carbapenem-induced lysis to further increase antibiotic productivity. Such approaches will enable the production of carbapenems from engineered microbes, and are likely applicable to heterologous biosynthesis of other bactericidal cell wall-targeting compounds.

2.2 RESULTS

2.2.1 HETEROLOGOUS PRODUCTION OF A CARBAPENEM ANTIBIOTIC

The antibiotic Car (**5**) is synthesized[18] from pyrroline 5-carboxylate (P5C, **1**), an intermediate of proline biosynthesis from glutamate (**Figure 2.1a**), and malonyl-CoA (**2**), a substrate of fatty acid synthesis. A previous study established the enzymes required for Car production by cloning the complete gene cluster from *Pectobacterium carotovorum* in *E. coli*[19]; however, titers of Car were not reported. We cloned the codon-optimized minimal Car pathway into a high-copy number plasmid (pCarCBA) which was transformed into *E. coli* BL21. Car production cultures were grown in MOPS-based minimal medium supplemented with glucose and glutamate.

We developed a novel LC/MS method to quantify Car pathway metabolites in culture supernatants. As the product **5** is relatively unstable (3 h estimated half-life[20]), we instead quantified hydrolyzed Car (hCar, **6**) as a measure of pathway productivity. An LC/MS signal from a compound matching the expected 171 *m/z* ratio of **6** was detected exclusively in cultures expressing the complete pathway (BL21 pCarCBA) under control of P_{lacUV5} (**Figure 2.1b**). Control cultures expressing incomplete Car pathways (BL21 pCarB and BL21 pCarAB) were used to identify LC/MS signals corresponding to the pathway

intermediates carboxymethylproline (CMP, **3**) and carbapenam (**4**) (173 *m/z* and 155 *m/z*, respectively). Fragmentation spectra of each compound further support our assignments **(Supplementary Figure S2.1).**

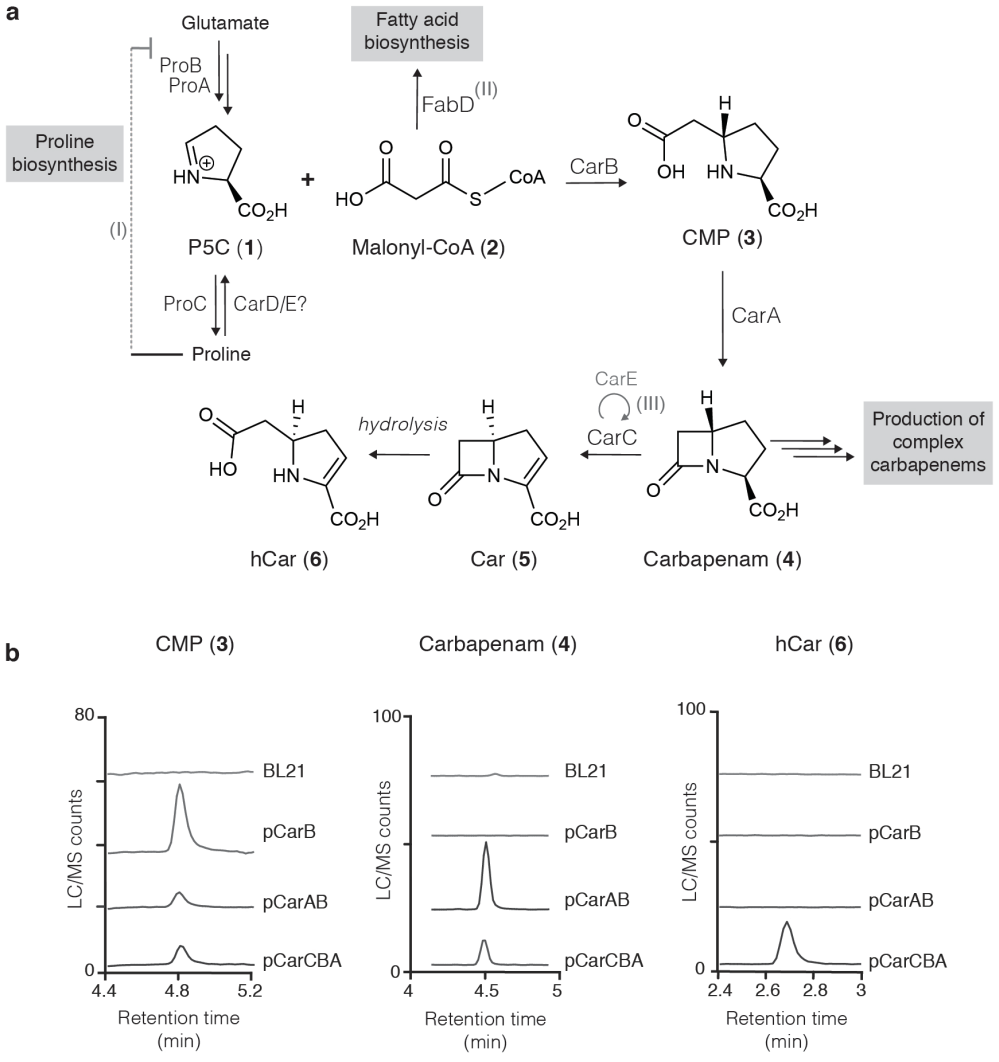


Figure 2.1 The Car biosynthesis pathway and LC/MS detection of synthetic intermediates and products. (a) The enzyme CarB joins P5C (**1**), an intermediate of proline synthesis, with the fatty acid precursor malonyl-CoA (**2**) to yield carboxymethylproline (CMP, **3**). CarA catalyses ATP-dependent cyclization to form the β-lactam ring, generating carbapenam (**4**), the precursor to all known naturally-occurring carbapenems. CarC catalyses two enzymatic steps: C5 epimerisation and C2-C3 desaturation to produce the bioactive carbapenem nucleus of Car (**5**). As with all carbapenems, the β-lactam ring of Car is susceptible to spontaneous hydrolysis, forming hCar (**6**). As indicated, the enzyme CarD and the ferredoxin CarE have been previously proposed to produce **1** from proline. Highlighted are the strategies employed to increase Car production. (**I**) increasing **1** by relieving allosteric inhibition by proline; (**II**) eliminating consumption of **2** by FabD; (**III**) regenerating CarC activity by expressing the ferredoxin CarE (our proposed mechanism). **(b)** Representative chromatograms of Car pathway metabolites **3**, **4** and byproduct **6** detected in cultures of *E. coli* pCarB, pCarAB, pCarCBA, and BL21. Fragmentation spectra are included in **Figure. S2.1**

2.2.2 METABOLIC ENGINEERING OF THE CAR PATHWAY

Expression of the *carDE* genes from the *P. carotovorum* Car gene cluster is known to substantially increase Car production[19,21]. CarD and CarE have sequence similarity with proline dehydrogenase from *Drosophila melanogaster* and [2Fe-2S] ferredoxins, respectively[19], leading to the hypothesis that CarD increases Car production by converting proline into the CarB substrate **1**, with CarE presumably acting as the reducing cofactor for CarD[17] (**Figure 2.1a**). We co-expressed both *carD* and *carE* with *carCBA* (BL21 pCarCBADE) and observed a 11-fold increase in Car productivity (defined as concentrations of **6** per unit biomass per unit time) (**Figure 2.2a**) compared to the minimal pathway pCarCBA. To further investigate the specific roles of CarD and CarE, we co-expressed each protein individually with CarCBA. While CarD did not significantly increase Car productivity when expressed without CarE, expression of CarE alone recovered the 11-fold productivity increase observed with CarDE expression (**Figure 2.2a**).

We next separated expression of *carE* from *carCBA* by cloning it into a separate operon controlled by an orthogonal promoter (P_{Tet}, strain BL21 pCarCBA_E), and titrated induction of CarE while maintaining constant induction of CarCBA. Car productivity correlates with increasing induction of CarE (**Fig. 2b**). The presence of the predicted [2Fe-2S] cluster of CarE was confirmed using whole-cell EPR spectroscopy[22,23]. An *in vivo* EPR spectrum obtained from *E. coli* BL21 overexpressing CarE displays a clear [2Fe-2S] cluster signal that is absent in whole-cell EPR spectra of wild-type *E. coli* BL21 (**Supplementary Figure S2.2a).** The observed *g*-values [2.04; 1.95; 1.89] closely correlate with the known [2Fe-2S] cluster signal of the spinach ferredoxin protein [2.05; 1.96; 1.89][24]. Mutations of cysteine residues (C43S and C46S) that form the FeS cluster suppressed the [2Fe-2S] signal (**Supplementary Figure S2.2b)** and eliminated the high-production phenotype (**Supplementary Figure S2.3a**), indicating that the FeS cluster is required to increase Car production. We next tested if CarE facilitates production of **1** by quantifying **3,** the product of CarB. Co-expression of *carD* with *carB* (strain BL21 pCarBD) increased production of **3** by approximately 70%*,* consistent with the hypothesized assignment of CarD as a proline dehydrogenase. However, the CarD-dependent increase in **3** does not require CarE, and co-expression of *carE* with *carB* (strain BL21 pCarBE) did not increase production of **3** compared to BL21 pCarB (**Supplementary Figure S2.3b**). Finally, a BLAST search[25] failed to detect a CarD homolog in the *E. coli* genome that may interact with CarE. We thus conclude that CarE does not influence any catalytic step upstream of CarA.

If CarE does not facilitate CarD activity, what could be the role of a ferredoxin in the Car pathway? CarE is unlikely to facilitate the synthesis of **4** from **3** by CarA, as this reaction has been characterized and is not known to require reducing equivalents.[26] Car production is severely limited by the activity of CarC, a redox-active Fe(II)- and 2-ketoglutarate-dependent enzyme, which

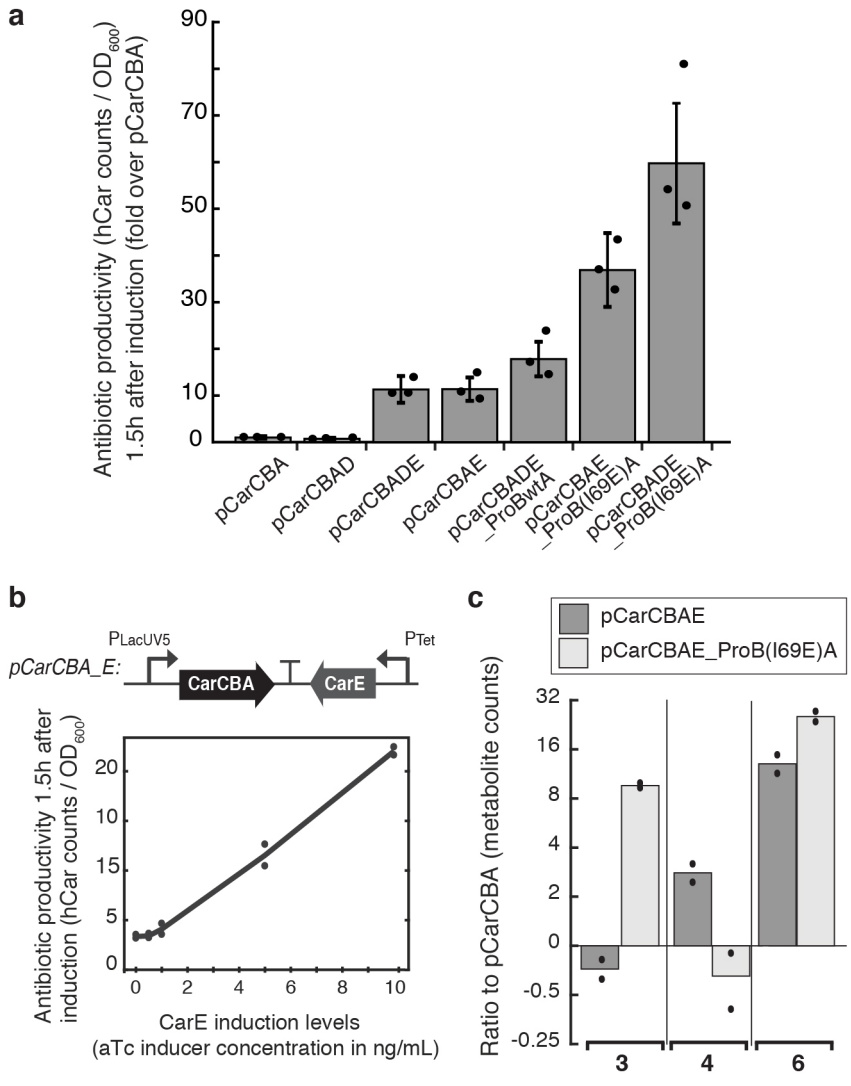


Figure 2.2 Metabolic engineering of the Car biosynthesis pathway in *E. coli*. (a) Successive metabolic engineering steps increased productivity by 60-fold over the minimal pathway (pCarCBA). Productivity is calculated from LC/MS counts of **6** in culture supernatants divided by the cell density (OD_{600}) 1.5 h after induction. Each bar represents the mean ± s.d. of three independent 5 mL cell cultures, grown separately from a same starting culture. **(b)** (Top) Schematic of plasmid pCarCBA_E encoding CarCBA and CarE controlled by orthogonal promoters P_{LacUV5} (IPTG-inducible) and P_{Tet} (aTc-inducible), respectively. (Bottom) Effect of varying concentrations of aTc (CarE induction) on **6** productivity (**6** counts/OD_{600}) recorded 1.5 h after induction. Solid line represents mean productivity of two biological replicates measured at each aTc concentration. **(c)** Mean ratios (normalized by LC/MS counts of BL21 pCarCBA) of pathway metabolites observed 2 h after induction of two 25 mL cultures. For all graphs, dots report values from individual replicates.

catalyzes both C5 stereoinversion and C2-C3 desaturation of **4** to yield **5**[27]. A

recent mechanism based upon extensive *in vitro* evidence proposes that stereoinversion by CarC is limited to a single turnover, as the reaction oxidizes the bound Fe(II) to Fe(III)[28]. Regeneration of Fe(II) by a reducing cofactor is thus required for subsequent C2-C3 desaturation and further stereoinversions. On the basis of our results, and the location of the *carE* gene within the native *car* operon, we propose that CarE is the primary reducing cofactor of CarC. CarE overexpression likely increases Car productivity by accelerating CarC reduction after stereoinversion. Further *in vitro* studies using purified CarC and CarE are needed to evaluate this proposal.

To further improve Car productivity, we sought to increase abundance of the CMP precursor **1**, an intermediate of the proline biosynthesis pathway. In *E. coli,* **1** is generated from glutamate by the enzymes ProB (glutamate 5-kinase) and ProA (glutamate 5-semialdehyde dehydrogenase). As with other amino acid biosynthesis pathways, proline inhibits its own synthesis via feedback inhibition of ProB[29](**Figure 2.1a**). Production of **3**, and ultimately carbapenems, may therefore be limited by low concentrations of **1**. We co-expressed the Car pathway with ProA and feedback-resistant mutants of ProB[29] (**Supplementary Figure S2.4)**. Expression of the mutant ProB I69E (BL21 pCarCBAE_ProB(I69E)A and BL21 pCarCBADE_ProB(I69E)A) further improved productivity of **6** by 3-fold (**Figure 2.2a**). In contrast, expression of wild-type (proline-inhibited) ProB and ProA resulted in a smaller productivity increase (**Figure 2.2a**) and did not increase titers of **3** (**Supplementary Figure S2.5**).

Co-expressing CarE and feedback-resistant ProBA improved productivity of **5** early after induction by nearly 60-fold compared to the minimal pathway (**Fig. 2a**). We quantified biosynthesis intermediates **3** and **4** to identify bottlenecks in our engineered pathway that may limit production of **5**. **Figure 2.2c** depicts concentrations of **3, 4** and **6** 2 h after induction in 25 mL cultures. As expected, expression of CarE appears to increase flux through the Car pathway by conversion of **4**, while expression of feedback-resistant ProB and ProA improves production of **3** by at least 13-fold over BL21 pCarCBAE, likely by increasing concentrations of precursor **1**. The accumulation of **3** in strains expressing feedback-resistant ProB(I69E) and ProA suggests that insufficient CarA activity acts as a bottleneck in our engineered pathway and identifies this enzyme as a target for future protein engineering.

2.2.3 IMPROVED ANTIBIOTIC PRODUCTIVITY TRIGGERS CELL LYSIS

Production of antibiotics in susceptible cells is expected to limit achievable titers by inhibiting biomass production (growth). In particular, production of β-lactam antibiotics, which target cell wall synthesis, should further limit titers by triggering lysis and arresting biosynthesis. OD_{600} measurements clearly indicate growth inhibition occurring very early (2 h) after induction of high-performing pathways (**Figure 2.3a**). Growth inhibition reduces maximum biomass achievable (as estimated by OD_{600}) by 9-fold compared to a control strain

(BL21 pCarAB). Car-producing cultures accumulate cell debris characteristic of cell lysis, which is also reflected by decreasing trends in OD_{600} apparent 3-5 h after induction (**Figure 2.3a**). Measurements of membrane permeability using propidium iodide (PI) confirm that permeability increases early after induction of the Car pathway and correlates with Car productivity, consistent with Car-induced lysis (**Figure 2.3b**). Measurements of cell viability, as determined from counts of colony forming units (CFU) 24 h after induction, confirm that cell death correlates with productivity (**Supplementary Figure S2.6)**.

Growth inhibition by **5** prevents the translation of productivity improvements into titer increases. Twenty five mL cultures of BL21 pCarCBAE exhibit 14-fold higher productivity than BL21 pCarCBA early after the pathway is induced, while reaching only 70% of the titer of **6** measured at 24 h. BL21

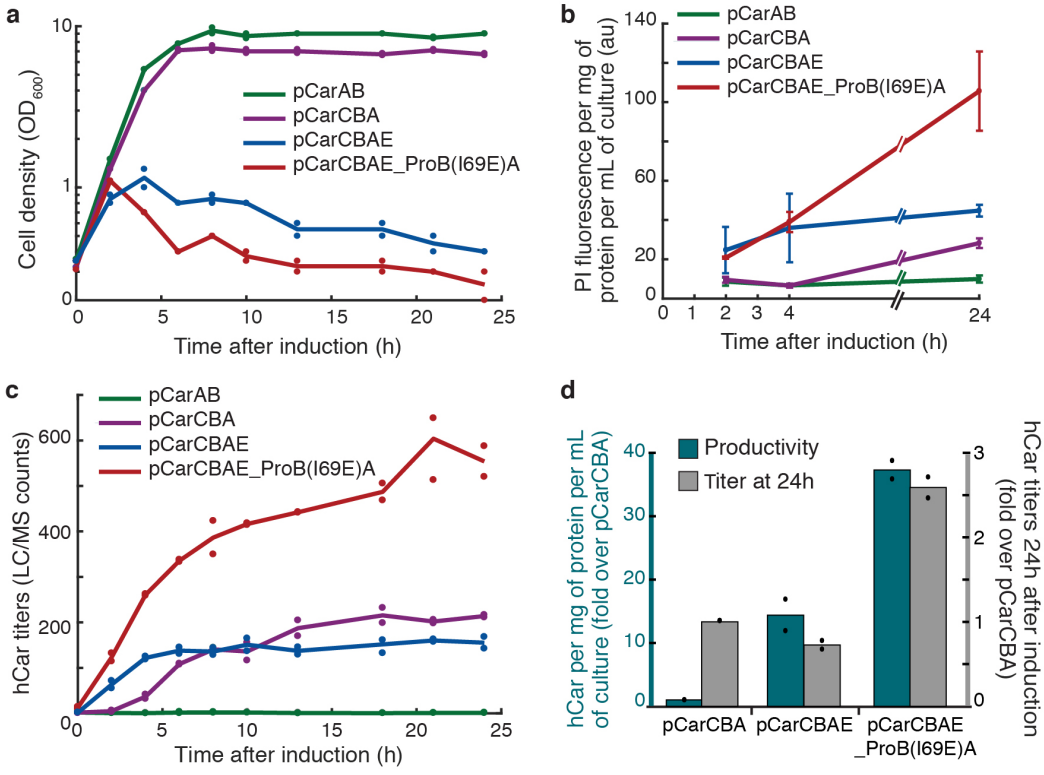


Figure 2.3 Car production causes lysis and limits achievable titers. (a) Growth curves of engineered strains after induction of Car pathway. **(b)** Cell permeability measurements in Car-producing cultures after induction. Each line represents the mean ± s.d. of three biological replicates. **(c)** LC/MS quantification of **6** after induction. Solid lines depict the mean of two biological replicates. **(d)** Comparison of productivity of **6** 2 h after induction with titers of **6** achieved at 24 h (normalized to values obtained for BL21 pCarCBA). Productivity of lysed cultures was estimated by titers of **6** per total protein extracted in culture samples collected 2 h after induction (green bars). Bars represent the mean of two biological replicates. For all graphs, dots report values from individual replicates.

pCarCBAE_ProB(I69E)A (37-fold higher productivity) improves 24 h titers of **6** by only 2.6-fold (**Figure 2.3c, 2.3d**). This is partly caused by the considerable reduction in biomass produced caused by growth inhibition and lysis by **5**. Production of **5** by cultures of BL21 pCarCBAE_ProB(I69E)A apparently continues during lysis, but at a decreasing rate, likely due to arrest of cell metabolism (**Figure 2.3a, 2.3c**).

2.2.4 INCREASED ANTIBIOTIC TOLERANCE IMPROVES CAR PRODUCTION

The toxicity of Car limits the achievable cell density of production cultures, which in turn severely limits antibiotic titers. We sought to engineer strains and fermentations to improve tolerance to **5** without compromising productivity. A straightforward approach to mitigate biomass limitation caused by Car toxicity is simply to delay expression of the Car pathway until late exponential growth, when a sufficient amount of biomass has been produced. Induction of BL21 pCarCBAE_ProB(I69E)A at a higher cell density (OD_{600} 1 rather than OD_{600} 0.4) increased both maximum biomass and **6** titer by nearly 2-fold (**Supplementary Figure S2.7a**). However, lysis was still observed in late-induced cultures as an OD_{600} decrease between 3 to 24 h (**Supplementary Figure S2.7b**).

In order to prevent lysis of Car-producing cells and extend Car production further, we explored natural mechanisms that confer tolerance to β-lactams. A phenotype known as persistence, in which cells are temporarily immune to antibiotic exposure, can be artificially induced by expression of growth-arresting toxin proteins[30]. Overexpression of the toxin HipA causes growth-arrest and confers β-lactam tolerance by triggering guanosine tetraphosphate synthesis (ppGpp)[31], which directly inhibits the phospholipid synthesis enzyme PlsB[32]. HipA-arrested cultures survive β-lactam exposure while remaining metabolically active, and are able to sustain production of the isoprenoid precursor mevalonate from a heterologous pathway for several days while resisting phage-induced lysis[31]. We tested whether growth arrest by HipA could also prevent Car-induced lysis. While HipA-arrested cultures sustained production of **5** and exhibited lower cell permeability (**Supplementary Figure S2.8a, S2.8d)**, higher titers were not achieved, likely due to decreased production of **3** (**Supplementary Figure S2.8c**).

We were encouraged by the improved tolerance of HipA-arrested cells against Car-induced lysis. β-lactam tolerance can also be achieved by direct inhibition of fatty acid synthesis (FAS), even without ppGpp accumulation[33]. Inhibition of FAS using the mycotoxin cerulenin, an inhibitor of FabF and FabB enzymes, starves phospholipid synthesis and confers β-lactam tolerance[33]. Furthermore, by inhibiting fatty acid elongation, cerulenin causes accumulation of **2**[34], a substrate of CarB. Inhibition of FAS by cerulenin could thus improve carbapenem production by *E. coli* via two mechanisms: by decreasing lysis, and by increasing availability of a precursor metabolite[35,36]. Treatment of Car-

producing cultures with 20 μg/mL cerulenin decreased cell permeability and Car-induced lysis (**Supplementary Figure S2.8a, S2.8b)**. Fluorescence readings from a genetic sensor[37] confirmed that cerulenin treatment increases **2** (**Supplementary Figure S2.9a**). Addition of cerulenin increases titers of **3** by nearly 5-fold (**Supplementary Figure S2.8c**). Although accumulation of **2** did not significantly improve **6** titers under these experimental conditions (**Supplementary Figure S2.8d**), observed increases in **3** confirmed that FAS inhibition improves flux into the Car pathway while alleviating Car-induced lysis. We thus decided to further optimize FAS inhibition to improve Car production.

As the cost of cerulenin makes its use in large-scale fermentations impractical, we sought a genetically-encoded trigger for FAS inhibition. We used a recently-developed synthetic protein degradation system to eliminate FAS enzymes which consume **2**[38]. The *mf*-Lon protease degrades proteins carrying a specific C-terminal tag (pdt). We appended a tag that confers a fast degradation rate (pdt#3)[38] to chromosomally-encoded fatty acid synthesis enzymes (FabB, FabF, and FabD), and induced expression of the *mf*-Lon protease. Similar to cerulenin treatment, induction of the *mf-Lon* arrested growth while leading to accumulation of **2** in BL21 *fabD*-pdt#3 (**Figure 2.4a, Supplementary Figure S2.9**) and increased **3** production in BL21 *fabD*-pdt#3 pCarCBAE_ProB(I69E)A (**Supplementary Figure S2.10**). Induction of FabD degradation reduced both cell lysis and membrane permeability of Car-producing cells, indicating that FAS inhibition via FabD degradation decreases Car toxicity (**Figure 2.4a, 2.4b**). Furthermore, FabD degradation increased Car production (normalized to culture volume) by 50% (**Figure 2.4c)**. Inhibition of FAS by FabD degradation also decreases apparent lysis caused by the complex carbapenem antibiotic imipenem (**Supplementary Figure S2.11)**.

To confirm that improved tolerance can directly lead to improved Car titers, we tested the stability of Car production in FAS-inhibited cells by supplementing cultures with fresh medium 1.5 h after induction. We hypothesized that addition of fresh medium would prolong productivity of metabolically-active cultures, either by restoring depleted nutrients or by diluting inhibitory waste products. No additional **5** was produced in control cultures after medium supplementation, suggesting that the cultures had been metabolically inactivated by lysis. However, biosynthesis activity continued in FAS-inhibited cultures, and total amount of **6** increased by 40% (**Figure 2.4c)**. Overall, Car production in FAS-arrested cultures supplemented with fresh medium improved by 2-fold compared to control cultures. We attribute this increase to a combination of antibiotic tolerance and increased concentrations of **2** available for synthesis of **3** by CarB.

2.2.5 ESTIMATING CAR TITERS TO BENCHMARK STRAIN PERFORMANCE

As authentic chemical standards for **5** and **6** are not available, estimating Car titers via LC/MS alone is not possible. Therefore, we used differential UV spectroscopy, a technique used to quantify carbapenems in both crude

fermentation broths and purified extracts[16,39,40]. Carbapenems exhibit UV absorption that is lost upon aminolysis of the chromophore by hydroxylamine[41]. Thus, carbapenem concentrations can be calculated by measuring the UV absorbance eliminated after hydroxylamine treatment. We confirmed the reliability of differential spectroscopy using an imipenem standard added to lysed *E. coli* cultures (**Supplementary Figure S2.12, Supplementary Table 2.1a**). Next, we used differential spectroscopy to quantify **5** in cultures of BL21 pCarBAE_ProB(I69E)A 3 h after induction, when we estimate that most **5** has not yet been hydrolysed. Using the extinction coefficient previously determined for Car[42], we calculate a titer of **5** (17.0 ± 5.1 mg/L) **(Supplementary Figure S2.13, Supplementary Table 2.1a)**.

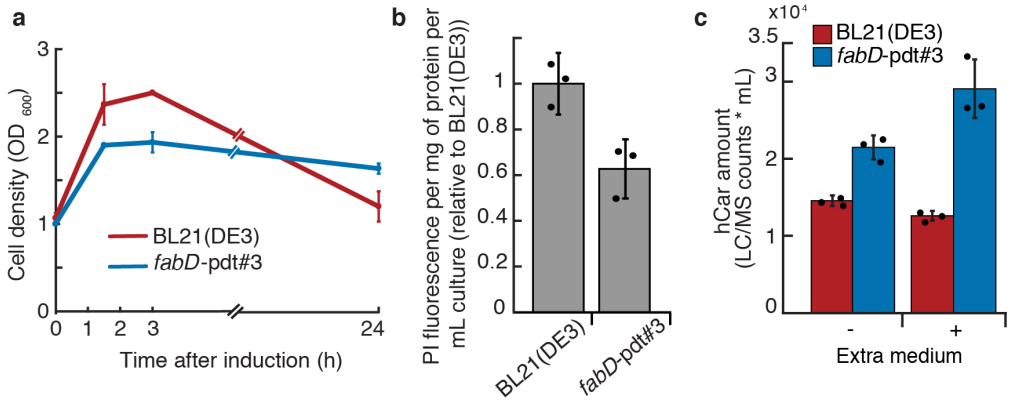


Figure 2-4 Inhibition of fatty acid synthesis increases antibiotic tolerance and Car pathway flux. (a) Growth curves of BL21(DE3) pCarCBAE_ProB(I69E)A after induction indicating lysis (45% OD_{600} decrease) in BL21(DE3), which is reduced in *fabD*-pdt#3 p*mf*-Lon strain (19% OD_{600} decrease). Protease-driven depletion of FabD in the strain *fabD*-pdt#3 p*mf*-Lon was simultaneously induced with Car production. **(b)** Cell permeability measurements 24 h after induction indicating decreased lysis in *fabD*-pdt#3 strains. **(c)** Total Car produced (**6** titer multiplied by culture volume) 24 h after induction. Cultures supplemented with 25 mL fresh production medium 1.5 h after induction are indicated with a +. Solid lines and bars represent the mean ± s.d. of three biological replicates. For all graphs, dots report values from individual replicates.

We sought to determine the total amount of **5** produced over a 24 h fermentation to enable comparisons with native carbapenem-producing species. However, the near-total hydrolysis of **5** after 24 h preludes the direct use of differential spectroscopy (**Supplementary Figure S2.13).** Therefore, we estimated total **5** produced over 24 h by multiplying the 3 h titer of **5** with the ratio of LCMS counts of **6** at 3 and 24 h after induction. In the absence of FAS arrest, BL21(DE3) pCarBAE_ProB(I69E)A produced 37.3 ± 16.7 mg/L of **5,** while FAS-arrested cells produced 54.1 ± 17.4 mg/L (detailed calculations in **Supplementary Table 2.1b**). These titers demonstrate a substantial improvement over carbapenem titers achieved by *S. cattleya*.

2.3 DISCUSSION

Our results demonstrate how *E. coli* can be harnessed as a heterologous platform for biosynthesis of bactericidal antibiotics. The genetic tractability and fast growth of *E. coli* enabled rational engineering and short fermentation times: in less than 24 h and despite low cell density, titers of **6** reached 54.1 ± 17.4 mg/L, whereas tank fermentation cultures of wild-type *S. cattleya* require at least 5 days to reach a final thienamycin titer of 4 mg/L[16]. While **5** is too unstable to be clinically useful, our pathway modifications, which increase flux to the universal carbapenem precursors **3** and **4,** can be used to improve production of more stable carbapenem antibiotics, either in *E. coli* or a carbapenem-producing *Streptomyces* host. The co-expression of carbapenem tailoring enzymes, either obtained from the variety of known carbapenem pathways or obtained via modification of existing enzymes[11], could generate novel derivatives and further expand carbapenem diversity[18].

Increasing the concentrations of carbapenem precursors **1** and **2** improved productivity, indicating that the activity of CarB, and thus flux through the carbapenem pathway, is limited by precursor availability. Increasing precursor concentrations has been repeatedly shown to improve biosynthesis of other antibiotic families[43,44], consistent with findings that fluxes through microbial biosynthesis pathways are often limited by precursor metabolites[45]. We have also identified a likely reducing partner for the enzyme CarC, an unusual enzyme with dual activities that is notorious for its low catalytic rates. Overexpression of CarE increased Car productivity by 11-fold, illustrating the importance of identifying and overexpressing reducing partners for redox enzymes in biosynthetic pathways.

The identification of additional reducing partners will likely prove necessary for heterologous expression of complex carbapenem pathways, which include several redox-active iron-sulfur (FeS) enzymes[46]. The requirement of FeS enzymes for complex carbapenem biosynthesis presents a barrier to heterologous carbapenem production in yeast, which are otherwise well-suited for producing β-lactam antibiotics due to their natural tolerance[9]. Engineering functional expression of bacterial FeS enzymes in eukaryotes has proved challenging due to apparent incompatibilities of bacterial FeS enzymes with eukaryotic FeS assembly pathways[47]. Until FeS enzymes can be reliably transferred between prokaryotes and eukaryotes, bacterial species may be more amenable hosts for heterologous carbapenem production.

Our approach for mitigating Car toxicity provides a strategy for production of growth-inhibiting compounds within susceptible hosts. Delaying induction of Car biosynthesis to allow biomass accumulation improved Car titer. Timed arrest of FAS synthesis, which confers tolerance against β-lactam antibiotics, prolonged Car production and improved titers further. As FAS arrest protects cells against β-lactam antibiotics, including complex carbapenems such as imipenem[48], this approach will likely prove effective for production of

clinically-relevant carbapenems. The use of fed-batch cultivation to reach high cell densities (i.e. 20-fold over densities achieved here) together with timed FAS arrest and continued nutrient feeding may bring carbapenem titers close to industrially-relevant levels. The use of self-inducing promoters[49] to trigger antibiotic production and FAS arrest would remove dependence of the present system on chemical inducers, which are not economical at large scales. Other methods for improving tolerance, such as mutating the cellular target of carbapenem, or expressing efflux channels, could further translate productivity improvements into increased titers. Combined with our engineered carbapenem pathway, such approaches could fully capitalize on the strengths of *E. coli* as a production host and bring natural and novel carbapenems closer to clinical use.

2.4 MATERIALS AND METHODS

2.4.1 STRAINS AND GROWTH MEDIUM.

E. coli BL21(DE3) was used for Car production experiments. We used cells freshly-transformed with plasmids encoding the Car pathway. Transformants were grown overnight on selective LB agar plates at 37°C, after which plates were stored at 4°C. Liquid cultures inoculated from colonies bearing the Car pathway showed poor growth if the colonies were kept longer than 48 h. Car production cultures were grown in MOPS-based minimal medium (8.372 g L^{-1} MOPS, 0.717 g L^{-1} Tricine, 2.92 g L^{-1} NaCl, 11 mg L^{-1} $MgCl_2 \cdot 7H_2O$, 0.56 μg L^{-1} $CaCl_2$, 200 μL micronutrient stock[50]) supplemented with 4 g L^{-1} D-glucose, 28.5 mM NH_4Cl, 10 μM $FeSO_4$ and 5 g L^{-1} potassium glutamate. The antibiotics kanamycin (25 μg/mL), ampicillin (50 μg/mL) and chloramphenicol (17.5 μg/mL) were added when appropriate. All strains and plasmids used in this work are listed in **Supplementary Table 2.2**.

2.4.2 PLASMID CONSTRUCTION.

All plasmids were constructed using Gibson assembly[51] in *E. coli* DH5α. Genes encoding the Car enzymes from *P. carotovorum* (CarABCDE) were codon-optimized for expression in *E. coli*. *proB* and *proA* were cloned from *E. coli* BL21(DE3) genomic DNA (primers in **Supplementary Table 2.3)**. The mutant variants I69E of ProB, and C43S/C46S of CarE were constructed by PCR site-directed mutagenesis using the primers P1 and P2 in **Supplementary Table 2.3.** Biosynthetic operons were assembled and cloned into the pBbE5k BglBrick backbone[52]. The plasmid pCarCBA_E was constructed from pCarCBA, by addition of the CarE expression unit under control of the inducible P_{Tet} promoter. The reverse sequence of the CarE inducible expression unit was placed at the 3' end of the bi-directional terminator present on the pBbE5k BlgBrick backbone (**Fig. 2.2b**). *hipA* was cloned from *E. coli* MG1655 genomic DNA and inserted into the pBbS2c BglBrick backbone to make pHipA (primers

in **Supplementary Table 2.3)**. To construct the *mf*-Lon protease expression vector, we introduced the codon optimised *mf-Lon* gene and strong ribosome binding site from pECL275[38] into the pBbA2c[52] BglBrick backbone. The resulting plasmid p*mf*-Lon contains a chloramphenicol resistance cassette. The derivative plasmid p*mf*-Lon-bis contains an ampicillin resistance cassette instead. All the strains and plasmids constructed in this work are listed in **Supplementary Table 2.2.**

2.4.3 CAR PRODUCTION PROTOCOL.

Individual colonies of engineered strains were picked into 5 mL selective production medium and incubated overnight with shaking at 37°C. From overnight cultures, culture triplicates were seeded at an initial OD_{600} of 0.06 either in 5 mL of selective production medium in culture tubes (small-scale assays), or in 25 mL medium in 250 mL Erlenmeyer flasks (shake-flask assays). Cultures were incubated at 37 °C with shaking (250 r.p.m). Car production was induced at OD_{600} 0.3-0.45 by addition isopropyl β-D-thiogalactopyranoside (IPTG) to 0.25 mM final concentration. Samples taken from production cultures were cleared by centrifugation and supernatant aliquots were collected and stored at –80 °C for LC/MS.

2.4.4 CarE TITRATION EXPERIMENT.

As the enzyme CarC is iron-dependent, we used an iron-deficient MOPS-based production medium (without $FeSO_4$ supplementation) to limit Car production from leaky expression of the Car pathway. Individual colonies of BL21(DE3) pCarCBA_E were grown overnight in 5 mL of iron-deficient production medium. Overnight cultures were diluted into 250 mL Erlenmeyer flasks containing 25 mL selective iron-deficient production medium to an initial OD_{600} of 0.06. Cultures were grown at 37 °C with shaking (250 rpm) until reaching an OD_{600} of 0.25. At this point, CarE expression was induced by addition of anhydrotetracycline (aTc). The cultures were further incubated for 2 h, after which CarCBA expression was induced with 0.25 mM IPTG and the medium supplemented with 10 μM $FeSO_4$. Cultures were then incubated at 37 °C with shaking and samples were collected 1.5 h after induction. Supernatant aliquots were collected and stored at –80 °C for LC/MS.

2.4.5 WHOLE-CELL EPR SPECTROSCOPY.

25 mL cultures used for EPR spectroscopy were incubated for 2-3 h after induction at 0.3 OD_{600}, and cell pellets recovered by centrifugation. Pellets were stored at –80 °C until analysis. Samples for EPR spectroscopy were prepared by resuspending the pellet in 200 μL sterile 50 mM Tris buffer (pH 8). The suspension was transferred into quartz glass EPR tubes and frozen in liquid nitrogen. X-band EPR measurements were performed using a Bruker ECS-106 EPR spectrometer with a National Instruments interface. Data acquisition was

performed using an in-house developed software written in LabVIEW and FORTRAN 90/95. EPR conditions were: microwave frequency, 9.388 GHz; microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1.27 mT; temperature, 27K. EPR spectra were analysed using programs previously described[53].

2.4.6 PROPIDIUM IODIDE (PI) FLUORESCENCE MEASUREMENTS.

A stock solution of 10 mM propidium iodide in DMSO was prepared and stored at –20 °C. 100 μL of culture sample was mixed with an equal amount of a 2X PI working solution (prepared by adding 3 μL of stock solution to 1 mL of water). Mixtures were incubated in transparent 96-well plates at room temperature in the dark. After 15 min incubation, PI fluorescence was measured using a BioTek Synergy HTX microplate reader, equipped with an excitation filter of 485/20 nm and an emission filter of 620/15nm with a gain setting of 40.

2.4.7 TOTAL PROTEIN QUANTIFICATION IN PRODUCTION CULTURES.

Due to Car-induced cell lysis, optical density measurements >2 h after induction no longer accurately measure total biomass accumulation. We therefore estimated biomass accumulation by measuring the total amount of protein present per volume of culture by chloroform-methanol protein precipitation. To 100 μL of culture were added 400 μL of methanol, 100 μL of chloroform and 300 μL of water. Mixtures were vortexed vigorously and centrifuged at 10,000 x g for 1 min. The upper MeOH/water layer was carefully removed without disturbing the interface, after which 300 μL of methanol were added to the remaining phase. Mixtures were then vortexed vigorously and centrifuged at 10,000 x g for 1 min. The supernatant was discarded and obtained pellets were dried by vacuum centrifuge. Protein pellets were resuspended in 10-100 μL binding buffer (6M urea, pH 7.2). The colorimetric Bradford assay[54] was used to measure protein concentrations.

2.4.8 CONSTRUCTION OF STRAINS WITH TUNABLE PROTEIN DEGRADATION.

The degradation tag pdt#3 was fused to the C terminus of the genes of interest in *E. coli* BL21(DE3) chromosomal DNA[38]. For each targeted gene (*fabB*, *fabD* and *fabF*), we generated PCR products that contained the pdt#3 tag amplified from pECT3 and 37-42 bp 5' extensions with homology to the C terminus, and 3' extensions with homology to the immediate 3' untranslated region of the gene of interest. The P1 and P2 primer sequences and full-length primers used to target *fabB*, *fabD* and *fabF* are found in **Supplementary Table 2.3**. Genomic pdt#3 insertions were performed using homologous recombination[55] by transforming the PCR products into *E. coli* BL21(DE3) containing pKD46. Successful insertions were verified by PCR. The kanamycin resistance cassette was subsequently removed using the plasmid pCP20. The resulting strains,

fabB-pdt#3, *fabD-pdt#3* and *fabF-pdt#3* were screened by PCR and verified by DNA sequencing.

2.4.9 RELATIVE MALONYL-COA QUANTIFICATION.

We used the transcription factor-based biosensor plasmid pCFR[37], which expresses red fluorescent protein (RFP) in response to intracellular malonyl-CoA. Strains transformed with pCFR were grown to OD_{600} 0.1, and 100 μL culture aliquots were placed on transparent 96-well plates in triplicate. RFP and OD_{600} were monitored using a BioTek Synergy HTX microplate reader, equipped with an excitation filter of 485/20 nm and an emission filter of 620/15nm with a gain setting of 40.

2.4.10 LCMS ANALYSIS.

Metabolite levels in culture supernatants were measured using LC/MS. As **5** is known to rapidly hydrolyse in acid (pH <3)[56], all samples were acidified before analysis to hydrolyse any remaining **5** into **6**. For each collected sample, 5 μL of supernatant was added to 195 μL ACN with 0.1% formic acid. The mixtures were vortexed, centrifuged at 15,000 x g for 2 min and incubated for 1 h prior analysis. 2 μL of the sample was injected onto an Agilent ZORBAX HILIC Plus column (100-mm length, 2.1-mm internal diameter, 3.5 μm particle size). Liquid chromatography separation was conducted at 30 °C using a LC/MS system (Agilent) consisting of a binary pump (G1312B), an autosampler (G7167A), a temperature-controlled column compartment (G1316A), and a triple quadrupole mass spectrometer (G6460C) equipped with a standard ESI source. The mobile phase was composed of 25 mM ammonium formate (solvent A) and 100% acetonitrile (solvent B). Samples were separated with a gradient from 95% to 60% of solvent B for 5 minutes at a flow rate of 0.5 mL/min, followed by a gradient from 60% to 50% for 2 minutes at a flow rate from 0.5 mL/min to 0.6 mL/min, 50% to 95% solvent B for 2 minutes at 0.7 mL/min, followed by a hold at 95% solvent B for 2 min at a flow of 0.5 mL/min. Peaks were analysed by mass spectrometry using ESI ionization in MRM mode. The precursor ions analysed for each compound (**3**, **4** and **6**) was determined by mass calculation based on their chemical formula. For each compound, fragmentation spectra and MRM settings are found in **Supplementary Table 2.4.**

2.4.11 ESTIMATING CAR TITERS.

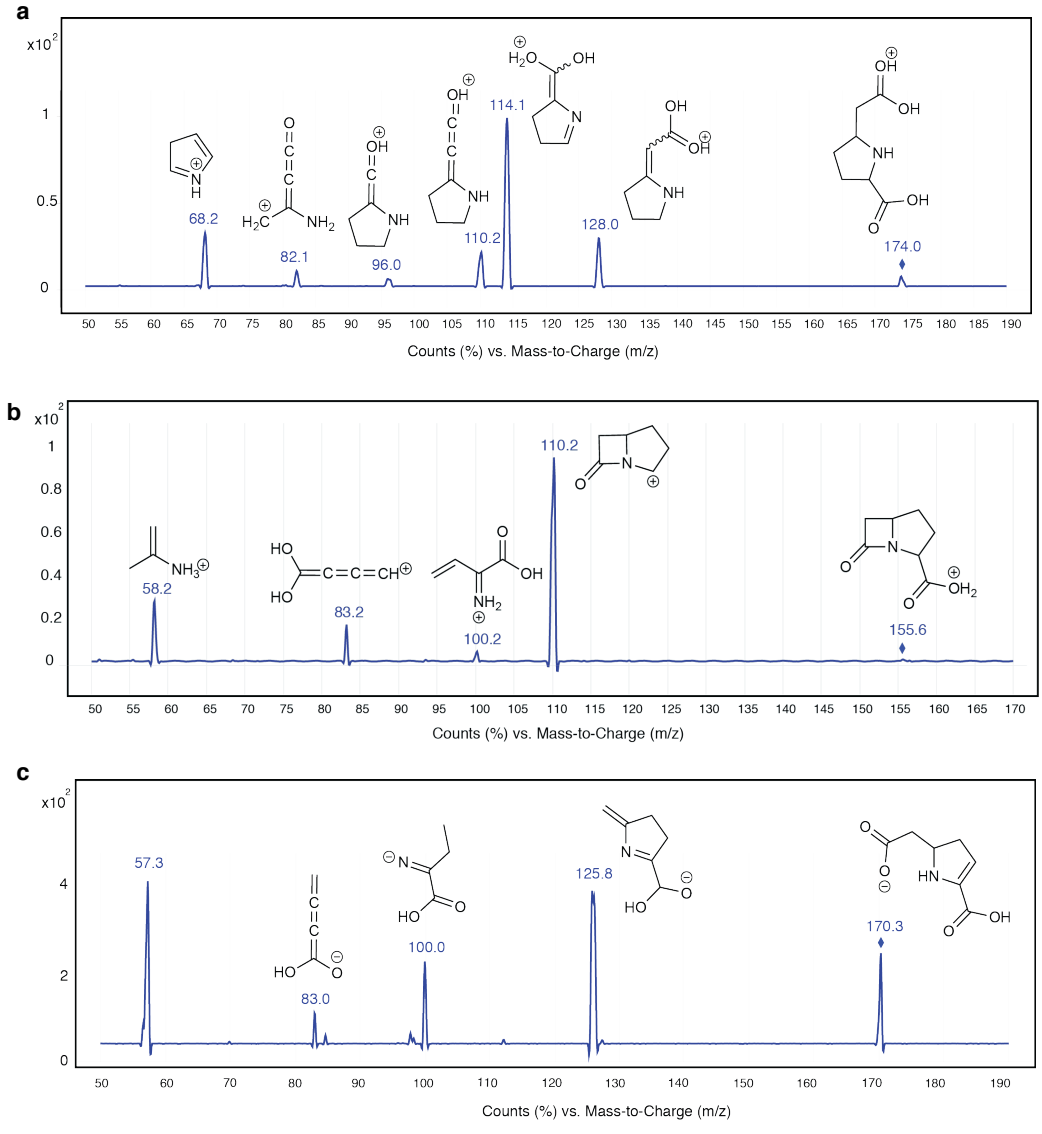
100 μL of culture supernatants were mixed with hydroxylamine (10 mM final concentrations; hydroxylamine solution freshly prepared from NH_2OH HCl, pH 7) in 10 mM KH_2PO_4 pH 7 for 1 h at room temperature. UV absorbance was measured using the pedestal mode of a NanoDrop™ 2000 Spectrophotometer. Car concentration is estimated from the average absorbance at 262 nm difference between hydroxylamine-treated and untreated samples using a molar extinction coefficient previously determined for Car[42] (4,500) and a

pathlength of 0.1 cm. Addition of hydroxylamine did not affect the absorbance spectra of supernatants taken from fosfomycin-lysed cultures that do not produce Car (BL21 pCarAB_ProB(I69E)A, **Supplementary Fig 2.12a**). We validated the use of differential UV spectroscopy by quantifying known concentrations of imipenem added to cultures in order to closely mimic the conditions of Car sampling. Imipenem absorbance (300 nm) was extinguished by hydroxylamine (**Supplementary Figure 2.12b, 2.12c**). Calculated values linearly corresponded to known concentrations of imipenem added, with 14% average error at concentrations of 2 mg/L or higher (**Supplementary Figure 2.12d** and detailed calculations in **Supplementary Table 2.1a**). For quantification of Car, overnight cultures from individual colonies of BL21 pCarCBAE_ProB(I69E)A were used to inoculate 25 mL production medium at a starting OD_{600} of 0.06 and incubated at 37 °C with shaking (250 r.p.m) in 250 mL Erlenmeyer flasks. Cultures were induced at OD_{600} 1.0 with 0.25 mM IPTG. 1 mL culture supernatant aliquots were collected 3 h (or 24 h) after induction and cleared by centrifugation. UV absorption spectra and detailed calculations are described in **Supplementary Figure 2.13a** and **Supplementary Table 2.1**. Consistent with the known instability of Car, no hydroxylamine-labile absorbance was detected in samples collected 24 h after induction of Car biosynthesis (**Supplementary Figure 2.13b**).

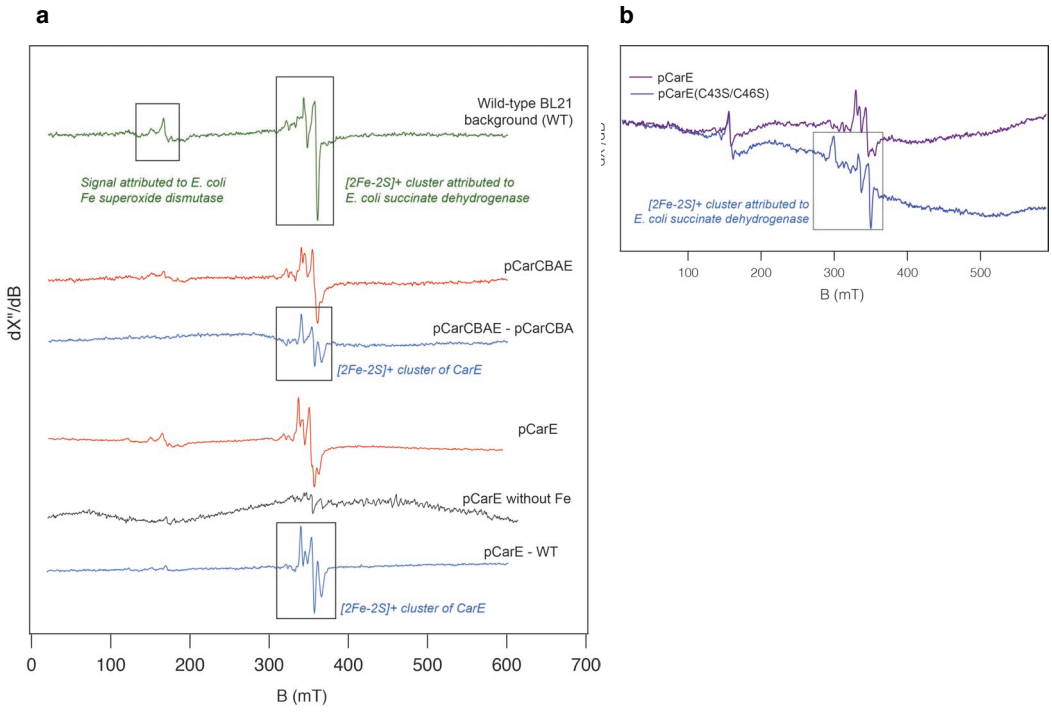
2.4.12 STATISTICS

Unless otherwise noted in the figure legend, bars and lines depict averages of independent cell cultures grown from independent bacterial colonies, by which we define biological replicates. Sample sizes are described in the figure legends. Error bars represent ± 1 standard deviation from the mean. Dots in figures report values obtained from independent biological replicates.

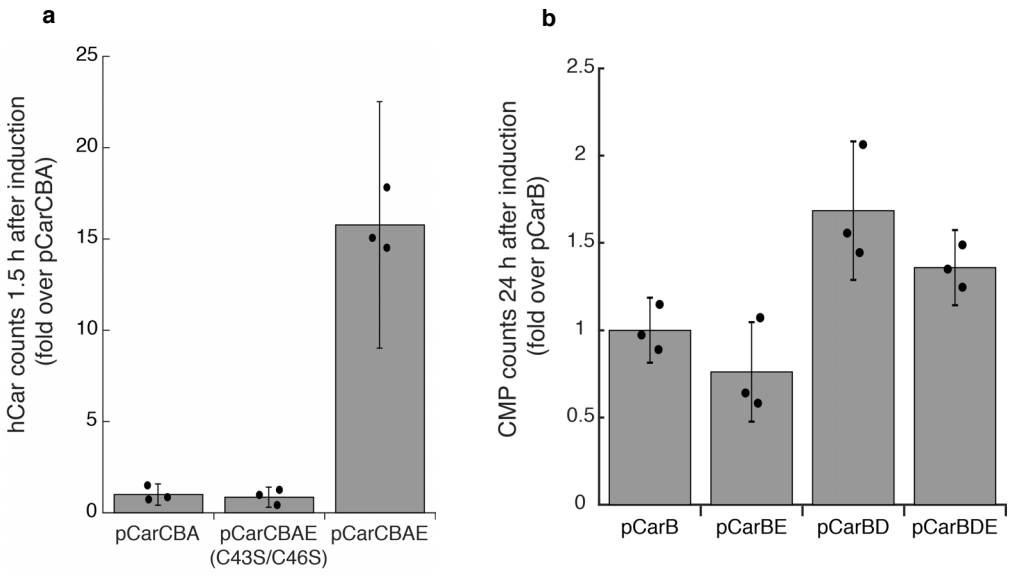
2.5 SUPPLEMENTARY INFORMATION



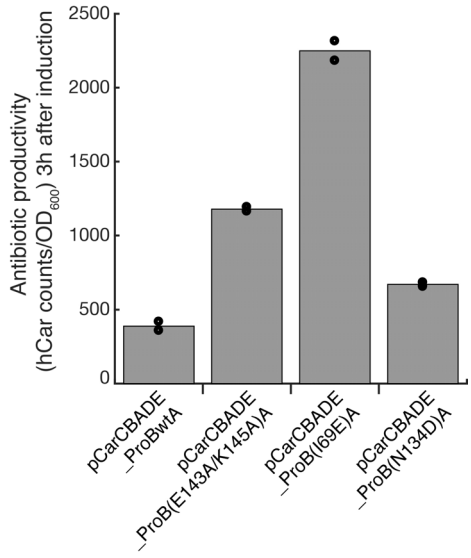
Supplementary Figure S2.1. **Fragmentation spectra of the Car pathway metabolites**. Assignment of potential fragments are indicated for each peak. **(a, b, c)** Spectrum of CMP (**3**), carbapenam (**4**), hCar (**6**), respectively. Peak annotation and fragment structures were modelled using the CFM-ID tool[1]. This experiment was routinely replicated, yielding similar results.



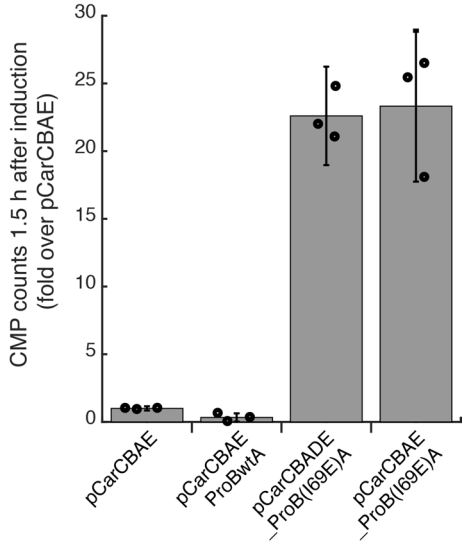
Supplementary Figure S2.2. **Whole-cell EPR spectra. (a)** A high background from *E. coli* proteins is present in all samples (green). The difference spectra between BL21 pCarCBAE-pCarCBA and pCarE-WT (red) show only one signal, which represents a $[2Fe\text{-}2S]^{+}$ cluster. The *g*-values attributed to the $[2Fe\text{-}2S]^{+}$ cluster of CarE are 2.04; 1.95; 1.89. The FeS cluster signal is suppressed in cells grown in iron deficient medium (black). **(b)** Mutations of CarE of cysteine residues (C43S/C46S) expected to participate in the FeS cluster suppressed the $[2Fe\text{-}2S]^{+}$ cluster of CarE, as we only observe the background signal from *E. coli* succinate dehydrogenase. These experiments were performed two times yielding similar results.



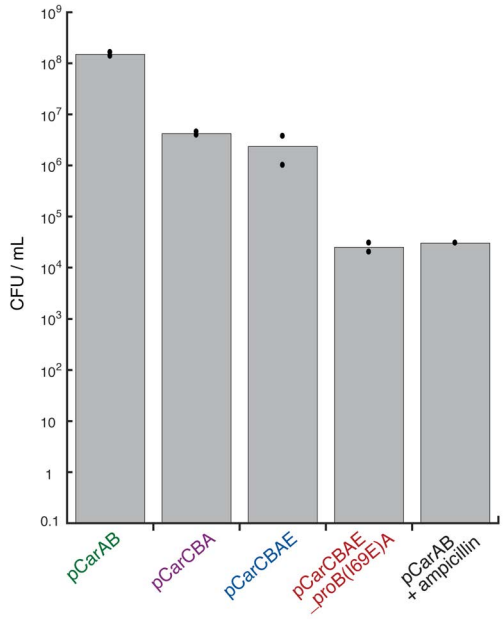
Supplementary Figure S2.3. **Elucidating the roles of CarE and CarD**. **(a)** Mutations of cysteine residues (C43S and C46S) that participate in the FeS cluster of CarE (BL21 pCarCBAE(C43S/C46S)) eliminate the high-production phenotype. Productivity is calculated from **6** LC/MS counts divided by the cell density (OD_{600}) recorded in culture supernatants 1.5 h after induction. **(b)** While the co-expression of CarD (a putative proline dehydrogenase) seems to slightly increase **3** productivity (1.7-fold increase), the expression of CarE (in strains BL21 pCarBE and pCarBDE) does not increase **3**. Productivity is calculated from **3** LC/MS counts divided by the cell density (OD_{600}) recorded in culture supernatants 24 h after induction. Data represent the mean ± s.d. of three biological replicates (5 mL cultures); dots represent each replicate.



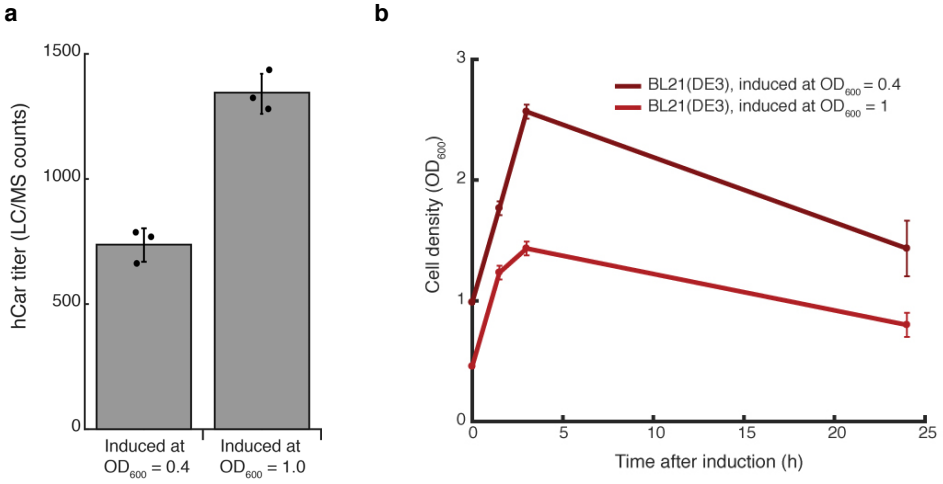
Supplementary Figure S2.4. **Relieving ProB feedback inhibition by proline.** Preliminary studies to evaluate antibiotic productivity (hCar counts / OD_{600} 3h after induction) in strains co-expressing the Car pathway with ProA and different feedback inhibition mutants of ProB. Bars represent the mean of two biological replicates (5 mL cultures); dots represent each replicate.



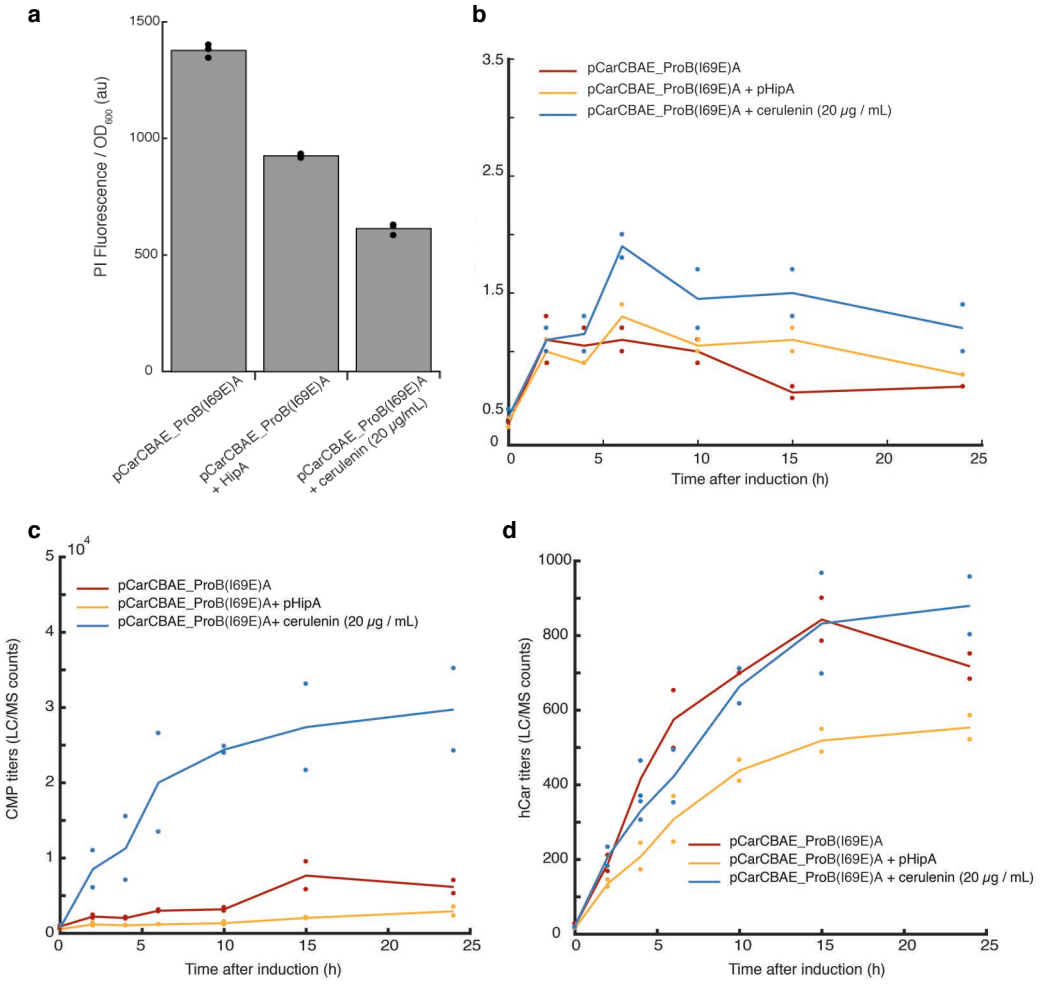
Supplementary Figure S2.5. **The overexpression of ProB(I69E) and ProA increases precursor metabolites.** CMP (**3**) titers (LC/MS counts) in culture supernatants of engineered strains 1.5 h after induction (used in **Fig 2a**). The co-expression of the Car enzymes with ProA and the feedback inhibition mutant ProB(I69E) increases **3** levels 23-fold relative to BL21(DE3) pCarCBAE. This effect is not present when expressing the wild-type ProBA enzymes, which are feedback-inhibited by proline. Data represent the mean ± s.d. of three independent 5 mL cell cultures grown from a single culture stock; dots represent each replicate.



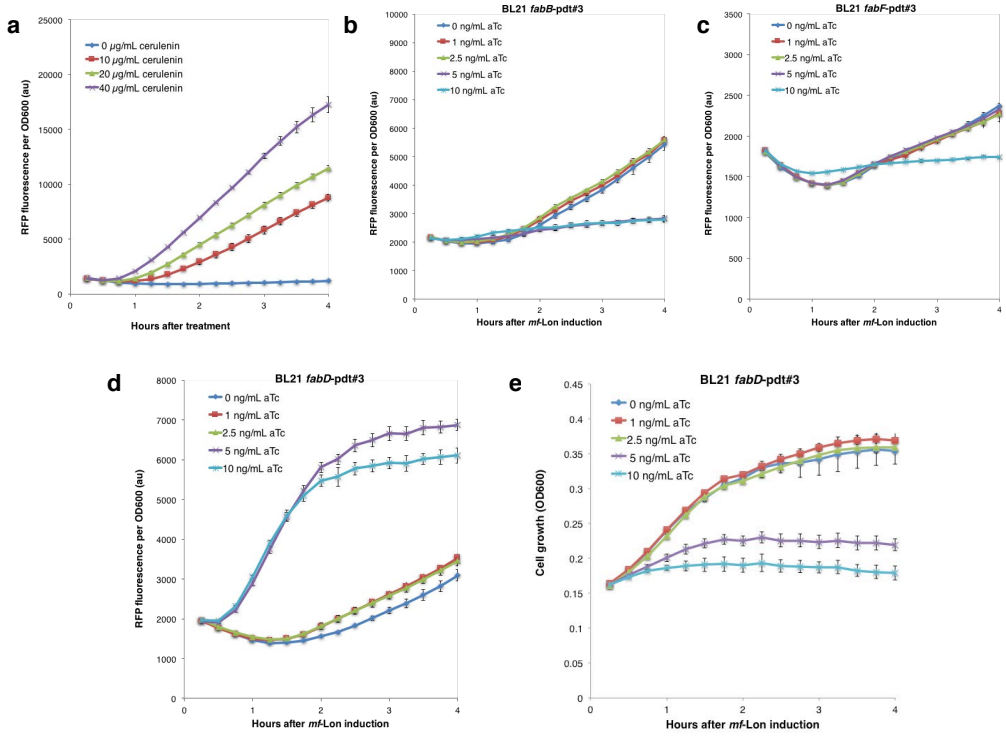
Supplementary Figure S2.6. **Car production provokes cell death**. Average colony forming units per mL of culture (CFU/mL) of engineered strains 24 h after induction in shake-flasks**.** Bars represent the mean of two biological replicates; dots represent each replicate.



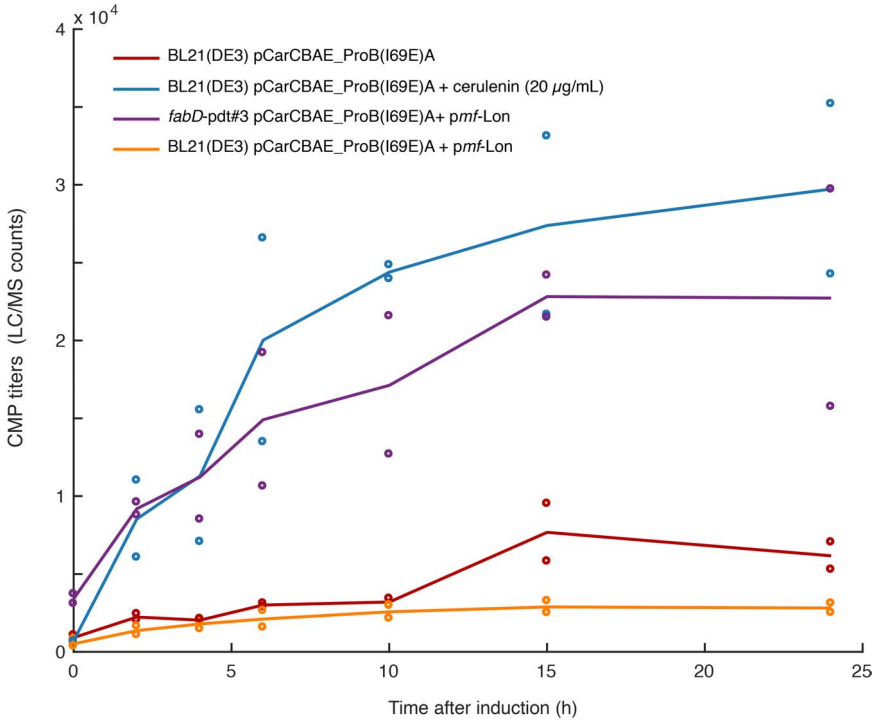
Supplementary Figure S2.7. **Biomass accumulation improves Car production.** Comparison of BL21(DE3) pCarCBAE_ProB(I69E)A cultures after induction at different cell densities (OD_{600} = 0.4 or 1.0) in shake-flasks**. (a)** Car titers recorded in cultures 24 h after induction. **(b)** Growth curves of culture after induction. Decay in cell density indicates lysis (45% OD_{600} decrease). Data represent the mean ± s.d. of three biological replicates (25 mL cultures). Dots represent each replicate.



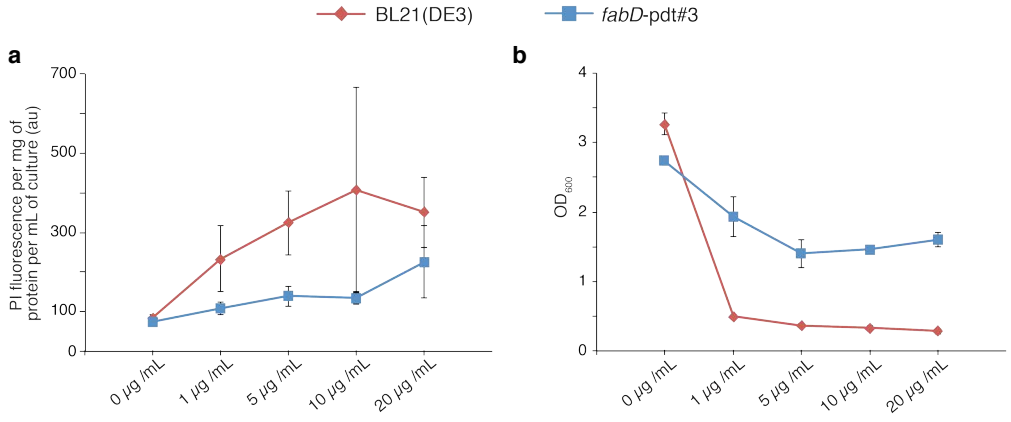
Supplementary Figure S2.8. **Car production in growth-arrested cultures**. Growth-arrest was provoked using two methods: HipA overexpression and FAS inhibition (cerulenin treatment). HipA overexpression was induced with 50 ng/mL aTc simultaneously with induction of Car pathway expression. 20 μg/mL cerulenin was added with induction of the Car pathway. (**a**) PI fluorescence per OD_{600} recorded in culture samples 24h after induction. Bars represent the mean of three independent measurements of the same sample; dots represent each replicate. (**b**) Growth curves of engineered cultures after induction measured by OD_{600}. (**c**) **3** titers after induction. FAS inhibition results in accumulation of **3**, whereas growth arrest by HipA decreases **3** titers. (**d**) Car production (**6** counts) after induction. Solid lines represent the mean of two biological replicates (25 mL cultures); dots represent each replicate.



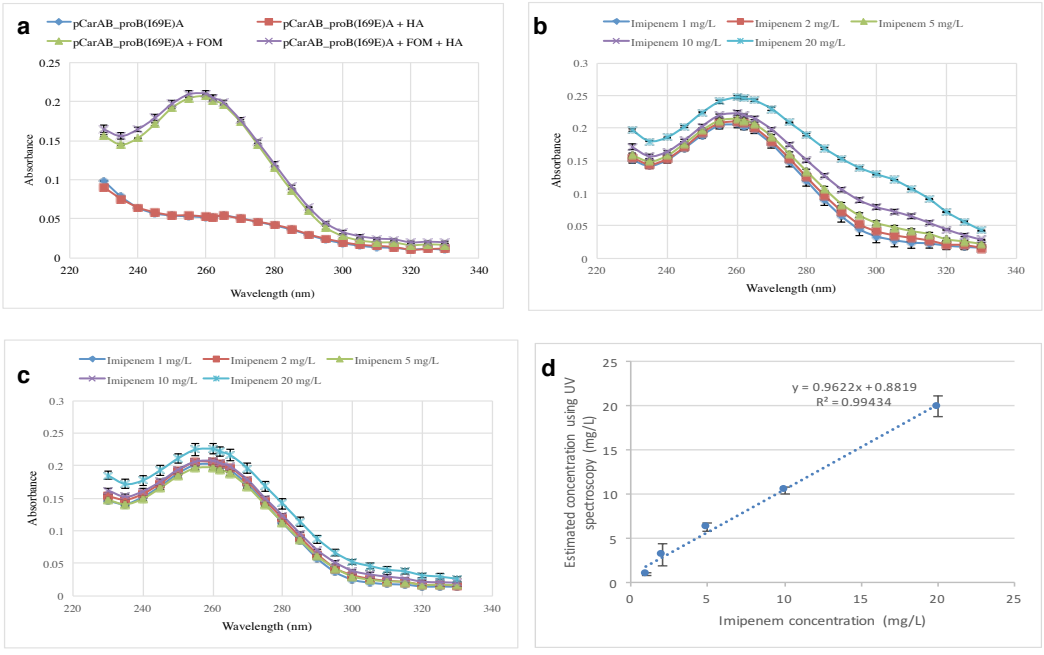
Supplementary Figure S2.9. **FAS inhibition results in a substantial accumulation of malonyl-CoA.** (**a**) Fluorescence of BL21 cells carrying the pCFR malonyl-CoA biosensor plasmid treated with cerulenin. (**b-d**) The strains *fabB-pdt#3, fabB-pdt#3 and fabD-pdt#3* strains were co-transformed with pCFR and p*mf*-Lon_bis. *mf*-Lon protease was induced with aTc. (**b**, **c**) Intracellular accumulation of **2** was not observed in strains *fabB-pdt#3 and fabF-pdt#3.* (**d**) Sufficient expression of *mf*-Lon protease triggers FabD degradation, provoking a significant accumulation **2**, resulting from FabD degradation, and growth arrest (**e**). Data represent the mean ± s.d. of three independent cell cultures grown from a single culture stock.



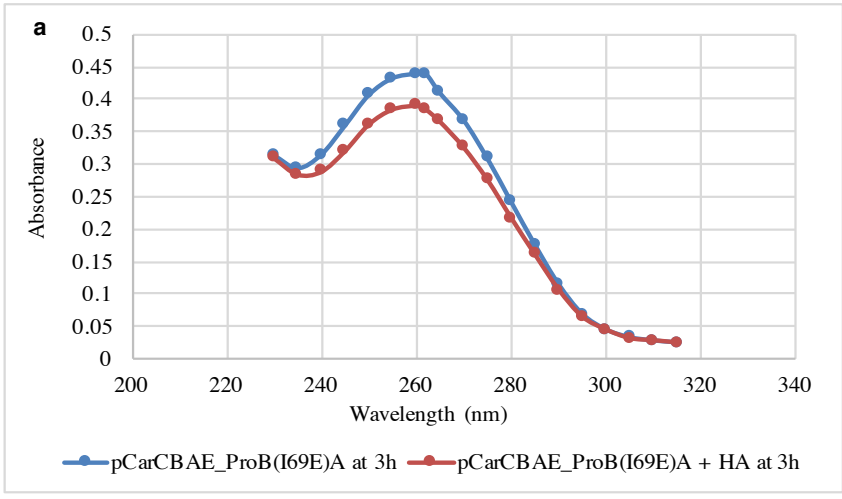
Supplementary Figure S2.10. **FAS inhibition increases the flux through the Car pathway.** LC/MS analysis of CMP (**3**) titers over time in culture supernatants. For *mf*-lon overexpression, cultures were induced with 10 ng/mL aTc simultaneously. Cerulenin (20 μg/mL) was added at induction. FAS inhibition significantly boosts the incorporation of **2** into the Car pathway (cerulenin treatment and FabD degradation in the strain FabD-pdt#3). Dots represent biological replicates. Solid lines represent the mean of two biological replicates (25 mL cultures); dots represent each replicate.

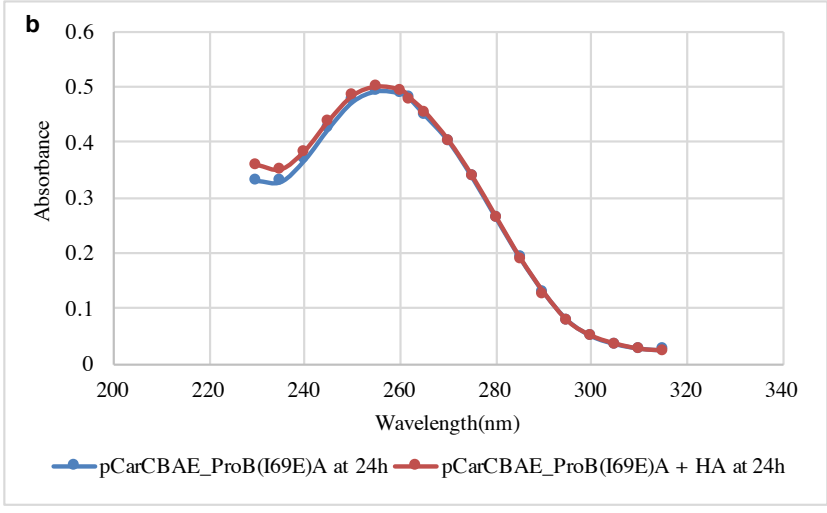


Supplementary Figure S2.11. **FAS arrest increases tolerance to the complex carbapenem mipenem.** Cultures of BL21(DE3) and *fabD*-pdt#3 p*mf*-Lon were grown until OD_{600} = 1.0 and treated with imipenem. Cultures of *fabD*-pdt#3 p*mf*-Lon were induced with 10 ng/mL aTc and incubated for 2.5 h to provoke growth-arrest prior to imipenem exposure. Measurements were performed 24 h after treatment. Data represent the mean ± s.d. of three biological replicates (25 mL cultures). FAS arrest reduces imipenem-induced lysis as measured by cell permeability (**a**) and optical density loss (**b**).



Supplementary Figure S2.12. Calibration of differential spectroscopy method for determination of carbapenem titers. BL21 pCarAB_ProB(I69E)A cultures were induced at OD_{600} = 1.0, split, and 50 μg/mL fosfomycin (FOM) was added to the culture medium at induction. The supernatant of lysed cultures was supplemented with defined concentrations of imipenem to validate the differential absorbance measurements. (**a**) Spectra of supernatants from non-lysed and lysed cultures 3 h after induction, before and after hydroxylamine treatment. (**b**) Spectra of lysed cultures supplemented with imipenem. (**c**) Spectra of lysed cultures supplemented with imipenem and treated with HA. (**d**) Linearity of imipenem concentration calculated with differential absorbance in culture supernatants supplemented with the antibiotic (detailed calculation in **Supplementary Table 1a**). Data represent the mean ± s.d. of three biological replicates (25 mL cultures).





Supplementary Figure S2.13. Typical UV spectra of supernatants from Car-producing cultures. (**a**) Culture supernatants collected 3 h after induction. A differential absorbance at 262 nm between hydroxylamine-treated and untreated samples is observed. (**b**) Culture supernatants collected 24 h after induction. No significant difference at 262 nm between hydroxylamine-treated and untreated samples is detected. Spectra displayed are the median of three biological replicates (25 mL cultures). These experiments were performed two times yielding similar results.

Supplementary Table 2.1a. Estimated antibiotic concentrations using differential UV spectroscopy. Absorbance measurements at 300 nm and 262 nm were used to estimate concentrations of imipenem and **5,** respectively. Concentrations c were calculated using the Beer-Lambert equation as follows: c = (A1-A2) / (ε * b); with b = 0.1 cm. Mean values and standard deviations of Car titer are derived from three biological replicates.

Sample	Differential absorbance		Extinction coefficient	Estimated antibiotic concentration (mg/L)	
	Mean	s.d.	ε in L $mol^{-1}cm^{-1}$	Mean	s.d.
Supernatant + Imipenem 1 mg/L	0.004	0.001	11500	1.009	0.137
Supernatant + Imipenem 2 mg/L	0.012	0.005	11500	3.170	1.300
Supernatant + Imipenem 5 mg/L	0.024	0.002	11500	6.342	0.514
Supernatant + Imipenem 10 mg/L	0.040	0.002	11500	10.461	0.419
Supernatant + Imipenem 20 mg/L	0.077	0.004	11500	19.989	1.142
Supernatant BL21 pCarCBAE_ProB(I69E)A at 3h	0.050	0.015	4500	17.032	5.128

Supplementary Table 2.1b. Car titer of BL21 pCarCBAE_ProB(I69E)A 3 h after induction (determined by differential spectroscopy) is multiplied by ratios of hCar LC/MS counts 24 h to hCar LC/MS counts at 3 h to determine total Car production at 24 h. Values are derived from three biological replicates.

Estimated titers in strains expressing pCarCBAE_ProB(I69E)A	LC/MS counts		Estimated antibiotic concentration	
	Mean	s.d.	Mean mg/L	s.d.
BL21(DE3) at 3h	411.1	44.8	17.0	5.1
BL21(DE3) at 24	899.2	280.9	37.3	16.7
Arrested fabD-pdt#3 at 24h	1306.0	48.7	54.1	17.4

Supplementary Table 2.2. List of strains and plasmids used in this study

Plasmid name	Description	Source
Plasmids		
pCarAB	Codon optimized CarAB enzymes in pBbaA5k backbone	This work
pCarCBA	Codon optimized CarCBA enzymes in pBbaA5k backbone	This work
pCarCBAE	Codon optimized CarCBAE enzymes in pBbaA5k backbone	This work
pCarCBAE_ProB(I69E)A	ProB(I69E)A and codon optimized CarCBAE enzymes (in that order) in pBbaA5k backbone	This work
pCarCBADE	Codon optimized CarCBADE enzymes in pBbaA5k backbone	This work
pCarCBADE_ProB(I69E)A	ProB(I69E)A and codon optimized CarCBADE enzymes (in that order) in pBbaA5k backbone	This work
pCarCBADE_ProBwtA	ProBwtA and codon optimized CarCBADE enzymes (in that order) in pBbaA5k backbone	This work
pCarCBA_E	Codon optimized CarCBA enzymes under the control of p_{LacUV5}, and CarE under control of p_{Tet}	This work
pCarE	Codon optimized CarE enzyme in pBbaA5k backbone	This work
pCarE(C43S/C46S)	Mutant variant of CarE (C43S/C46S) in pBbaA5k backbone	This work
pCarCBAE(C43S/C46S)	Codon optimized CarCBA enzymes and mutant variant of CarE (C43S/C46S) in pBbaA5k backbone	This work
pCarBD	Codon optimized CarBD enzymes in pBbaA5k backbone	This work
pCarBDE	Codon optimized CarBDE enzymes in pBbaA5k backbone	This work
pCarBE	Codon optimized CarBE enzymes in pBbaA5k backbone	This work
pHipA	HipA toxin in pBbS2c	This work
pmf-Lon	Codon optimized mf-Lon protease in pBbA2c	This work
pmf-Lon-bis	Codon optimized mf-Lon protease in pBbA2a	This work
pECL275	Source of codon optimized mf-Lon protease	D.E. Cameron, MIT
pECT3	Source of pdt#3 tag	D.E. Cameron, MIT
pCFR	Malonyl-CoA biosensor	V. Libis, Rockerfeller University
Strains		
Escherichia coli DH5α	*E. coli* strain for cloning and plasmid amplification	Invitrogen
Escherichia coli BL21(DE3)	*E. coli* strain for antibiotic production and HipA overexpression. Source of *proBA.*	Invitrogen
Escherichia coli MG1655	Source of *hipA*	ATCC
Escherichia coli fabD-pdt#3	*E. coli* strain BL21(DE3) containing FabD-pdt#3 fusion carrying pmf-Lon	This work
Escherichia coli fabB-pdt#3	*E. coli* strain BL21(DE3) containing FabB-pdt#3 fusion carrying pmf-Lon	This work
Escherichia coli fabF-pdt#3	*E. coli* strain BL21(DE3) containing FabF-pdt#3 fusion carrying pmf-Lon	This work

Supplementary Table 2.3. List of primers used in this study

Primer name	Full sequence
P1-proBA	AGGAGGAAAAAAATGAGTGACAGCCAGACG
P2-proBA	TTACGCACGAATGGTGTAATCAC
P1-hipA	AGAACAGCAAAATCTGGAGTGGTA
P2-hipA	TCACTTACTACCGTATTCTCGGCT
P1-I69E	TGAGCACCTGGGTTACCCGGAACTGCCAGCGACTGAAGC CTCGAAACAACTGCTGGC
P2-I69E	TTGTTTCGAGGCTTCAGTCGCTGGCAGTTCCGGGTAACCC AGGTGCTCACG
P1-CarE	CCGGTGTATCCAAGGTGCGCCTGACGTCCGGCAACGTCA ACATGGATCATTCTGGTGGG
P2-CarE	ACGTTGCCGGACGTCAGGCGCACCTTGGATACACCGGAG TAACCAGAAGCACAGCGATAA
P1-*fabB*	GGCGGCACCAACGCCACGCTGGTAATGCGCAAGCTGAAA GATGCGGCGAACAAAAACGAA
P2-*fabB*	GATGCGACGCTGGCGCGCCTTACCCGACCTACGGCGAAT TATGTAGGCTGGAGCTGCTT
P1-*fabD*	TGAACGAACCTTCAGCGATGGCAGCGGCGCTCGAGCTTG CGGCGAACAAAAACGAA
P2-*fabD*	CAGTGCGATTTTTCCTTCAAAATTCATGATTTTCCTCTTTTA TGTAGGCTGGAGCTGCTT
P1-*fabF*	GCTTCGGTGGCACTAATGGTTCTTTGATCTTTAAAAAGATC GCGGCGAACAAAAACGAA
P2-*fabF*	CGCAAGCGGACCTTTTATATGGGTGGGAAATGACAACTTA TGTAGGCTGGAGCTGCTT

Suplementary Table 2.4. MRM settings for analysis of the Car pathway metabolites

Compound name	Formula	Mass	Precursor ion	Product ions	Dwell	Collision Energy(V)	Polarity
CMP	$C_7H_{11}NO_4$	173	174	128 114	100	12 12	Positive
Carbapenam	$C_7H_9NO_3$	156	157	110 83	100	20 20	Positive
hCar	$C_7H_9NO_4$	171	170	126 100	60	8 8	Negative

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3

SYNTHETIC BIOLOGY STRATEGIES TO ENHANCE METALLOENZYME ACTIVITY IN BACTERIA

Bacteria are routinely engineered to synthesize high-value chemicals from renewable materials through synthetic biology and metabolic engineering. Microbial biosynthesis relies on the activity of enzymes that catalyze the reactions involved in the production of valuable products. Metalloenzymes are one of the most ubiquitous family of enzymes in natural product biosynthesis pathways across kingdoms. These enzymes employ metal-containing cofactors to mediate a wide range of remarkable and challenging biochemical reactions. However, a number of challenges remain in the full exploitation of their catalytic potential in microbial biosynthesis. Indeed, the activity of metalloenzymes strongly depends on post-translational modifications ensured by highly specific proteins, such as maturation pathways or reduction systems, which remain poorly characterized. Consequently, traditional metabolic engineering approaches that focus on pathway gene expression and carbon flux alone are often insufficient to implement and optimize pathways that contain metalloenzymes. This review highlights the biotechnological relevance of metalloenzymes, and discusses novel synthetic biology strategies to advance their industrial application. Strategies to enhance metalloenzyme activity and enable their functional heterologous expression include: (1) optimizing specific maturation machineries; (2) improving catalytic stability; and (3) enhancing electron transfer pathways.

This chapter is based on a review article in preparation for submission: Helena Shomar, Gregory Bokinsky, 'Synthetic biology strategies to enhance metalloenzyme activity in bacteria'

3.1 INTRODUCTION

Synthetic biology and metabolic engineering have greatly advanced our ability to engineer microorganisms to produce natural products of commercial and economic value[1]. Significant progress has been made towards developing novel microbial cell factories for the large-scale production of valuable compounds from renewable resources[2]. Indeed, microbial biosynthesis has the potential to develop cost-effective and sustainable manufacturing technologies for the commercial supply of a huge variety of valuable biomolecules[3,4]: pharmaceuticals, cosmetics, fine and commodity chemicals, fragrances, flavors or even biofuels. Many relevant natural products are secondary metabolites naturally produced at low levels by eukaryotic or prokaryotic organisms that are incompatible with scalable and economical bioprocesses, and for which genetic tools remain unavailable. Therefore, considerable efforts have been made to reconstruct heterologous biosynthetic pathways within non-native and well-characterized industrial hosts, such as *Escherichia coli*[5], Streptomycetes[6] or yeast[7], as an alternative to extraction from natural producers. Heterologous pathway engineering commonly involves controlled pathway gene expression to effectively balance enzymatic levels, as well as metabolic interventions to optimize the availability of pathway precursors and cofactors, which when combined improve production yields[8]. However, reconstructing and optimizing heterologous biosynthetic pathways within microbial hosts can be a difficult task. Indeed, many interesting multi-enzymatic pathways are not fully elucidated. Worse yet, many enzymes lose catalytic activity when expressed in foreign hosts. In such cases, the simple expression of biosynthetic enzymes and the use of traditional metabolic engineering approaches are often insufficient to ensure product synthesis, hence limiting the development of relevant heterologous bioprocesses.

Metalloenzymes, one of the most ubiquitous and versatile family of enzymes, participate in the biosynthesis of many valuable compounds. These enzymes employ metal-containing cofactors to catalyze some of the most remarkable and challenging biochemical reactions in Nature[9]. The activity of many metalloenzymes depends on the supply and proper incorporation of the metal cofactors, which usually involve post-translational modifications coordinated by specific maturation proteins. Moreover, the catalytic cycle of metalloenzymes often requires efficient electron supply to their active site from specific electron donors. Therefore, specific maturation pathways and reduction systems must be implemented and optimized in heterologous hosts to enable the functional expression of foreign metalloenzymes. The dependence of metalloenzymes on such peripheral molecular systems, for which many remain poorly characterized, has been one of the main limitations to their application in heterologous biosynthesis.

Considerable efforts to advance microbial biosynthesis have been made by enabling the functional heterologous expression of biosynthetic

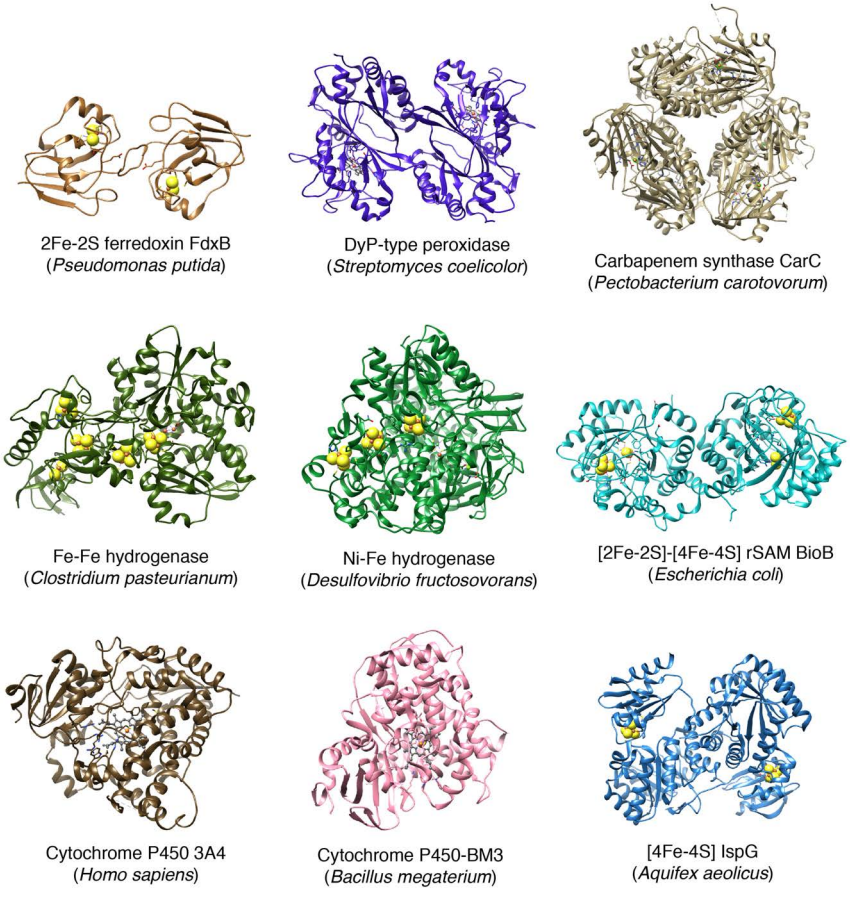

Figure 3.1 Structural diversity of metalloenzymes. Crystal structures of diverse types of metalloenzymes bound to their metal-cofactors. PBD entries corresponding to each structure are listed in Supplementary Table 1.

metalloenzymes, as well as improving their catalytic efficiency (both within natural and foreign hosts). This typically involves several challenges, including: (i) many metalloenzymes lose activity in heterologous hosts; (ii) the cofactor requirements for catalytic activity are not fully understood (iii) many interactions with maturation pathways and electron transfer systems within native/foreign organisms remain unelucidated (iv) some metalloenzymes are inactivated by oxygen and might require additional proteins for their protection and/or (re)activation. Therefore, traditional metabolic engineering approaches that improve carbon flux alone are often insufficient to circumventing the challenges in the reconstruction and optimization of metabolic pathways that rely on metalloenzymes. Additional engineering strategies to enhance peripheral molecular systems involved in metalloenzyme activity, such as maturation and electron transfer pathways, have been proven effective (and necessary) in overcoming these obstacles. This review aims to highlight the biotechnological

relevance of metalloenzymes in natural product biosynthesis, the challenges in their use, and some successful engineering strategies developed to enable/improve metalloenzyme activity in native and foreign bacterial hosts for large-scale production of natural products.

3.2 BIOTECHNOLOGICAL RELEVANCE OF METALLOENZYMES: POTENTIAL AND CHALLENGES

3.2.1 EXAMPLES OF BIOSYNTHESIS PATHWAYS OF HIGH-VALUE CHEMICALS THAT REQUIRE METALLOENZYMES

Many enzymes involved in the biosynthesis of relevant natural products contain metallocofactors, generally composed of metals such as iron, copper, nickel, manganese, zinc or molybdenum[10]. These cofactors can be metal ions bound to specific amino acids, or comprised within more complex cofactors such as heme, and homo- or hetero-nuclear clusters (i.e. [Fe-Fe], [Fe-Ni], [Fe-S] and [Fe-Mo] clusters)[10]. The great variety of metallocofactors bound to different protein structures (**Figure 3.1**) also accounts for the wide diversity of reducing potentials and diversity of reactions. Because metalloenzymes facilitate a huge variety of reactions including oxidative transformations, epimerization, hydroxylation, epoxidation, methyl transfers, alkylation or oxygenation, they hold a tremendous potential for microbial biosynthesis[9,11]. **Table 3.1** highlights metabolic pathways of prominent biotechnological relevance that rely upon reactions catalyzed by metalloenzymes.

Table 3.1. Biotechnological relevance of metalloenzymes

Name/Type of metalloenzyme	Metal site composition	Compound or compound family	Native pathways	Biotechnological relevance	Source organism	Ref
IspG, IspH	[4Fe-4S] cluster	Isoprenoid precursors (for over 40 000 compounds)	MEP pathway	Pharmaceuticals,fragances, flavors, biofuels, polymers,	Most bacteria and plastids	12
IlvD	[4Fe-4S] cluster	Isobutyraldehyde isobutanol	Isoleucine and valine biosynthesis	Biofuels	Most bacteria	13, 14
BioB	B_{12}-Radical SAM [2Fe-2S] and [4Fe-4S] clusters	Biotin	Biotin biosynthesis	Food supplements, pharmaceuticals,cosmetics, animal feed	Most bacteria	15
ThnK, ThnL, ThnP	B_{12}-Radical SAM [4Fe-4S] clusters	Thienamycin and carbapenem derivatives	Thienamycin biosynthesis	Antibiotics	*Streptomyces cattleya*	16

PoyB, PoyC, PoyD	Radical SAM [4Fe-4S] clusters	Polytheonamide cytotoxins	Polytheonamide biosynthesis	Antibiotics	Sponge-associated uncultivated *Theonella swinhoei*	17
Fom3	B_{12}-Radical SAM [4Fe-4S] cluster	Fosfomycin	Fosfomycin biosynthesis	Antibiotics	*Streptomyces webmorensis*	18
GenK, GenD1	B_{12}-Radical SAM [4Fe-4S] cluster	Gentamicin	Gentamicin biosynthesis	Antibiotics	*Micromonospora* genus	19, 20
YtkT (radical SAM/FeS)	Radical SAM [4Fe-4S] cluster	Yatakemycin	Yatakemycin biosynthesis	Antitumor	*Streptomyces* sp. TP-A0356	21
[2Fe-2S] Rieske-type oxygenases	Iron-sulfur Rieske domain and non-heme Fe(II)-binding motif	Hapalindole-type products	Hapalindoles biosynthesis	Antimycotic insecticidal	Cyanobacteria	22
Hydrogenases	[FeFe]- or [NiFe] active site. Typically contain multiple Fe-S subclusters	Hydrogen (H_2) gas	Hydrogen production	Biofuels	Archaea and Bacteria	23, 24
Cytochrome P450s	Heme cofactor	Wide range of natural products	Secondary metabolism	Fragrances, flavors, pigments, fine chemicals, pharmaceuticals	Prokaryotes and Eukaryotes	25
DyP-type peroxidases	Heme cofactor	Utilization of lignocellulosic biomass	Lignin degradation	Lignocellulose biorefineries	Bacteria and fungi	26
Methane monoxygenases (pMMO, sMMO)	sMMO: diiron center pMMO: mononuclear and dinuclear copper sites	Biological activation of methane	Methane oxidation	Methane bioconversion	Methanotrophic bacteria Methylococcaceae	27
L-tyrosine hydroxylase / L-DOPA dioxygenase	Heme cofactor/ mononuclear non-heme iron(II) site	Lincosamides, PDBs, and hormaomycin	Bacterial secondary metabolism	Antibiotics	Actinomycetes	28
CarC	Mononuclear non-heme-iron(II) site	Carbapenem antibiotic	Carbapenem biosynthesis	Antibiotics	*Pectobacterium carotovorum*	29
Tyrosinase	Dinuclear copper site	Melanin and other phenolic compounds	Melanin biosynthesis	Pharmaceuticals,cosmetics, battery technologies	Bacteria	30

3.2.2 CHALLENGES RELATED TO METALOENZYME-DRIVEN BIOSYNTHESIS

Substantial challenges remain to their full exploitation for large-scale biosynthesis. The intrinsic structural and mechanistic features of metalloenzymes largely account for the difficulties associated with their use in biosynthetic pathways. Here we discuss the main challenges that arise in enabling/enhancing the activity of overexpressed metalloenzymes (notably in heterologous hosts), and that have directly limited their application in developing commercially viable bioprocesses.

Specific maturation proteins and chaperones are often required for activity

Metalloenzyme activity directly depends on the efficiency of metal-cofactor incorporation into inactive apoenzymes. This process often requires native enzyme-specific post-translational modifications mediated by specialized proteins[10]. For instance, the activation of most enzymes containing metallic clusters, such as FeS enzymes[31] or [NiFe]- and [FeFe]- hydrogenases[23], involve complex maturation pathways comprised of several helper proteins that assemble and deliver the clusters to the apoenzyme via sequential ligand exchange reactions. Therefore, maturation pathways rely on highly specific protein-protein interactions and conformational changes that can vary depending on the orgainsms[31]. For example, at least three different FeS-cluster biogenesis machineries (ISC, SUF and NIF systems) are found in prokaryotes, and their distribution, protein composition and specialized functions can differ across bacterial species[32]. Moreover, specific proteins used for metalloenzyme maturation may vary depending on growth and environmental conditions. For instance, in *E. coli* the protein ErpA delivers a FeS cluster to the 4Fe-4S enzyme IspG in the presence of oxygen, while IscA mediates this delivery under anaerobic conditions[33]. In addtition, some metalloenzymes are sensitive to oxidative stress[34] or O_2 inactivation[23] and necessitate specialized proteins for the protection and/or (re)activation of their catalytic metallocenter. Therefore, the identification and optimization of specific maturation/stabilizing machineries are essential in ensuring metalloenzyme activity.

Metalloenzyme catalysis relies on efficient electron supply

The activity of most metalloenzymes requires electrons delivered by reducing partners, such as small molecule electron carriers (i.e. NADH, NADPH) or cognate electron transfer proteins (ETPs), to their catalytic metallocenter. The efficiency of the electron transfer between protein partners is highly dependent on specific protein-protein contacts, redox potential and protein concentrations[35]. Therefore, the performance of many metalloenzymes is directly coupled to the expression of native protein-specific ETPs that efficiently sustain catalytic activity. The delivery of the electrons to the active site has often been demonstrated to be a rate-limiting step associated with the

biosynthetic efficiency of overexpressed metalloenzymes, and therefore constitutes a major limitation to pathway optimization. For instance, the methylerythritol phosphate pathway (MEP) pathway for isoprenoid biosynthesis is known to be limited by the activity of two 4Fe-4S enzymes, IspG and IspH[12]. IspG/H both require an effective reducing partner that shuttles electrons from NADPH via a specific protein electron carrier[36]. Diverse studies have demonstrated that electron supply to these enzymes is a limiting factor in increasing the flux through the MEP pathway[36,37]. Insufficient electron transfer to FeS enzymes IspG and IspH could explain why the
mevalonate pathway is favored for engineered isoprenoid synthesis, despite the higher theoretical carbon yield of the MEP pathway[38].
The efficiency of another major family of industrially relevant metalloenzymes is notoriously limited by its dependence on efficient redox chains that transfer electrons from NADPH: the heme-containing cytochrome P450 monooxygenases[25] (P450s). Indeed, achieving high P450 activity often necessitates improving electron transfer, which requires the identification and overexpression of natural reducing partners that support catalysis. However, identifying cognate redox partners is a challenging task, largely due to the fact that most genes that encode them are not adjacent to P450 genes[39]. Therefore, difficulties in identifying efficient redox partners to sustain the activity of many discovered P450 biocatalysts remains a major barrier to their full exploitation for microbial biosynthesis.

Many metalloenzymes are inactive when expressed within foreign hosts

As discussed previously, electron transfer and/or maturation pathways are limiting factors in the activity of overexpressed metalloenzymes within native hosts. This problem is much more severe within heterologous hosts, where these essential pathways may be entirely absent or incompatible with foreign metalloenzymes. Indeed, the dependence of these enzymes on post-translational modifications for protection against oxidative stress, holoenzyme maturation and effective electron transfer, strongly affects their functional expression within foreign hosts. Since these post-translational modifications are governed by highly specific molecular interactions and protein-protein contacts, they are sensitive to molecular variabilities that exist between phylogenetically distant species[40]. As a result, many metalloenzymes are often incompatible with foreign maturation and electron transfer pathways, and display reduced or no catalytic activity when expressed within heterologous organisms. The functional compatibilities between the systems associated with metalloenzyme activity remain poorly characterized, making their transferability across different organisms unpredictable. This has greatly hindered the reconstruction and optimization of relevant pathways that rely on heterologous metalloenzymes within commonly used bacterial strains.

If the activation machineries required for a given metalloenzymes are unknown, the optimization of their activity can be a difficult task, which limits the

opportunities for heterologous metabolic engineering. For instance, aerobic methanotrophy relies on the activity of two metalloenzymes for which the corresponding activation machineries remain unknown: the copper-dependent membrane protein pMMO (particulate methane monooxygenase) or the iron-dependent cytosolic protein sMMO (soluble methane monooxygenase). Heterologous methanotrophy in highly characterized organisms such as *E. coli* has proposed to fully capitalize on the use of methane as low-cost and sustainable carbon feedstock[41]. However, efficient methane oxidation with recombinant MMOs in non-native hosts has not yet been demonstrated[42,43].

3.3 INCREASING THE AMOUNT OF FUNCTIONAL METALLOENZYME WITHIN PRODUCTION HOSTS

The quantity of functional metalloenzymes expressed in a given host depends on the proper maturation, folding and stability of their protein subunits, as well as on the efficient insertion of the metal-cofactor(s) necessary for catalytic activity[10]. Indeed, the activation of expressed apoenzymes (without a metal cofactor) often requires essential post-translational modifications coordinated by diverse helper synthesis and incorporation of the metallic redox cofactors/clusters into specific apoproteins. Other additional post-translational modifications (i.e. proteolytic cleavage, conformational changes) of apo and/or holoenzymes can participate to enzymatic activation[10]. Therefore, engineering strategies to improve the maturation, activation and catalytic stability of metalloenzymes can be successful for enabling or enhancing biosynthetic pathways (**Figure 3.2**).

3.3.1 AVAILABILITY OF METAL COFACTOR DETERMINES HOLOPROTEIN LEVELS

The cellular availability of the metal cofactors is an important determinant of holoprotein stability and activity. Engineering synthetic pathways to enhance the intracellular availability of metal cofactors can significantly increase the levels of functional metalloproteins and improve enzymatic activity. The heme supply in *E. coli* was improved by increasing the endogenous production of its precursor 5-aminolevulinic acid (ALA), which subsequently improved the activity of the heterologous heme-containing dye-decolorizing peroxidase (DyP) from *B. subtilis*[44]. Using a similar approach, the production of active radical SAM methyltransferases (such as ThnK, PoyC or Fom3) can be greatly enhanced by increasing intracellular concentrations of the exogenous cobalt-containing cobalamin cofactor, required for activity. The overexpression of proteins

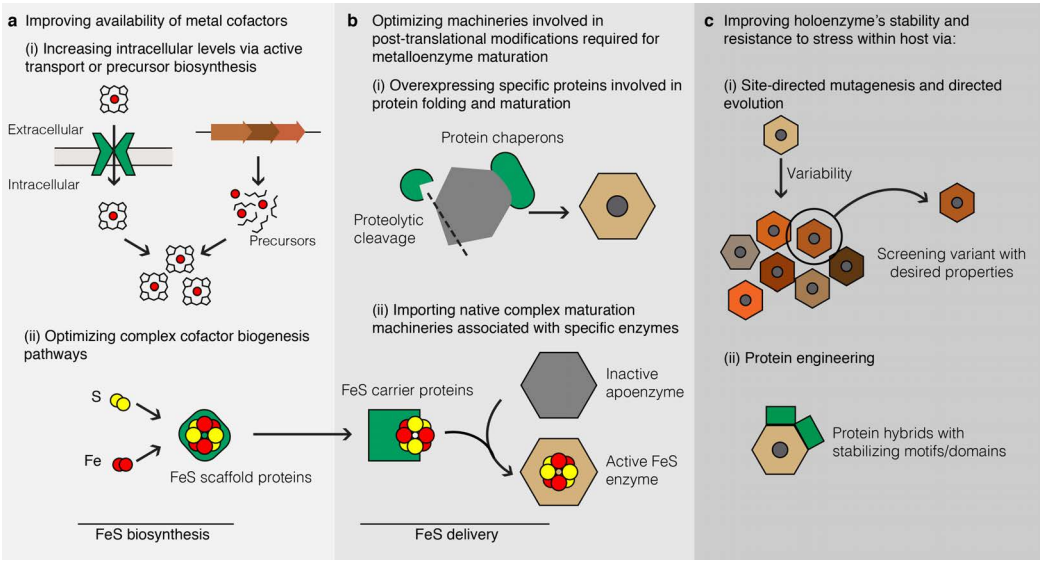

Figure 3.2 Strategies to enhance metalloenzyme maturation and stability in bacterial hosts. The levels of active holo-metalloenzyme can be improved by: **a.** Increasing the cellular availability of metal-containing cofactors required for activity. The availability of such cofactors can be improved by optimizing machineries and pathways that optimize their import from the envinronment and/or biosynthesis. **b.** Implementing and optimizing the molecular machineries involved in post-translational modifications and complex maturation pathways. **c.** Protein modifications that enhance the molecular stability and resistance to oxidative stress within the cellular environment of the host.

involved in cobalamin uptake and trafficking[45] improved yields of folded rSAM enzymes, and could be employed to improve pathways that depend on such enzymes.

3.3.2 METALLOENZYMES REQUIRE SPECIFIC MATURATION PATHWAYS

The difficulties in performing heterologous methane oxidation using the iron-dependent sMMO from native methanogens illustrate the crucial importance of proper folding and protein conformation in metallozymatic activity. Indeed, *in vivo* studies of the individual subunits of sMMO in foreign bacteria suggest that the lack of sMMO activity in foreign organisms is linked to improper folding and assembly of the subunits[46,47]. Recent reports of successful methane oxidation with *M. capsulatus* sMMO in *E. coli* described the co-expression of GroES/GroEL protein-folding chaperones from native or diverse heterologous organisms[48]. Moreover, the same approach is reported to improve the conversion of ethane into ethanol by foreign sMMO in *E. coli*. These results show that well-known molecular chaperones, such as GroEL, Hsp70, and Hsp40 that stabilize the overall quality of the proteome, can assist folding and maintain the active conformation of some overexpressed heterologous metalloenzymes[48,49]. While the use of these chaperones demonstrates that improved maturation can result in improved activity, they do not constitute

specific and precise tools for the activation of metalloenzymes in foreign hosts. Therefore, engineering strategies to optimize specific maturation pathways for key metalloenzymes may be necessary to develop relevant biochemical pathways in heterologous hosts, including methane oxidation with sMMO.

Many metalloenzymes require a set of multi-protein systems that generally assemble the metal cofactors/clusters, stabilize the proper conformation of the target apo-enzyme, and assist in the incorporation and activation of the metallocenters through specific protein–protein interactions. Consequently, the functionality of a given metalloenzyme in heterologous organisms depends on the co-expression of functional and compatible maturation pathways. In some cases, simple molecular systems composed of protein chaperones are necessary for enzyme activation, and are often located adjacent to the biosynthetic genes[49]. The *Streptomyces* copper-containing tyrosinases (MelC2), involved in the production of melanin, require a single protein-specific chaperone (MelC1) to assist protein folding and Cu incorporation[50]. *In vivo* enzymatic activity of MelC2 in *E. coli* has been demonstrated by co-expression of MelC1[30]. Similarly, the chaperone XdhC is essential to the functional expression of the molybdenum-containing xanthine dehydrogenase (XDH) from *Rhodobacter capsulatus* in *E. coli*[51]. However, several metalloenzymes often require the expression of elaborate maturation pathways that involve precise successive molecular steps mediated by numerous specific accessory proteins[50]. For instance, the activities of [Fe-Fe] and [NiFe]-hydrogenases rely on specific maturation pathways comprised of several proteins[23] to ensure proper folding/assembly of enzyme subunits, cofactor/cluster incorporation, and proteolytic processing in some cases[52]. Various studies have demonstrated heterologous *in vivo* activity of hydrogenases from diverse organisms when co-expressed with their respective maturation proteins in *E. coli* [53–56]. Heterologous H_2 biosynthesis illustrates the success co-expressing maturation pathways required for metalloenzyme activation into foreign production hosts.

The vast family of FeS enzymes also requires complex maturation pathways with sequential steps mediated by specific protein components (Figure 2). First, the FeS cluster is synthetized by scaffold proteins that assemble Fe and reduced sulfur atoms, which are mobilized from L-cysteine by a cysteine desulfurase. Subsequently, the cluster is delivered to target apoenzymes by specific FeS carrier proteins[31]. In a given prokaryote, these pathways can be encoded by up to three operons called SUF, ISC, or NIF. Although the different FeS cluster assembly and delivery systems rely on protein-protein interactions, their components show some promiscuity as they ensure the maturation of multiple FeS enzymes within the same species[31], and sometimes foreign FeS enzymes[57]. In the context of optimizing biosynthetic pathways of commercial interest, reliable tools for the robust activation and enhancement of native/foreign FeS enzymes are needed. The enhanced expression of the *isc* operon, either by direct overexpression of the native *isc*

genes[58] or by deletion of the negative regulator IscR[37], has been used for instance to improve the catalytic activities of IspG/H from the MEP pathway in *E. coli*. Importing orthologs of relevant metalloenzymes from foreign organisms could improve biosynthetic pathways by circumventing kinetic limitations of native enzymes, as demonstrated by the improved isoprenoid production resulting from the introduction of functional IspG/H from *T. elongatus* into *E. coli*[37]. However, many FeS enzymes when expressed in heterologous hosts do not retain enzymatic activity, strongly suggesting that uncharacterized maturation incompatibilities across microbial species exist.

As many pathways of biotechnological interest rely on diverse families of FeS enzymes (Table 1), enzyme inactivity within foreign organisms still constitutes a major obstacle to the heterologous biosynthesis of high-value compounds. Reliable methods to activate FeS enzymes (as well as other metalloenzymes) within foreign hosts must be developed to fully capitalize on their biocatalytic potential. The co-expression of the ISC operon from *Azetobacter vinelandii* with heterologous FeS enzymes is a common approach used by biochemistry groups to ensure expression of cluster-containing enzymes. This use further demonstrates that heterologous maturation machineries can function within foreign hosts and activate foreign FeS enzymes[16,45,57]. Yet, the *A. vinelandii* pathway cannot activate all FeS enzymes, hence frustrating the development of synthetic biology applications. Little is known about functional redundancy between FeS maturation pathways *in vivo* across different organisms, as the components and mechanisms required for FeS assembly and incorporation appear to differ across species[31]. In addition, activating FeS proteins is all the more important because some act as essential ETPs (e.g ferredoxins) for activating foreign metalloenzymes[59].

3.3.3 PROTEIN ENGINEERING TO ENHANCE ENZYME STABILITY

Protein engineering is another common strategy to activate and enhance the catalytic properties of biosynthetic metalloenzymes, through modifications that improve their stability and folding. For instance, early difficulties in activating eukaryotic P450 enzymes in microbial hosts were related to unsuccessful incorporation into the cell membrane required for activity. N-terminus engineering to truncate or replace the transmembrane domain with hydrophilic tags has been a successful method to improve the solubility and functionality of eukaryotic P450s and related ETPs, notably in *E. coli*[60,61]. Although these N-terminal modifications can result in decreased enzyme activity, further protein engineering strategies can be employed to improve the kinetic properties of modified P450s.

The cluster stability and catalytic activity of some metalloenzymes is greatly hindered by oxidative stress caused by molecular oxygen and/or reactive oxygen species (ROS). Nature has evolved specific amino-acid features and structural arrangements that improve the tolerance of metalloenzymes to oxidative damage and that are essential to their stability[34]. A

study demonstrated for example that oxidative stress is one of the main barriers to the functionality of the bacterial FeS enzymes IspG and IspH in yeast[62], implying that cluster stability after initial FeS cluster assembly is a limiting factor in metalloenzyme transferrability. Engineering protein hybrids that contain stabilizing motifs (such as chains of aromatic residues) could improve the oxygen tolerance of foreign enzymes, and for instance enable the activity of metalloenzymes from anaerobic organisms in aerobic environments.

Additionally, site-directed mutagenesis and directed evolution can be fast and reliable methods to engineer metalloenzyme variants with improved stability and catalytic properties within foreign organisms[63]. In combination with structural and mechanistic studies, these techniques enable rational design of enzymes with improved stability in the host's environment and without compromising the protein's function[64]. By employing this approach, evolved variants of a bacterial DyP-type peroxidase from *Pseudomonas putida* showed improved kinetic stability, increased resistance against inactivation by hydrogen peroxide, as well as reduced proteolysis in the cytoplasm of *E. coli*[26].

3.4 ENGINEERING ELECTRON TRANSFER PATHWAYS TO IMPROVE METALLOENZYME ACTIVITY

The catalytic activity of redox-active metalloenzymes often requires electrons provided by either small-molecule electron carriers (i.e. NADH, NADPH), or by electron transfer proteins (ETPs) that mediate electron flow between specific electron donors and acceptors. Engineering peripheral systems involved in electron supply to bottleneck metalloenzymes can be an effective strategy to improve pathway performance. This approach is especially relevant when the overexpressed metalloenzymes display low activity, and/or if direct manipulations of a given metabolic pathway (e.g. balancing multienzymatic expression) are proven insufficient to further increase product formation. Hence, both the identification and optimization of cognate electron transfer pathways that sustain catalytic activity, including their protein components and redox recycling systems, are crucial to enhancing metalloenzyme efficiency (**Figure 3.3**).

3.4.1 IDENTIFYING REDUCING PARTNERS REQUIRED FOR METALLOENZYME ACTIVITY

Most metalloenzymes are electronically coupled to small-molecule reduced cofactors (such as NADH, NADPH, FAD or ubiquinol). In some cases, these cofactors can directly deliver electrons to the enzyme[65,66], but the large majority of metalloenzymes receive electrons from ETPs, such as ferredoxins, flavodoxins or reductases[23,35,65]. ETPs compose redox chains that control the flow of electrons from reduced cofactors to specific target enzymes[35]. Indeed,

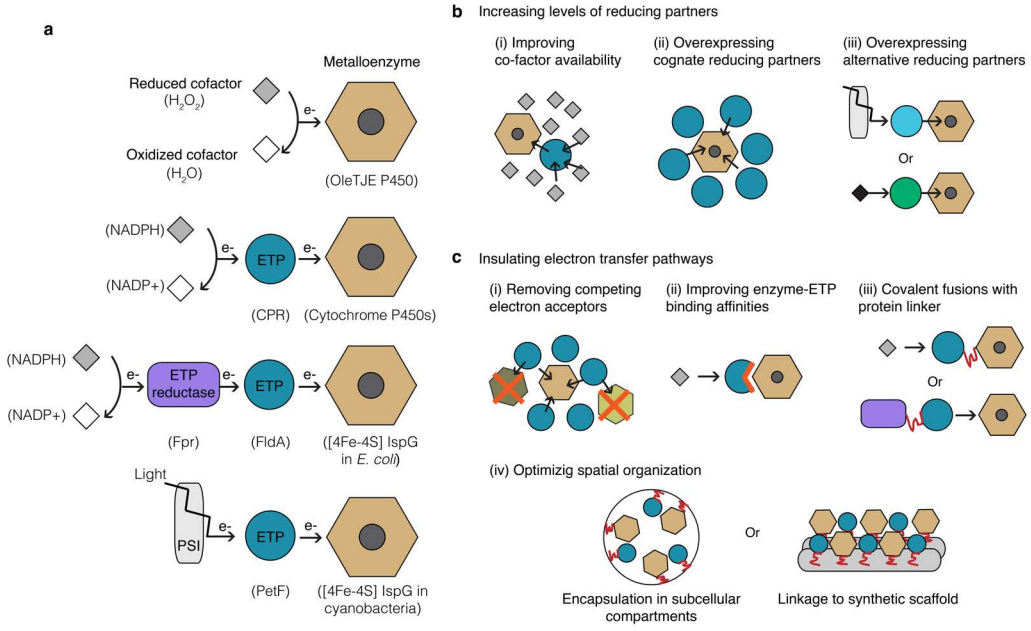

Figure 3.3 Electron transfer pathways for metalloenzymes and strategies to enhance electron supply *in vivo*. a. Schematic representation of different natural electron transfer systems coupled to metalloenzyme activity. Most metalloenzymes are electronically coupled to one or multiple ETPs that shuttle electrons from cellular cofactors or photosynthetic chains (such as PSI). Examples of cognate proteins and co-factors involved in each system are given in parenthesis. b. Increasing the intracellular levels of natural and/or alternative electron donors can enhance electron supply to given metalloenzymes. **c.** Engineering approaches to insulate electron transfer pathways can be employed to optimize and direct electron flow.

electrons from a given electron donor, travel from lower to higher potential ETPs, until reaching a final acceptor protein. The efficiency of electron transfer pathways depends upon the reduction potential and local concentration of ETPs, as well as protein binding affinities defined by highly specific residue-residue contacts at their interaction surface. Because ETPs are genetically encoded, redirecting and optimizing electron flow to given metalloenzymes can be achieved by tightly regulating their expression, improving interaction affinities between protein partners, or controlling their allosteric conformations[35]. Therefore, the identification of associated reducing partners is often necessary to improve the performance of given metalloenzymes, especially within heterologous hosts.

Structural and functional biochemical studies have been successfully employed to identify the small-molecule cofactors and ETPs that are naturally coupled to metalloenzyme activity. For metalloenzymes participating to secondary metabolic pathways, specific ETPs are often directly found within the same biosynthetic cluster. If the specific ETPs associated with a given metalloenzyme are unknown, and/or are not contained within the same biosynthetic gene

cluster, a bioinformatics analysis can identify potential redox partners in the genomes of native or heterologous organisms. Moreover, since ETPs display some functional promiscuity and can mediate electron transfer to a diversity of interacting proteins, cellular and biochemical assays can be employed to identify surrogate ETPs for target enzymes[35].

For instance, the activity of most cytochrome P450s relies on the identification of effective electron transfer pathways to deliver electrons from NADPH[65]. However, the genes coding for these natural redox partners are rarely adjacent to the P450 genes, which often limits their identification despite advanced genome annotations. This direct dependence on electron transfer pathways has greatly limited the use of pathways that rely on the activity of heterologous P450s[39,67]. Surrogate ETPs based on the cross-reactivity of P450s have been developed using well-characterized (often) heterologous electron transfer systems, and have been used to develop whole-cell P450-based biocatalysts[39,68–70]. In addition, novel redox partners for P450s can be found using genome mining tools. For example, the class I bacterial cytochrome P450 enzymes involved in glycopeptide antibiotic biosynthesis require a ferredoxin reductase and a FeS ferredoxin, which are not present in the native gene clusters. A combination of *in silico* analyses of the *A. balhimycina* DSM5908 genome and *in vitro* assays allowed identifying 2 novel ferredoxins that can mediate electron transfer to P450 enzymes involved in vancomycin and balhimycin biosynthesis[59]. The same study also demonstrates that ferredoxins from diverse species (in this case *E. coli* and *A. balhimycina* and spinach) can support the activity of P450 enzymes from different organisms. This functional flexibility can be an advantage in optimizing electron supply for bottleneck enzymes to increase production.

3.4.2 INCREASING THE AMOUNT OF REDUCING PARTNERS TO ENHANCE ELECTRON FLUX

Improving availability of small-molecule cofactors

Once the natural electron donors for biosynthetic enzymes of interest are identified, a general untargeted approach employed to increase electron transfer rates through a given pathway, is to optimize the regeneration and availability of the associated redox co-factor(s)[71]. For instance, strategies that improve NADPH pools result in increased performance of pathways limited by NADPH-dependent metalloenzymes such as cytochrome P450s[72] or Fe-S cluster enzymes[36].

Overexpression of known ETPs to direct electron flow

More precise and targeted approaches to increase the electron flow through given biosynthetic metalloenzymes involve the manipulation of redox chains composed of specific ETPs. Increasing the levels of expression of native ETPs can enhance electron supply to metalloenzymes, therefore regenerating their active redox state more rapidly and optimizing the production of compounds of

interest. Increasing the intracellular concentrations of the associated electron transfer system improved activity of the FeS enzymes IspG/H, leading to improved MEP pathway performance[36]. Overexpression of the flavodoxin protein FldA, and the flavodoxin/ferredoxin NADP+ reductase Fpr, which are thought to increase electron flux to IspG/H from NADPH, improved titers of an isoprenoid product by 3-fold. In cyanobacterial species, the activity of IspG/H is modulated by a different set of ETPs, the ferredoxin PetF and its associated ferredoxin-NADP+ reductase PetH. Isoprene production was increased by 1.5-fold in Synechocystis sp. PCC 6803 resulting from the overexpression of PetF/H[73]. Improved metabolic flux through the MEP pathway in E. coli was achieved by the incorporation of heterologous IspG/H from Thermosynechococcus elongatus combined with the expression of PetF/H from the same organism[37] (2.7-fold improvement).

The same approach has been employed to optimize the performance of secondary metabolic pathways that require enzyme-specific ETPs. For example, the identification and expression of a putative reducing partner was effective in boosting heterologous carbapenem production in E. coli[74]. This recent study expressed the ferredoxin CarE from the native biosynthetic cluster and showed that it likely acts as a reducing partner for the mononuclear non-heme iron oxygenases CarC, notorious for being single-turnover. The overexpression of CarE improved the catalytic rates of CarC and increased antibiotic productivity by 11-fold, thereby disrupting a major bottleneck in the carbapenem pathway. The same approach will be necessary when expressing complex antibiotic pathways that are known to include several redox-active FeS enzymes, such as the thienamycin pathway.

However, since ETPs can mediate electron transfer between a variety of different redox partners, their overexpression or accumulation could lead in some cases to excessive redox activities and the accumulation of reactive oxygen species, causing global imbalances that hinder cell viability and production[35,61,67,75]. For instance, high levels of expression of the reductase associated with the cytochrome P450 enzyme CYP71AV resulted in decreased viability and product formation in yeast[75]. The same study demonstrated that reducing reductase expression levels and reducing the formation of uncoupled electrons (by introducing the cytochrome b5 from the native organism) increased production by almost 2-fold. In E. coli the highest reported titer of oxygenated taxanes involving P450-mediated biochemistry was achieved by modulating P450/reductase ratios and levels[61]. These works exemplify the importance of tuning the expression of reducing partners (relative to their target enzymes) to optimize electron flux without compromising cell viability.

Expressing surrogate ETPs

The ability of ETPs to interact with multiple acceptor proteins, can be harnessed to design surrogate electron transfer circuits (often derived from heterologous organisms) for targeted metalloenzymes[76]. For instance, some

ferredoxins participate as electron carriers in both the photosynthetic chain (mediating electron transfer between photosystem I (PSI) and electron acceptors) and in P450 systems by delivering electrons from NADPH. The functional promiscuity of ferredoxins has for instance encouraged efforts to develop alternative light-driven electron transfer pathways for P450-catalyzed biosynthesis that allow NADPH-independent activity[77,78]. Other approaches to bypass co-factor dependence and capitalize on solar energy to drive cytochrome P450 activity have been developed in vivo using artificial electron donors[79]. The modularity of ferredoxins to mediate electron transfer between different partner proteins, and their functionality across a wide range of heterologous hosts, make them useful tools for engineering synthetic redox chains to drive metalloenzyme activity. However, the proportion of electrons that are shuttled between given ETPs and compatible electron acceptors is still difficult to predict[35].

3.4.3 INSULATION STRATEGIES FOR ELECTRON TRANSFER PATHWAYS

Removing competing reactions

Preventing electron transfer to other biochemical processes in the cell can further increase the flow of electrons from electron carriers to metalloenzymes. An approach to redirect electron flow into a pathway of interest is to remove competing electron acceptors from the host. Agapakis et al. increased hydrogen production in E. coli by 40% by deleting a gene encoding a protein that was suspected to interact with the synthetic electron transfer pathway of [Fe-Fe] hydrogenases[55]. Moreover, as protein-protein binding affinities control electron flow between redox partners, targeted mutations in ETPs can reduce or suppress their interaction with competing binding partners, hence redirecting electron flow to biosynthetic metalloenzymes and improving product yields[55,80]. For instance, targeted mutations of a hydrogenase at the interaction surface to enhance the charge-complementarity with a partner ferredoxin improved hydrogen production up to 2-fold, compared to the wild-type hydrogenase[55].

Covalent fusions with redox partner

Other methods to enhance electron transfer between desired protein partners are based on increasing their relative local concentration within the cell. Indeed, incrementing the chance of binding between enzymes and redox partners is an efficient strategy to direct the flux into biosynthetic pathways of interest. Improved enzymatic activity and pathway performance can be achieved by directly fusing metalloenzymes to their reducing partner using genetic engineering tools. Some microorganisms have naturally evolved enzyme-ETP fusions with high catalytic activity, such as the remarkable $P450_{BM3}$ from Bacillus megaterium (a natural P450-reductase fusion)[81]. Such chimeric protein complexes can be synthetically constructed by fusing the desired partners through protein linkers. Fusions of relevant cytochrome P450 enzymes and native/heterologous reducing partners have been traditionally

used to sustain and boost their catalytic activity [67,78,82–86]. For instance, the direct fusion of the hydrogenase from C. acetobutylicum with a ferredoxin resulted in a maximum 4.4-fold increase in biological hydrogen production[55]. However, it is difficult to predict how these covalent fusions will impact enzymatic activity, which depends on the resulting conformational changes, overall configuration and linker length[55,87,88].

The physical linkage of metalloenzymes with electron carriers can also be used as a strategy to engineer alternative or orthogonal electron transfer pathways to improve electron flow to desired biosynthetic pathways. For instance, alternative electron donors have been engineered in E. coli to uncouple the activity of the [NiFe]-hydrogenase-3 (Hyd-3) from its natural electron donor formate. An engineered Hyd-3 covalently attached to a ferredoxin from Thermotoga maritima accepts electron from pyruvate instead, and sustains in vivo hydrogen production when co-expressed with a pyruvate-ferredoxin oxidoreductase (PFOR)[89]. This approach can be particularly attractive for enzymes which activity is driven by costly or toxic electron donors such as H_2O_2[90], and for which alternative redox partners could be more appropriate. H_2O_2-indepent activity of the P450 monoxygenase OleTJE from Jeotgalicoccus sp. ATCC 8456 was engineered by covalent fusion with a Rhodococcus P450 reductase domain, hence coupling its activity to the NADPH pool[90].

Moreover, protein fusions can be employed to engineer versatile electron transfer pathways with reduced complexity by attaching multiple interacting ETPs. Attempts to enhance the catalytic activity of P450 enzymes, led to the design of chimeric electron transfer systems based on the fusion of the protein Fpr from E. coli (for NADPH oxidation) with the flavodoxins FldA from E. coli[91] or YkuN from B. subtilis[87](for P450 heme-iron reduction). In these studies, the genetic fusion of ETPs led to improved electron transfer to a variety of P450 enzymes, compared to the electron transfer systems composed of separate proteins. The promiscuity of this system can mediate electron transfer to a variety of P450s with high efficiency, and therefore may serve as a surrogate redox system for orphan P450 enzymes.

Spatial organization of enzymes and partner ETPs

Since the physical interaction between partner proteins is critical for electron transfer, their spatial organization within the cell can be optimized to boost electron flux through desired metabolic pathways. Indeed, mediating the spatial organization of electron donor and acceptor proteins increases their chance of physical contact, and reduces cross-talk with other cellular pathways[92]. Molecular scaffolds can be designed to attach desired redox partner proteins and increase pathway yields in vivo. This approach has been applied to control the spatial organization of hydrogenases and ferredoxins to improve hydrogen production in E. coli. Attachment of a hydrogenase and a ferredoxin on a synthetic protein scaffold improved hydrogen production by 3-fold[55]. The use of

rationally designed RNA structures with protein-docking sites to optimize spatial organization and electron transfer boosted hydrogen yields by 50-fold[93]. Other strategies based on subcellular compartmentalization to encapsulate metabolic pathways can be employed to enhance electron transfer and pathway performance[94].

3.5 DISCUSSION AND PERSPECTIVES

To fully capitalize on the catalytic potential of metalloenzymes for the commercial biosynthesis of high-value chemicals, a number of engineering strategies have been pursued to enable and/or enhance their activity, particularly in heterologous microbial hosts. While synthetic biology and metabolic engineering approaches have been traditionally focused on balancing metabolic pathways (e.g. controlling metabolites and enzyme levels) and directing carbon flux towards production, they are often insufficient to optimize the steps catalyzed by metalloenzymes. Because the activity of these enzymes depends on specific redox chains for electron supply and/or complex post-translational maturation processes, successful engineering efforts have been focused on optimizing such peripheral pathways.

Here, we have presented novel synthetic biology strategies that have been employed to redirect electron flow through specific metalloenzymes, and to increase the amount of active enzyme within native and heterologous hosts. The combination and further development of these technologies has the potential to develop novel whole-cell bacterial biocatalysts with economically viable yields. However, major challenges and bottlenecks remain for the full exploitation of these engineering strategies, in particular the lack of fundamental knowledge on cognate complex maturation pathways (such as the mechanistic differences between bacterial FeS biogenesis systems) and interacting redox partners, as well as our limited understanding on how their alteration globally affects cellular metabolism and homeostasis. Indeed, these issues are particularly reflected in our inability to predict the transferability of given metalloenzymes across bacterial species, and to anticipate the components that will be required for their activation in foreign hosts. To understand and characterize these processes and their mechanisms, significant research at the intersection between biochemistry, metabolomics, functional genomics and synthetic biology is necessary. For instance, cellular assays could be developed to screen the *in vivo* functionality of metalloenzymes within foreign hosts, and to address the barriers to their transferability and optimal activity. Indeed, little is known about how incompatible metalloenzyme activation pathways are across evolutionarily distant bacterial species, and about the nature of the incompatibilities that represent a barrier to their functional expression: do they concern effective redox partners, maturation pathways and/or protein stability? Furthermore,

shedding light on the biochemical requirements to metalloenzyme activity could also be employed to reconstruct relevant bacterial pathways within industrial eukaryotic hosts such as *S. cerevisiae*. For instance, several barriers to bacterial MEP pathway functionality in yeast have been linked to the low activity and oxygen sensitivity of the bacterial FeS enzymes IspG and IspH[62]. The functional expression and optimization of bacterial metalloenzymes in eukaryotes could greatly benefit to the biological production of valuable compounds.

Recent advances in synthetic biology have enabled the *de novo* construction of artificial metalloenzymes by incorporating catalytic metallocofactors (including abiotic cofactors) into designed protein scaffolds. These artificial enzymes can catalyze a remarkable range of natural and synthetic reactions[95]. The biosynthetic potential of artificial metalloenzymes is tremendous, yet the same issues encountered with natural metalloenzymes often limit their activity, including electron transfer efficiency[96] and catalytic stability[95]. Deeper understanding of the maturation and functional requirements of natural metalloenzymes will likely be of significant relevance for improving artificial or bioinspired catalysts, and even optimizing hybrid catalytic complexes[97]. Moreover, designing custom FeS proteins (i.e. ferredoxin-like artificial proteins) that mediate electron transfer could be of considerable interest for engineering novel redox chains to support metalloenzyme activity[98].

Besides their potential for microbial biosynthesis, metalloenzymes participate in a wide range of key biological processes across kingdoms, including some of significant technological importance, such as antiviral mechanisms[99] or antibiotic resistance[100]. Therefore the relevance of new technologies developed in characterizing their mechanism and addressing the barriers related to their activity goes beyond applications to microbial cell factories.

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4

EXPRESSION OF SMALL ELECTRON AND IRON-SULFUR CLUSTER CARRIERS ACTIVATE HETEROLOGOUS IRON-SULFUR ENZYMES IN *E. COLI*

Iron-sulfur (FeS) cluster enzymes have tremendous untapped potential for biotechnology and microbial biosynthesis. These highly versatile enzymes catalyze a wide range of reactions involved in the synthesis of a huge variety of valuable natural products. Unfortunately, the dependence of FeS enzymes upon external cofactors often limits their catalytic activity when expressed in foreign hosts, completely precluding the use of many biosynthetic pathways. To exploit the catalytic potential of FeS enzymes, we have developed an experimental assay to report the functionality of foreign FeS enzymes within the common host Escherichia coli. By testing the functionality of enzyme orthologs, we show that FeS enzymes are typically inactive when expressed within evolutionarily-distant species. We demonstrate that inactive FeS enzymes can be recovered by the co-expression of 'plug adapter' proteins, which correspond to foreign FeS biogenesis and electron carrier proteins. Finally, we explore the applicability of these 'plug adapters' to engineer the production isoprenoids and the antibiotic fosfomycin in E. coli.

This chapter is based on a research article in preparation by Helena Shomar, Elena Fernandez Fueyo and Gregory Bokinsky.

4.1 INTRODUCTION

Iron-sulfur (FeS) cluster enzymes contain prosthetic metalloclusters composed of iron and sulfur ([2Fe-2S], [3Fe-4S] or [4Fe-4S]) that can facilitate a variety of biochemical reactions, by acting as catalytic or redox-active centers[1]. These ubiquitous and highly versatile enzymes mediate a wide range of reactions, including dehydration, substrate activation, oxygenation, methyl transfers, epimerization, or electron transfer[2]. FeS enzymes participate in essential biochemical processes and pathways required for the biosynthesis of a huge variety of valuable natural products, including biofuels[3], fragrances and flavors[4], fixed nitrogen[5], and pharmaceuticals such as antibacterial[6,7] or anticancer[8] compounds. Therefore FeS enzymes hold a tremendous potential for biotechnology and microbial biosynthesis.

The catalytic activation of FeS enzymes requires a set of post-translational modifications (PTMs) partly ensured by multiprotein FeS biogenesis pathways[9] (ISC and SUF in *E. coli*) that assemble FeS clusters and insert them into apoproteins (**Figure 4.1a**). FeS assembly is a multi-step process, in which sulfur atoms are mobilized from cysteine, reduced, and assembled with iron into a cluster bound by a scaffold protein complex. The FeS cluster is then either delivered directly to client FeS enzymes, or further transferred to proteins known as A-type carriers (ATCs such as IscA, SufA, ErpA, NfuA). Specific FeS enzymes obtain FeS clusters from dedicated ATCs depending upon growth and environmental conditions. For instance, in *E. coli* the FeS enzyme IspG (4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase) obtains its FeS cluster from the A-type carrier protein ErpA in the presence of oxygen, while during anaerobic growth, IscA is able to deliver clusters directly to IspG[10]. Moreover, many FeS enzymes are oxidized as part of their catalytic cycle, and require reduction with electrons from the cellular reductant pool (e.g. NADH or NADPH). Hence the catalytic activity of FeS enzymes is often coupled to a reduction system which shuttle electrons from a reduced donor to the oxidized FeS cluster, via electron transfer proteins (ETPs) such as flavodoxins or ferredoxins. For instance, *E. coli* IspG is regenerated by the flavodoxin FldA[11], whereas the ferredoxin PetF fulfills this role in Cyanobacteria[12]. ETPs are themselves reduced by ferredoxin/flavodoxin reductases (e.g. Fpr in *E. coli*).

The dependence of FeS enzymes upon external cofactors and complex maturation pathways often limits their catalytic activity, which creates bottlenecks in biosynthetic pathways that rely upon them. Moreover, many FeS enzymes show little-to-no activity when expressed in foreign hosts, which precludes their use in heterologous compound biosynthesis. Indeed, as foreign FeS enzymes require PTMs for their activation, they must successfully interact with the host proteins that mediate the PTMs. Because the protein residues that mediate these interactions are more likely to diverge with increasing phylogenetic distance from the recipient, such enzymes may sustain activity

only within closely-related species. The 'complexity hypothesis' predicts the functional dependence on protein connectivity greatly constrains the transferability of FeS enzymes[13], making their activity within foreign hosts unpredictable. Therefore, importing cognate FeS biogenesis and electron transfer pathways could be an efficient strategy to activate FeS enzymes within foreign hosts, surmounting the transferability barrier. An *in vivo* platform to study the transferability and possible activation of flux-limiting FeS enzymes in a common host would allow us to understand the barriers to heterologous expression of FeS enzymes, and eventually realize the biosynthetic potential of industrially relevant FeS-dependent pathways.

Isoprenoids are highly versatile compounds of great commercial value, as they can be used to produce a large number of industrially relevant products such as biofuels, pharmaceuticals, food additives, solvents or polymers[14]. The universal isoprenoid precursors, dimethylallyl diphosphate (DMAPP) and its isomer isopentenyl pyrophosphate (IPP), are naturally produced via two distinct routes: the methylerythritol phosphate (MEP) pathway and the mevalonate-dependent (MEV) pathway[15]. Although the MEP pathway is the most carbon-efficient isoprenoid biosynthetic route[16], its use for the industrial production of isoprenoids in *E. coli* has remained unsatisfactory[17] as its flux is greatly limited by [4Fe-4S]-cluster enzymes that catalyze the final two reactions[18]: IspG and IspH (4-hydroxy-3-methylbut-2-enyl diphosphate reductase). The ability to reliably import orthologs of the flux-limiting FeS enzymes of the MEP pathway into production hosts may enable the discovery of foreign IspG/IspH orthologs with improved kinetics and stability. Such foreign orthologs may be key to raising the performance of the MEP pathway to realize its theoretical yields, and to finally surpass the MEV pathway as the pathway of choice for isoprenoid biosynthesis in prokaryotic hosts.

Here, we describe a simple complementation assay that quickly reports the functionality of foreign FeS enzymes within *E. coli*, including 44 orthologs of the enzyme IspG. Our experimental data shows that FeS enzymes are typically inactive when expressed within evolutionarily-distant species. We demonstrate that inactive FeS enzymes can be recovered by co-expression of foreign FeS biogenesis and electron carrier proteins, which can act as 'plug adapters' to activate foreign FeS within *E. coli*. These complementation experiments suggest that FeS inactivity is caused in some cases by incompatible interactions with the hosts FeS maturation pathway proteins or ETPs. Our complementation experiments shed light on the nature of these incompatibilities, and demonstrate how these can be circumvented. Additionally, we explored the catalytic potential of activated IspG orthologs to achieve higher flux within the context of a biosynthetic pathway for production of the isoprenoid compound bisabolene[19]. We additionally used our 'plug adapters' to activate the radical *S*-adenosylmethionine [4Fe-4S]-cluster enzyme Fom3 from the biosynthesis pathway of the antibiotic fosfomycin[20] from *Streptomyces wedmorensis*. We report the first heterologous production of

fosfomycin in *E. coli*, by co-expression of the biosynthetic cluster from *S. wedmorensis* with our engineered 'plug adapter'. The further development of such 'plug adapters' will enable and enhance the *in vivo* activities of relevant FeS enzymes, and expand the microbial synthesis of high-value chemicals.

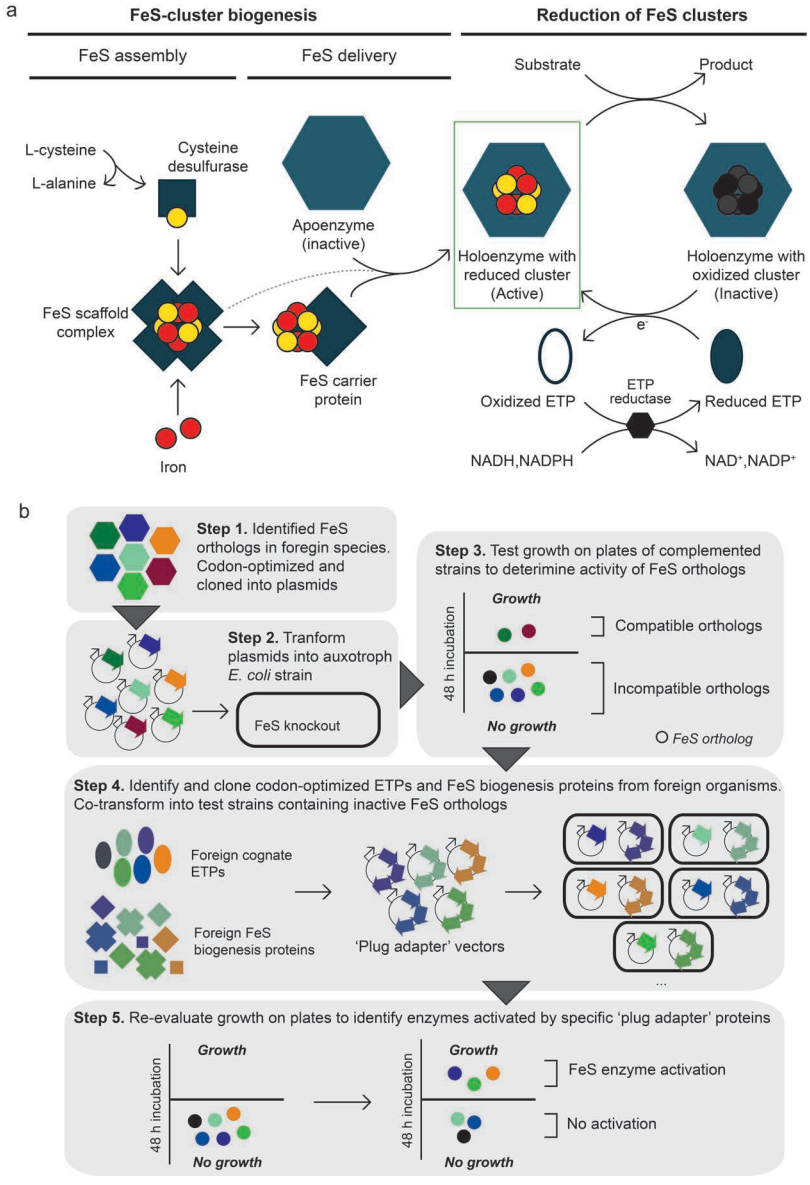

Figure 4.1 Schematic representation of the general principles of FeS enzyme activitation in bacteria. (a) The synthesis of active FeS holoenzymes is carried-out by multi-protein FeS-cluster biogenesis systems (SUF, ISC and NIF systems), which are based on the same basic principles. The FeS cluster is formed on a protein scaffold complex that assembles reduced iron (red circles) and sulfur (yellow circles) atoms (delivered by a cysteine desulfurase). A carrier protein then receives and transfers the assembled cluster to a target apoprotein, hence

producing an active holoenzyme (highlighted by green square). Some apoenzymes can receive cluster directly from the scaffold protein complex (dotted line). The supply of electrons to the metallocenter by electron transfer proteins (ETPs), coupled to reduced cellular cofactors, is often required to sustain the activity of many FeS enzymes. **(b)** Complementation experiments to test FeS activity in *E. coli*. Strains lacking a conditionally-essential FeS enzyme are complemented with foreign orthologs of those enzymes. Growth of the complemented strains (in the absence of the necessary nutrient) indicates that the foreign ortholog is active within *E. coli*. Inactive FeS enzymes are recovered by co-expression of ETPs or FeS biogenesis pathways (steps 4-5).

4.2 RESULTS

4.2.1 TESTING FUNCTIONALITY OF FOREIGN FeS ENZYME ORTHOLOGS IN *E. COLI*

We constructed knockout strains lacking conditionally-essential FeS enzymes: NadA (quinolinate synthase), BioB (biotin synthase), ThiC (HMP-P synthase), IlvD (dihydroxy-acid dehydratase) and IspG and IspH (from the MEP pathway). We identified the corresponding orthologs in the genomes of two foreign bacterial strains from different phyla: *Bacillus subtilis* and *Streptomyces cattleya*. These genes were codon-optimized and cloned into separate low-copy and anhydrotetracycline (aTc) inducible expression vectors (pBbS2k) that were transformed into the corresponding knockout strains. The functionality of the FeS enzymes from *B. subtilis* and *S. cattleya* in *E. coli* were tested using a complementation assay: if an overexpressed foreign ortholog restores the growth of the auxotroph in the absence of the required nutrient (if it complements the auxotrophy), this indicates that the FeS ortholog is transferable and functional within the *E. coli* host (Figure 1b). The native *E. coli* enzymes were also cloned for use as a positive control in complementation assays.

The expression of the enzymes IlvD, BioB and IspH from both *B. subtilis* and *S. cattleya* restored the growth of the knockout strains, while only the ThiC ortholog from *B. subtilis* complemented the *thiC* knockout (Table 1). However, over-expression of NadA and IspG orthologs failed to complement the auxotrophy of the corresponding knockout strains, likely due to insufficient enzymatic activity to support cell growth.

4.2.2 EXPLORING COMPATIBILITY OF ISPG ORTHOLOGS FROM DIFFERENT BACTERIAL SPECIES

Our preliminary complementation assays revealed that some IspG orthologs are inactive within E. coli (Table 1), which prompted us to further explore its functional transferability.. Indeed, the catalytic activity of the native [4Fe-4S]-cluster enzyme IspG is known to greatly limit the flux of the MEP pathway, hindering its use for the mass production of isoprenoids21. The activity of E. coli IspG in aerobic conditions relies on the native A-type carrier protein ErpA

for FeS acquisition10 (which delivers FeS clusters built by both the ISC and SUF systems under aerobic conditions), and on an electron transfer system composed of the flavodoxin FldA and its reductase Fpr. In addition to previous efforts to improve native IspGs (e.g. through overexpressing electron carriers FldA and Fpr18) one method for disrupting the bottleneck step catalyzed by IspG is to identify orthologs of IspG from other species. This may identify heterologous orthologs with improved kinetics and stability, which could directly increase MEP pathway performance.

	Origin of complementing FeS enzyme					
	E. coli MG1655		*Bacillus subtilis str.168*		*Streptomyces cattleya*	
Strain	- aTc	+ aTc	- aTc	+ aTc	- aTc	+ aTc
ΔbioB	-	+	-	+	-	+
ΔilvD	-	+	-	+	-	+
ΔthiC	-	+	-	+	-	-
ΔnadA	-	+	-	-	-	-
EC_ΔispH	-	+	-	+	-	+
EC_ΔispG	-	+	-	-	-	-

Table 4.1 Complementation of *E. coli* knockout strains by expression of the corresponding FeS enzyme ortholog. Each knockout strain was transformed with an aTc-inducible expression vector encoding for the corresponding FeS enzyme ortholog, and tested for growth on suitable medium plates with or without aTc. After 24-48h of incubation at 30°C, complementation was measured as the ability (**+**) or inability (**-**) to grow, compared to the wild-type strain *E. coli* MG1655.

To investigate the catalytic properties of orthologs of this rate-limiting enzyme, we decided to explore the functional transferability of foreign IspGs in *E. coli*. For this purpose, we expanded our experimental approach to study the *in vivo* activity of IspG orthologs from 44 different organisms arbitrarily selected across diverse bacterial phyla. The genes encoding foreign IspGs were codon optimized and cloned into pBbS2k. The complete list of FeS orthologs used in this study can be found on **Supplementary Table S4.1**. Using our complementation assay with the *ispG* knockout strain, we show that only 7 out of 44 IspG orthologs (16%) are sufficiently active within *E. coli* to support growth. From this minority of active orthologs, 5 belong to the Proteobacteria phylum (**Figure 4.2a**): 3 from Gammaproteobacteria (protein sequence similarity between ~78-93% identity to *E. coli* IspG) and 2 variants from Betaproteobacteria (~29% identity). We assume that the conditional lethality is related to a lack of IspG activity that can be due to insufficient FeS incorporation or supply of electrons, resulting from uncharacterized molecular incompatibilities with the native FeS maturation

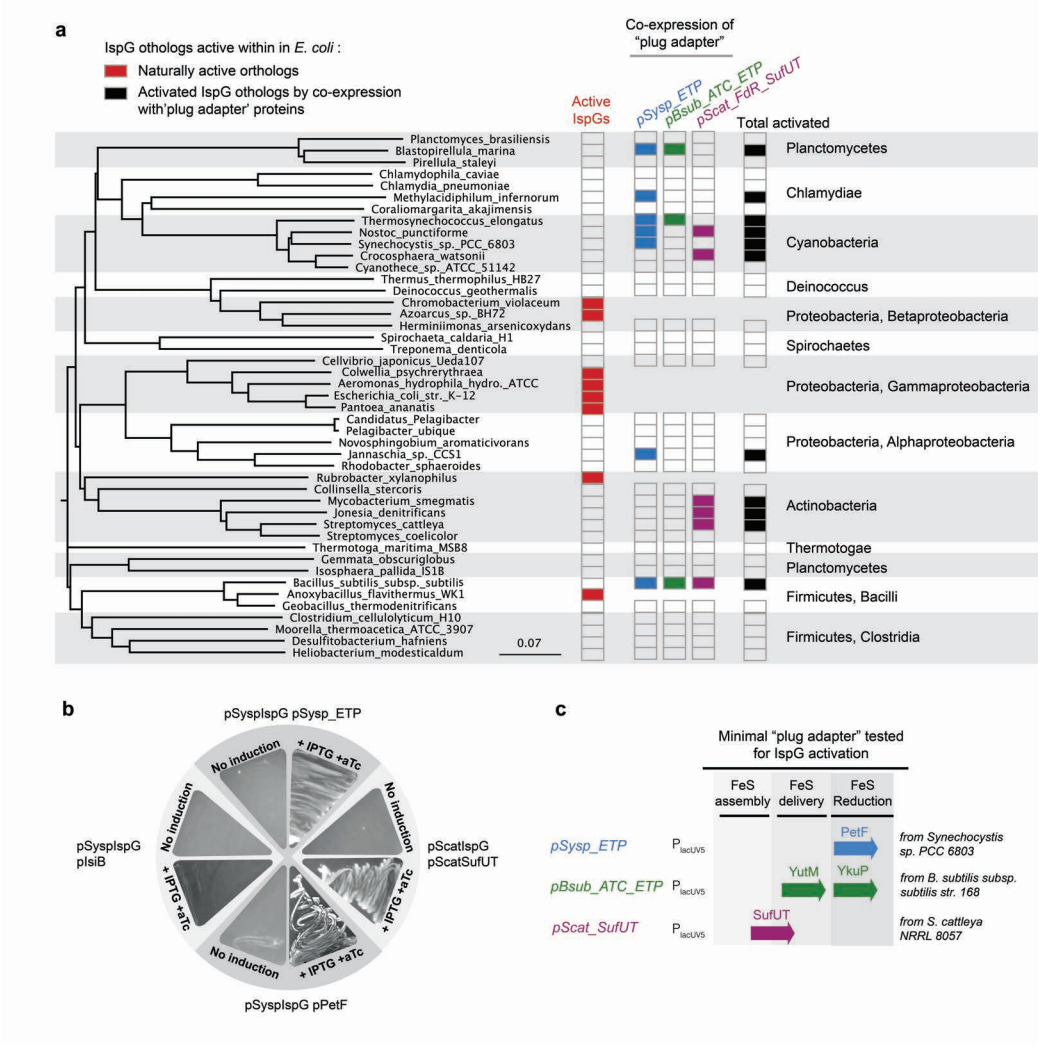

Figure 4.2 Protein 'plug adapters' enable heterologous activation of foreign IspGs in *E. coli*. Complementation assays of the *E. coli ispG* knockout strain (EC_ΔIspG) expressing a codon-optimized IspG ortholog. Co-expression with 'plug adapter' vectors was performed for inactive IspGs. Growth was tested on LB agar plates (containing IPTG and aTc), and compared to the wild-type strain *E. coli* MG1655 **(a)** Phylogenetic tree based on a multiple amino acid sequence alignment (MUSCLE) of the IspG variants from different selected bacterial organisms. Empty rectangles represent a lack of complementation. Colored rectangles represent the ability to grow when (co-)expressing the indicated vectors. **(b)** Pictures displaying growth on agar plates of EC_ΔIspG co-transformed with the indicated pIspG and 'plug adapter' vectors. **(c)** 'Plug adapter' vectors, expressing the minimal set of proteins from three different organisms that were proven successful in activating of IspG orthologs in *E. coli*.

and/or electron transfer pathways from *E. coli*. Although protein-protein interactions between FeS enzymes and interacting protein partners are

unpredictably selective, our *in vivo* assays in combination with the phylogenetic tree based on a MUSCLE[22] (Multiple Sequence Comparison by Log-Expectation) alignment of the selected IspG sequence, indicate that FeS enzymes are typically inactive when expressed within evolutionarily distant species. Our data supports the complexity hypothesis, which predicts that enzymes that require contacts with other proteins will sustain activity only within closely-related species[13].

4.2.3 ACTIVATING INCOMPATIBLE ISPG ORTHOLOGS IN *E. COLI*

In light of our IspG compatibility studies, we sought to identify the barriers to the functional expression of foreign IspGs within in *E. coli*, and explore how these could be circumvented to enable their heterologous activity. Given that the inactivity of foreign IspGs may be due to incompatibilities with the host's FeS biogenesis or electron transfer proteins, we investigated whether cognate proteins from foreign organisms could be used as context-dependent biological parts to enable heterologous IspG activity. Consequently, we sought to identify native proteins potentially involved in delivering FeS clusters and electrons to FeS enzymes that could act as specific 'plug adapter' proteins for IspG activation in *E. coli*. Studies of FeS cluster biochemistry and genetics have revealed some of the key molecular actors involved in FeS biogenesis and electron supply to FeS enzymes within their natural contexts[9,12]. Although the main principles of these processes are known, the specific molecular machineries and their characteristic interactions remain poorly understood for most organisms[23]. Moreover their functionality in foreign organisms has been poorly characterized. We hypothesized that the identification and co-expression of native biological parts potentially involved in FeS activity would activate and sustain foreign IspGs in *E. coli*. In order to identify FeS biogenesis and electron transfer proteins, we have performed an *in silico* analysis in combination with BLAST homology searches of the genomes of seven different organisms holding IspG orthologs that we have determined to be inactive: *B. subtilis*, *Coraliomargarita akajimensis, Rhodobacter sphaeroides, S. cattleya, Synechocystis sp. PCC 6803, Thermotoga maritima* and *Thermus thermophilus.* Supplementary **Table 4.2** summarizes the identified putative proteins involved in FeS assembly (SUF operons) or FeS delivery (ATCs, homologs of ErpA, SufA, IscA or NfuA), and a few selected ETPs (ferredoxins and flavodoxins) from the genomes of the selected organisms.

To identify which type of proteins potentially involved in FeS activity is capable of activating the IspG orthologs from their native organisms in *E. coli*, we constructed 'plug adapter' vectors to co-express them (**Figure 4.1b**). All the genes encoding for the given proteins were codon-optimized (except for those from *B. subtilis*) and cloned on separate plasmids compatible with those used to express IspG, and under control of an IPTG (isopropyl β -d-thiogalactopyranoside) inducible promoter P_{lacUV5} (pBbA5a). We constructed vectors expressing identified ETPs that may be implicated in electron supply to

IspG, and/or ATCs that may be required to deliver FeS clusters to foreign apoenzymes: pETP, pATC or pATC_ETP vectors listed in Supplementary **Table 4.3**. Next, we co-transformed *ispG* knockout strains harboring an IspG ortholog expression vector with a 'plug adapter' plasmid from the same organism; the co-transformants were subsequently used for complementation assays. Complementation results are shown in Table 2. Although the co-expression of most of our designed 'plug adapter' vectors did not restore growth, the expression of ATC and ETP orthologs from *B. subtilis* and *Synechocystis* activated the IspG orthologs from *B. subtilis* and *Synechocystis sp. PCC 6803* respectively. Therefore, lack of activity of some of these orthologs is consistent with a lack of effective interactions between foreign FeS enzymes and the proteins involved in FeS maturation and/or electron transfer pathways from the *E. coli* host.

Origin of IpGs orthologs	Type of "plug adapter" corresponding to each strain		
	pETP	pATC	pATC_ETP
Bacillus subtilis subsp. subtilis str. 168	-	-	+
Coraliomargarita akajimensis DSM 45221	-	-	-
Rhodobacter sphaeroides ATCC 17025	-	-	-
Streptomyces cattleya NRRL 8057 = DSM 46488	-	-	-
Synechocystis sp. PCC 6803	+	-	+
Thermotoga maritima MSB8	-	-	-
Thermus thermophilus HB27	-	-	-

Table 4.2 Complementation of *E. coli* ispG knockout strain (EC_ΔIspG) by co-expression of IspG orthologs with 'plug adapter' vectors from the same foreign organism. The ispG knockout was co-transformed with an IspG ortholog expression vector (aTc-inducible) and a 'plug adapter' vector (IPTG-inducible) encoding putative FeS activating enzymes from one of the listed bacterial organisms. Growth was tested on LB agar plates containing IPTG and aTc. Complementation was measured as the ability (+) or inability (-) to grow, compared to the wild-type strain *E. coli* MG1655.

Remarkably, the co-expression of the pSysp_ETP vector, carrying only ETPs from *Synechocystis sp. PCC 6803*, the ferredoxin PetF and the flavodoxin IsiB, was sufficient to activate the IspG ortholog from the same strain (SyspIspG). To further investigate whether PetF or IsiB is responsible for the activation of SyspIspG, we co-expressed each protein individually (using vectors pPetF and pIsiB), and tested them for complementation. While the co-expression of IsiB did not restore growth, the co-expression of PetF alone was sufficient to effectively activate SyspIspG (**Figure 4.2b**). Homologs of the ferredoxin PetF are known to supply electrons from photosystem I to cyanobacterial IspG enzymes[12], and have been used to sustain heterologous activity of IspG/H orthologs within *E. coli* [24]. Therefore we suspect that PetF is a functional electron donor to the IspG variant from *Synechocystis sp. PCC 6803*,

and constitutes an effective 'plug adapter' for this enzyme in *E. coli*. The electron donor of PetF remains unknown in *E. coli*.

We next tested whether co-expression of PetF could recover the activity of other inactive IspG orthologs. Our results show that pPetF is an effective 'plug adapter' for 6 additional IspGs (**Figure 4.2a, Table 4.3**), including from 2 other Cyanobacterial species, and hence demonstrate that ETPs show some functional promiscuity across bacterial species. Successful complementation resulting from the overexpression of heterologous ETPs indicates that such activated foreign IspGs are able to obtain FeS clusters from *E. coli* FeS biogenesis pathways, suggesting that the generation of holo-IspG is not a limitation to their transferrability. We thus hypothesize that for these IspG orthologs, insufficient electron supply resulting from molecular incompatibilities with the host's ETPs is the main barrier to their functional heterologous expression. In this case, we hypothesize that FeS biogenesis machineries from *E. coli* are compatible with this orthologs and ensure holoenzyme formation. Moreover, these specific foreign ETPs may additionally contribute to improving the regeneration of IspGs inactivated by oxidative damage in this chassis, hence benefiting isoprenoid synthesis.

Interestingly the activation of some the same IspG orthologs was achieved using a 'plug adapter' vector from *B. subtilis*, for which both the A-type carrier YutM and the flavodoxin YkuP (respective homologs of ErpA and FldA) were found to be required (Table 3). Indeed, the expression of pBsub_ATC_ETP successfully restores growth of auxotrophs expressing the IspG orthologs from *B. subtilis*, *Blastopirellula marina* and *Thermosynechococcus elongatus*, which are also activated by expression of pPetF (**Figure 4.2a**). The requirement for both ATC and ETP to maintain essential levels of active IspG in this case, suggests that individually the FeS delivery by YutM or electron supply by YkuP are insufficient to drive substantial IspG activity to sustain growth, or that YkuP might be required to maintain the reduced active state of the FeS cluster inserted by YutM. Further biochemical studies to characterize the interactions between IspG, YutM and YkuP are needed in order to underlying mechanisms involved in the activation of FeS orthologs by the YutM-YkuP 'plug adapter'.

Taking into account these considerations, we sought to discover a 'plug adapter' for activating the IspG ortholog from *S. cattleya* (ScatIspG). Indeed this strain and other Streptomycetes hold rate-limiting FeS enzymes involved in the biosynthesis of relevant chemicals, such as the antibiotics thienamycin[7] and fosfomycin[25]. Hence, the identification of proteins from *S. cattleya* required for the activation of ScatIspG could be harnessed to expand our understanding of FeS maturation in Streptomycetes species and potentially be applied for the activation of other biosynthetic FeS enzymes. Considering the fact that the co-expression of the specific ATCs (ScatATC1 and ScatATC2) and ETPs (ferredoxin ScatFdR and flavodoxin ScatFld) previously tested did not activate ScatIspG, we decided to investigate whether other untested proteins potentially

involved in FeS activation from *S. cattleya* could enable its activation in *E. coli*. Therefore, we codon-optimized and cloned 3 additional ferredoxins to generate pScat_FdR, as well as the proteins SufU and SufT from *S. cattleya* to generate pScat_SufUT and pScat_FdR_SufUT. The protein SufT contains a DUF59 domain of unknown function that has been proposed to be involved in the maturation of FeS enzymes within several organisms[26], while SufU is a scaffold protein involved in FeS biogenesis[9,27]. We show that pScatSufUT_FdR can activate ScatIspG and five other IspG orthologs, including from three Streptomycetes species (**Figure 4.2a, Table 4.4**). Complementation assays of the *ispG* knockout expressing these activated orthologs show that the co-expression of ScatSufUT is sufficient to activate the IspGs from Streptomycetes *S. cattleya* and *Jonesia denitrificans* (**Table 4.4, Figure 4.2b**), indicating that the incompatibilities related to the activity of ScatIspG in *E. coli* reside in its interaction with the SufU and SufT proteins involved in the biogenesis of the FeS cluster. However, the other activated orthologs require the co-expression of ETPs from pScat_FdR_SufUT. In all cases the vector pScat_FdR did not restore growth (Table 4). Our results suggest that that these orthologs might hold weaker interactions with the proteins ScatSufUT, which can be compensated by the co-expression of the ferredoxins. These ETPs could enhance electron transfer to the IspG orthologs as well as participate in maintaining the active redox state of the FeS cluster generated by ScatSufUT, hence maintaining essential levels of active IspG.

Altogether, our results demonstrate that the heterologous activation of FeS enzymes can be achieved by importing cognate proteins involved in FeS biogenesis and electron supply. Collectively, the co-expression of our discovered 'plug adapters' from only 3 bacterial species activated 11 out of 37 IspGs that we found to be inactive in *E. coli*. Moreover, we show that orthologs from different organisms require a different set of proteins involved in FeS activity (**Figure 4.2c**): while some enzymes can be activated by the co-expression of compatible ETPs alone (i.e. IspGs activated with pPetF), others require proteins that participate in the assembly and/or delivery of the FeS cluster (i.e. ScatIspG which is activated by SufUT alone). Interestingly, we also observe that distinct adapter proteins are able to activate the same IspG orthologs (such as the ones from *B. marina, Nostoc punctiform*, *T. elongatus* and *B. subtilis*). For instance, we show that inefficient electron transfer is likely the main barrier to the functional expression of IspG from *N. punctiform* (NpunIspG) in *E. coli*, as co-expression with the ferredoxin PetF is sufficient for restoring growth. However, activation of NpunIspG can also be achieved by co-expression with ferredoxins from *S. cattleya*, but only in presence of the proteins ScatSufUT, involved in FeS biogenesis (**Table 4.4**). This result reveals that the absence of complementation, resulting from poor molecular interactions between IspG orthologs and foreign FeS activation machineries, can be compensated by the expression of additional functionally compatible foreign proteins involved in other processes. While we show that some

functional promiscuity exists between FeS enzymes and FeS activation machineries from different organisms, their molecular compatibility and effective interactions (based on protein-protein contacts) remain unpredictable and poorly characterized.

		Origin of "plug adapter" proteins						
		Synechocystis sp.PCC 6803			*Bacillus subtilis str.168*			
	Name vector	pSysp_ATC_ETP	pSysp_ATC	pSysp_ETP	pBsub_ATC_ETP	pBsub_ATC	pBsub_ETP	
	Proteins expressed in "plug adapter" vector	PetF IsiB		PetF IsiB	YkuP		YkuNOP	Electron transfer proteins
		SyspATC1 SyspATC2	SyspATC1 SyspATC2		YutM	YutM		A-type carrier proteins
Origin of IpGs orthologs	*Bacillus subtilis subsp. subtilis str. 168*	+	-	+	+	-	-	
	Blastopirellula marina DSM 3645	+	-	+	+	-	-	
	Jannaschia sp. CCS1	+	-	+	-	n.d.	n.d.	
	Methylacidiphilum infernorum V4	+	-	+	-	n.d.	n.d.	
	Nostoc punctiforme	+	-	+	-	n.d.	n.d.	
	Synechocystis sp. PCC 6803	+	-	+	-	n.d.	n.d.	
	Thermosynechococcus elongatus BP-1	+	-	+	+	-	-	

Table 4.3 Identification of foreign 'plug adapter' proteins involved in the activation of IspGs orthologs in *E. coli*. Complementation of *E. coli* ispG knockout strain (EC_ΔIspG) by co-expression of IspG orthologs with 'plug adapter' vectors from B. subtilis and Synechocystis sp. PCC 6803. The ispG knockout was co-transformed with an IspG ortholog expression vector (aTc-inducible) and a 'plug adapter' vector (IPTG-inducible). Growth was tested on LB agar plates containing IPTG and aTc. Complementation was measured as the ability (**+**) or inability (**-**) to grow, compared to the wild-type strain E. coli MG1655. The conditions marked as 'n.d' were not determined.

In order to enable the activation of any given FeS ortholog in *E. coli*, a combination of biochemical studies, genome analysis and complementation assays are needed to accelerate the identification of molecular incompatibilities and effective 'plug adapters' proteins. Additional characterization of protein-protein interactions and structural analysis of IspG orthologs activated by our discovered 'plug adapters' may reveal some of the underlying criteria that determine the functional compatibility between FeS enzymes and foreign proteins, as well as fundamental principles of FeS biochemistry.

4.2.4 TESTING HETEROLOGOUS ISPGS FOR BISABOLENE PRODUCTION

To maximize the biotechnological impact of our ability to activate foreign ispG othologs, we sought to employ our technology for improving MEP-based biosynthesis of high-value chemicals. Indeed, successfully imported IspG orthologs from foreign organisms may improve MEP pathway performance by circumventing limitations of the native *E. coli* enzyme. To evaluate the activities of both native and functional foreign IspG orthologs in *E. coli,* we have engineered an MEP-based biosynthesis pathway for the production of

bisabolene, a sesquiterpene that can serve as both a biofuel precursor and fragrance molecule[28]. We have constructed IPTG-inducible MEP pathway expression vectors, each carrying a different IspG ortholog, to be tested in combination with aTc-inducible bisabolene production vectors expressing the bisabolene synthase from *Abies grandis*[29], IspA and IDI from *E. coli*, as well as native electron carriers or discovered 'plug adapters' associated with IspG activity (**Figure 4.3a**, vectors listed **in Supplementary Table S4.3**). These engineered vectors were co-transformed into *E. coli* BL21, and the resulting strains were used to prepare bisabolene production cultures. We tested whether this biosynthesis platform would allow us to identify foreign IspG orthologs with superior biochemical traits, which would result in higher bisabolene titers.

	Name vector	pScat_ETP	pScat_ATC	pScat_ATC_ ETP	pScat_FdR	pScat_FdR _SufUT	pScat _SufUT	
	Proteins expressed in "plug adapter" vector	ScatFld1 ScatFld2		ScatFld1 ScatFld2	ScatFdR1 ScatFdR2 ScatFdR3	ScatFdR1 ScatFdR2 ScatFdR3		Electron transfer proteins
			ScatATC1 ScatATC2	ScatATC1 ScatATC2				A-type carrier proteins
						ScatSufU ScatSufT	ScatSufU ScatSufT	FeS assembly proteins
Origin of IpGs orthologs	*Streptomyces cattleya NRRL 8057 = DSM 46488*	-	-	-	-	+	+	
	Jonesia denitrificans DSM 20603	-	-	-	-	+	+	
	Mycobacterium smegmatis str. MC2 155	-	-	-	-	+	-	
	Bacillus subtilis subsp. subtilis str. 168	-	-	-	-	+	-	
	Crocosphaera watsonii WH 8501	-	-	-	-	+	-	
	Nostoc punctiforme	-	-	-	-	+	-	

Table 4.4 Identification of foreign 'plug adapter' proteins from *S. cattleya* involved in the activation of IspG orthologs in *E. coli.* Complementation of *E. coli ispG* knockout strain (EC_ΔIspG) by co-expression of IspG orthologs with 'plug adapter' vectors from *S. cattleya*. The *ispG* knockout was co-transformed with an IspG expression vector (aTc-inducible) and a 'plug adapter' vector (IPTG-inducible). Growth was tested on LB agar plates containing IPTG and aTc. Complementation was measured as the ability (**+**) or inability (**-**) to grow, compared to the wild-type strain *E. coli* MG1655

First, we confirmed the biological production of bisabolene using a control strain overexpressing an incomplete MEP pathway without the enzymes IspG and IspH (BL21 pMEP_neg pBIS_idi_ispA), which produced 0.5 mg bisabolene per L per OD_{600} of culture (**Figure 4.3b**). However, cultures of strains overexpressing the native IspG from *E. coli* (BL21 pMEP_EcIspG pBIS_idi_ispA) produced undetectable levels of bisabolene. A similar drop in product titers caused by overexpression of IspG has been previously observed in various studies[18,30,31], and could result from unexpected feedback effects on the activity of upstream and downstream pathway enzymes, or the potential

accumulation of inhibitors or toxic metabolites. Based on recent studies that demonstrate improved MEP pathway performance by overexpression of native ETPs FldA and Fpr[18], we constructed a bisabolene production vector that additionally contains the genes *fldA* and *fpr* from *E. coli* (pBIS_idi_ispA_FldA_Fpr), and tested it in combination with pMEP_EcIspG. Improved electron transfer to EcIspG by co-expression of FldA and Fpr significantly boosted bisabolene productivity by 7.7-fold compared to the control strain BL21 pMEP_neg pBIS_idi_ispA (**Figure 4.3b**), and reaching titers of 16.5 mg product per L of culture (**Supplementary Figure S4.1b**).

Next, we tested the ability of our engineered 'plug adapters' to activate IspG orthologs in the context of MEP-based bisabolene production. Since we have demonstrated that the activities of these orthologs rely upon specific foreign proteins, we have combined their incorporation into the MEP pathway with the overexpression of the corresponding 'plug adapter' proteins. A bisabolene production vector containing the 'plug adapter' from *B. subtlilis* (pBIS_idi_ispA_YutM_YkuP) was used to evaluate the activities of the IspG orthologs from *B. subtlilis, B. marina* and *T. elongatus*. Moreover, an additional bisabolene production vector carrying the ferredoxin PetF from *Synechocystis sp. PCC 6803* (pBIS_idi_ispA_PetF) was constructed to test the activities of the IspGs from *Synechocystis sp. PCC 6803*, *B. subtlilis, B. marina*, *Methylacidiphilum infernorum* and *T. elongatus*. For all MEP pathway vectors containing these IspG orthologs, we tested production in combination with the expression of pBIS-idi-ispA (without any 'plug adapter' protein) as a control.

Although our complementation assays indicate that the A-type carrier and flavodoxin proteins from *B. subtilis* improve the catalytic activity of some IspG orthologs, their overexpression in the context of our biosynthetic platform did not enhance bisabolene production (**Figure 4.3a**). Indeed, for all the MEP pathway vectors containing an IspG ortholog previously activated with YutM and YkuP, the co-expression with pBIS_idi_ispA_YutM_YkuP significantly decreased bisabolene production compared to the strains co-expressing pBIS_idi_ispA (**Supplementary Figure S4.1a**). As overexpression of YutM and YkuP does not significantly affect cell growth (**Supplementary Figure S4.2**), it is unclear at this point how these proteins interfere with bisabolene biosynthesis. Since ATCs and ETPs are known to display some functional promiscuity, potential cross-reactivity with other enzymatic pathways of the *E. coli* host could decrease the flux through the MEP pathway.

We find that the co-expression of the ferredoxin PetF results in significantly increased bisabolene titers when combined with IspG orthologs from *Synechocystis sp. PCC 6803* or *T. elongatus* (vectors pMEP_SyspIspG and pMEP_TeloIspG). However, bisabolene production is slightly reduced in all the other strains (**Supplementary Figure S4.1a**). We assume that the boost in

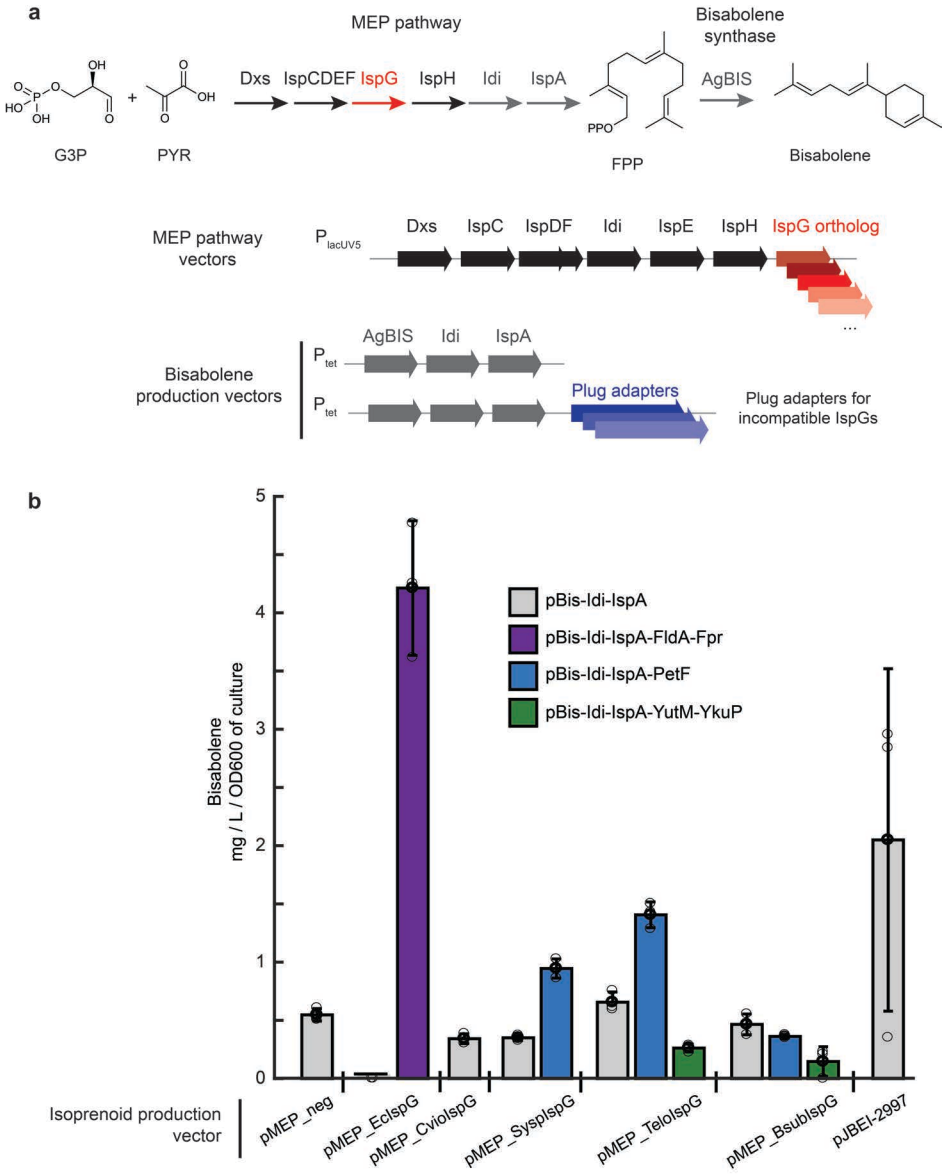

Figure 4.3 Using protein 'plug adapters' to test activity of foreign IspGs for bisabolene production (a) The native MEP pathway (comprised of the enzymes Dxs and IspCDEFGH) combined with isoprenyl diphosphate isomerase (Idi) and farnesyl diphosphate synthase (IspA), synthetize farnesyl diphosphate (FPP) from the precursors glyceraldehyde-3-phosphate (G3P) and pyruvate (PYR) in *E. coli*. The enzyme AgBIS converts FPP into bisabolene. Schematic of inducible vectors constructed to engineer bisabolene production in *E. coli* are presented. **(b)** Bisabolene productivity (mg bisabolene per L of culture per OD_{600}) recorded 24 h after induction with aTc and IPTG. Bars represent the mean productivity ± s.d. of three biological triplicates from strains co-transformed with the indicated vectors.

bisabolene productivity (defined as mg bisabolene per L per OD_{600} of culture) observed with PetF expression in the strains BL21 pMEP_SyspIspG and BL21 pMEP_TeloIspG (2.7 and 2.1-fold increase respectively) sults from enhanced

specific electron transfer to cyanobacterial IspGs. Electron transfer to IspG orthologs could be further increased by the co-expression of the native ferredoxin-NADP oxidoreductase PetH[12,32] from *Synechocystis sp. PCC 6803.*
To evaluate the catalytic potential of foreign IspGs that were determined to be active and compatible with *E. coli* FeS pathways, we produced bisabolene using MEP pathway vectors containing the compatible IspG orthologs from *Chromobacterium violaceum* (CvioIspG), *Rubrobacter xylanophilus* (RxylIspG) and *Pantoea ananatis* (PanaIspG). The strain overexpressing CvioIspG (BL21 pMEP_CvioIspG pBIS_idi_ispA) produced 3.2 mg per L of culture, resulting in a 1.7-fold increase compared to the control strain BL21 pMEP_neg pBIS_idi_ispA (**Supplementary Figure S4.1**).

Finally, we have compared bisabolene titers obtained from our MEP-based isoprenoid production platform with a synthetic MEV pathway from *S. cerevisiae* (vector pJBEI-2997), which was previously used to produce bisabolene in *E. coli*[29]. We confirmed that the molecular nature of the biosynthetic bisabolene obtained with both MEV and MEP-based isoprenoid pathways was identical using GC/MS and a commercial bisabolene standard (**Supplementary Figure S4.3**). **Figure 4.3a** shows that than our best MEP-based producer (strain BL21 pMEP_EcIspG pBIS_idi_ispA_FldA_Fpr) displays a 2-fold higher productivity than cultures of BL21 pJBEI-2997 pBIS_Idi_ispA. However, when comparing the bisabolene titers obtained with these same strains, cultures expressing the MEV pathway produced 10% higher titers up to 18.1 mg per L of culture (**Supplementary Figure S4.1b**). Additional efforts to improve the pathway performance of and cell growth of our MEP-based isoprenoid production platforms will be necessary in order to surpass titers obtained with the MEV pathway. Strategies for further enhancing flux through such recombinant MEP pathways could require the co-expression of cognate oxidoreductases (such as Fpr or PetH) to increase electron supply to IspG/H, as well as balancing protein levels of MEP pathway enzymes and 'plug adapter' proteins. Altogether these results demonstrate the difficulties in predicting how the introduction of enzyme orthologs with different biochemical traits alters flux through complex multi-enzyme pathways.

4.2.5 ACTIVATION OF A HETEROLOGOUS RADICAL SAM FES ENZYME FOR FOSFOMYCIN BIOSYNTHESIS

The radical S-adenosylmethionine (sSAM) FeS superfamily of enzymes use SAM and a [4Fe–4S] cluster to catalyse challenging radical reactions that are involved in the biosynthesis of diverse high-value chemicals[33–35]. Many relevant antibiotic biosynthetic pathways found in difficult-to-culture organisms[6], or in organisms that are not suited for industrial fermentation[36], contain one or several rSAM FeS enzymes. A method that can reliably activate these enzymes within foreign hosts, such as *E. coli,* must be found before these promising natural products are to ever be produced on any scale whatsoever using heterologous biosynthesis[37]. In particular, rSAM FeS enzymes that participate

in important antibiotic pathways are found in a variety of Streptomyces species[7,8,25], and therefore the activation of such enzymes in *E. coli* would be of significant relevance. Our complementation assays of the *thiC* knockout expressing the ThiC ortholog from *S. cattleya* (ScatThiC) found this rSAM FeS enzyme to be inactive in *E. coli* (**Table 4.1**). However co-expression of the 'plug adapter' vector pScat_FdR_SufUT restored the growth of the knockout, indicating heterologous activation of the rSAM ScatThiC enzyme (**Supplementary Figure S4.4**). Encouraged by these results, we sought to test the applicability of our 'plug adapters' to engineer heterologous biosynthesis of the antibiotic fosfomycin in *E. coli*.

Fosfomycin is a broad-spectrum antibiotic that irreversibly inhibits the cytosolic enzyme MurA (UDP-N-acetylglucosamine enolpyruvyl transferase) essential for cell wall biosynthesis[38]. Nevertheless, its clinical use had remained restricted in favor of other antibacterial drugs since its approval in the 1970s[39]. Due to its potency, unique structure and lack of cross-resistance with other common antibiotic compounds, fosfomycin has been proposed as an efficient alternative to treat multi-drug resistant infections[39,40]. Fosfomycin is synthetized in *S. wedmorensis* from PEP and requires the action of at least 6 enzymes, including the rSAM cobalamin-dependent FeS enzyme Fom3 (**Figure 4.4a**). This enzyme was recently shown to catalyze the methylation of HEP-CMP to form HPP-CMP[20,25], and is notorious for displaying poor solubility and cofactor incorporation upon expression in *E. coli*[41,42]. Consequently, heterologous activity of Fom3 in *E. coli* has not been reported.

We sought to test the efficacy of our discovered 'plug adapter' from *S. cattleya* to enable functional expression of Fom3 and heterologous biosynthesis of fosfomycin in *E. coli*. We cloned the codon-optimized fosfomycin pathway from *S. wedmorensis* into an IPTG-inducible high-copy number plasmid (pFom34D12C), and constructed additional plasmids carrying 'plug adapter' proteins from *S. cattleya* in the same operon (to make the plasmids pFom34D12C_ScatSufUT, pFom34D12C_ScatFdR_SufUT and pFom34D12C_ScatFdR). Moreover, since the activity of Fom3 relies on a cobalamin cofactor that is not naturally synthetized by the host, we have engineered a vector for overexpression of proteins that improve cobalamin uptake and transport in *E. coli,* based upon a recent result demonstrating improved folding of cobalamin-dependent radical SAM enzymes[42] (pBtuCEDFB). Fosfomycin production was performed using *E. coli* NCM3722 cultures co-transformed with pBtuCEDFB and a fosfomycin pathway vector, grown in defined medium supplemented with cobalamin. Detectable levels of fosfomycin were obtained in the control culture NCM3722 pBtuCEDFB pFom34D12C, successfully demonstrating heterologous production of the antibiotic in *E. coli*. The co-expression of the ferredoxins and SufUT proteins

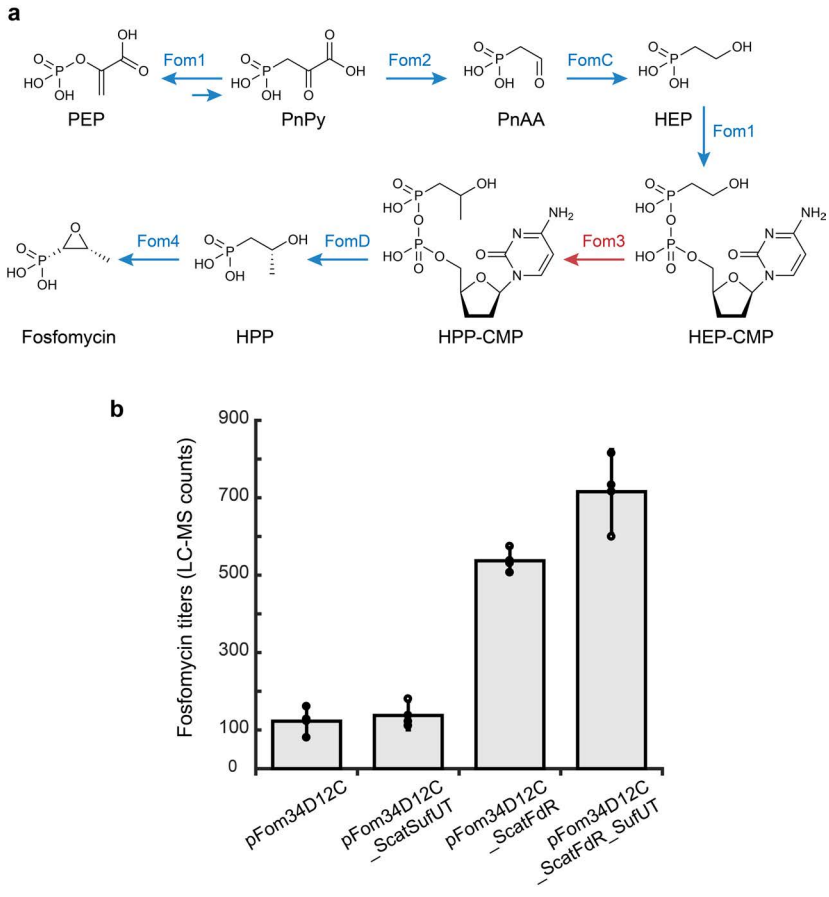

Figure 4.4 Protein 'plug adapters' increase fosfomycin biosynthesis in *E. coli.* (a) In *S. wedmorensis*, the enzyme Fom1 produces phosphonopyruvate (PnPy) from phosphoenolpyruvate (PEP), which is subsequently converted into phosphonoacetaldehyde (PnAA) by the decarboxylase Fom2. The alcohol dehydrogenase FomC then produces 2-hydroxyethyl-phosphonate (HEP) from PnAA. Recently it has been proposed that the cytidylyltransferase domain of Fom1 catalyzes the synthesis of cytidylyl-2-hydroxyethyl-phosphonate (HEP-CMP) from HEP, which is subsequently methylated by Fom3 (in red) to yield cytidylyl-2-hydroxypro- pylphosphonate (HPP-CMP). The enzyme FomD has been proposed to hydrolyze HPP-CMP into (S)-2-hydroxypropylphosphonate ((S)-HPP). Finally, the peroxidase Fom4 produces fosfomycin from (S)-HPP. **(b)** Fosfomycin titers (LC-MS counts) recorded 4 h after induction (by addition of IPTG and aTc) in cultures of *E. coli* NCM3722 co-transformed with pBtuCEDFB and different fosfomycin pathway vectors. Bars represent the mean ± s.d. of three biological replicates.

from *S. cattleya* with the fosfomycin pathway (strain NCM3722 pBtuCEDFB pFom34D12C_ScatFdR_SufUT) further increases antibiotic titers by 5.8-fold, compared to the strain NCM3722 pBtuCEDFB pFom34D12C (**Figure 4b**). Early estimations of antibiotic titers obtained with our best producers indicate a production of fosfomycin in the range of 0.1 mg per liter of culture. Measurements of the pathway intermediate HPP-CMP indicate that the 'plug

adapter' proteins increase flux through the fosfomycin pathway by improving the catalytic activity of Fom3 (**Supplementary Figure 5**). Moreover, our results suggest that the boost in fosfomycin production is mainly due to the overexpression of the ferredoxins from *S. cattleya*, instead of ScatSufUT. Further studies are needed in order to identify which of these ferredoxins specifically improves electron transfer to Fom3 in this context.

4.3 DISCUSSION

Here we present a simple complementation assay to test the activity of foreign FeS enzymes in *E. coli*. Our results demonstrate that FeS enzymes are often inactive when expressed within evolutionarily-distant species, likely due to molecular incompatibilities with the host's FeS biogenesis and electron transfer pathways. Our results illustrate that interactions between FeS enzymes and interacting protein partners required for activity remain unpreductable. Next we show that the identification and expression of biological parts from heterologous organisms involved in FeS activity can be used as 'plug adapters' to activate incompatible foreign FeS enzymes in *E. coli*. The co-expression of 'plug adapter' proteins from only 3 different bacterial species was sufficient to activate 30% of the incompatible IspG orthologs reported in this study. These results validate our approach for using genome mining to find FeS biogenesis proteins and ETPs that drive FeS catalysis, and establish a proof-of-concept for how foreign FeS orthologs can be activated within *E. coli*.

Our complementation experiments can contribute to improving our understanding of the biochemical barriers to horizontal gene transfer of FeS enzymes. The identification of effective 'plug adapter' proteins that activate FeS orthologs sheds light on the nature of the molecular incompatibilities between FeS enzymes and foreign FeS activating proteins. We show that insufficient electron transfer resulting from incompatible interactions with *E. coli*'s ETPs is the main barrier to the functional expression of some FeS orthologs, while others require the co-expression of compatible proteins involved in FeS biogenesis. However other factors that are not linked to specific molecular incompatibilities could result in a lack of complementation. Indeed, additional factors such as inadequate protein expression, protein insolubility, or database sequencing errors, should be taken into account. Moreover, many potential 'plug adapter' ETPs are FeS proteins (i.e. ferredoxins), and might also require the action of other specific unidentified proteins for their activation in heterologous hosts. Increasing the throughput of our experimental approach, in combination with extensive genome mining, can in principle be a straightforward strategy to greatly expand the range of activated FeS enzymes within foreign hosts. In order to enable the activation of any given FeS ortholog in *E. coli*, a combination of biochemical studies, genome analysis and complementation assays are needed to accelerate the identification of

molecular incompatibilities and effective 'plug adapters' proteins. Additional characterization of protein-protein interactions, and structural analysis of activated IspG orthologs, may reveal some of the underlying criteria that determine the functional compatibility between FeS enzymes and foreign proteins, as well as fundamental principles of FeS biochemistry.

The principles established in this project to enable the functional expression of heterologous FeS enzymes in *E. coli* were applied to importing foreign biosynthetic enzymes involved in the production of high-value chemicals (e.g. isoprenoids and antibiotics). In particular, we show that the identified 'plug adapter' proteins from *S. cattleya* can activate foreign rSAM enzymes, and improve flux through the fosfomycin pathway by improving the catalytic activity of the enzyme Fom3. These results are a significant breakthrough in microbial biosynthesis, as fosfomycin production in *E. coli* had not been previously demonstrated. Given that many foreign rSAM enzymes participate in the biosynthesis of antibiotic, anticancer, and antifugal compounds, we hope our work will lead to many other efforts to reconstruct and optimize these pathways within tractable, fermenter-friendly hosts. Further studies will be required to investigate the potential collateral effects of overexpressing protein 'plug adapters' on the general metabolism and physiology of the host.

Because of a wide array of high-value natural products require FeS enzymes for their biosynthesis, engineered 'plug adapters' could be harnessed as key enabling technologies for the reliable use of FeS enzymes in biotechnology. Indeed, this might enable the exploitation of many FeS-dependent pathways that are currently incompatible with industrially-relevant species, such as *E. coli* and other widely-used platform strains. In the long-term, the bio-based manufacturing of FeS enzyme-dependent chemicals from sustainable resources will impact many sectors across the global economy.

4.4 MATERIALS AND METHODS

4.4.1 CONSTRUCTION OF KNOCKOUT STRAINS

In order to construct *ispG* and *ispH* knockout strains, we have first constructed the *E.coli* strain MG1655_IMBS that expresses the enzymes of the lower MEV pathway from *S. cerevisiae* required to produce DMAPP and IPP from mevalonate. The codon-optimized operon encoding mevalonate kinase (MK), phosphomevalonate kinase (PMK) and mevalonate diphosphate decarboxylase (PMD) under the control of a P_{BAD} promoter was amplified from pJBEI-2999, and assembled by PCR to a FRT-flanked kanamycin resistance (KanR) cassette from pKD13. The resulting DNA fragment was further amplified with primers containing homology arms directed into the *intA* gene (primers in **Supplementary Table S4.4**). The operon was introduced into the genome of *E. coli* MG1655 using homologous recombination with a strain harboring

pKD46. After verifying genomic insertions with PCR, we have removed the KanR cassette using the plasmid pCP20. Deletions of *ispG*/*H* in the resulting strain MG1655_IMBS can be rescued by supplementing the growth medium with mevalonate. We have generated PCR products that contained the FRT-flanked KanR cassette amplified from pKD13 and 30-40 bp 5' extensions with homology to regions flanking *ispG* and *ispH* respectively. Recombination was performed to generate the knockouts upon electroporation of the PCR products in MG1655_IMBS cells containing pKD46. After removal of the KanR cassette with pCP20, the resulting strains EC_ΔispG and EC_ΔispH were verified by DNA sequencing and complementation assays with mevalonate. For constructing the knockout strains ΔnadA and ΔilvD we have amplified the FRT-flanked KanR cassette from pKD13 with 5' homology extensions directed to the *nadA* and *ilvD* genes and transformed the resulting PCR products into *E. coli* MG1655 containing pKD46. We have verified the conditional lethality of the resulting knockouts with complementation assays using minimal medium, and removed the KanR cassette with pCP20. Gene deletions were verified by DNA sequencing.

4.4.2 VECTORS FOR COMPLEMENTATION ASSAYS

All expression vectors were constructed using BglBrick standard vectors as backbones (pBb plasmids) in *E. coli* DH5α. Genes encoding the FeS enzyme orthologs (*bioB*, *ilvD*, *thiC*, *nadA*, *ispH* and *ispG*) were codon optimized for expression in *E. coli*, except for the variants cloned from *Bacillus subtilis str.168* (NC_000964) genomic DNA (primers in **Supplementary Table S4.4**). All the FeS enzyme genes were cloned into pBbS2k between the EcoRI and XhoI sites. To construct the vectors expressing FeS biogenesis proteins and electron carriers, we have codon optimized the selected foreign genes for expression in *E. coli*, and designed synthetic operons with strong ribosome binding sites. All these helper proteins were placed on pBbA5a between the EcoRI and XhoI sites. *yutM* and *ykuNOP* were cloned from *B. subtilis str.168* (NC_000964) genomic DNA and inserted into pBbA5a with Gibson assembly to make pYutM and pYkuNOP respectively using the primers P1 and P2 in **Supplementary Table 4**. These plasmids were used as templates to construct pYkuP_YutM using Gibson assembly.

4.4.3 MEP PATHWAY AND BISABOLENE PRODUCTION VECTORS

We have used Gibson assembly to construct all plasmids in *E. coli* DH5α. Genes encoding the MEP pathway enzymes were cloned from *E. coli* MG1655 genomic DNA, then assembled and cloned into pBbA5k to form pMEP_EcIspG. Subsequently this plasmid was used to construct derivative MEP pathway vectors where the *ispG* gene from *E. coli* was replaced with genes of foreign IspG orthologs amplified from the respective expression vectors. The plasmid pBIS_idi_ispA was constructed by introducing the codon optimized bisabolene

synthase gene from pTrcAgBIS (Addgene) with *idi* and *ispA* from pBbA5c-MevT(CO)-T1-MBIS(CO, ispA) (Addgene) into the pBbE2a backbone. Subsequently this plasmid was used as a backbone to construct all the bisabolene production vectors listed in **Supplementary Table S4.4**: *fldA* and *fpr* were cloned from *E. coli* BL21(DE3) genomic DNA; the operon containing *ykuP* and *yutM* was amplified from pYkuP_YutM; *petF* and *petH* from *Synechocystis sp. PCC 6803* were codon optimized for expression in *E. coli*. All the primers used to amplify genes from genomic DNA are listed in **Supplementary Table S4.4**.

4.4.4 COMPLEMENTATION OF KNOCKOUT STRAINS WITH FES ORTHOLOGS AND PLUG ADAPTERS.

To test the functionality of FeS enzyme variants from foreign organisms in *E. coli*, we transformed the plasmids expressing enzyme orthologs of NadA, BioB, IlvD, ThiC, IspG and IspH into the respective auxotroph strains. Transformants were grown on selective LB agar plates at 37°C overnight. For the strains EC_ΔispG and EC_ΔispH plates were supplemented with 1 mM MVA. We tested complementation of the NadA, BioB, IlvD and ThiC knockouts by striking single transformant colonies on selective MOPS-based minimal medium[43] agar plates (supplemented with 4 g L^{-1} D-glucose, 28.5 mM NH_4Cl, 10 μM $FeSO_4$ and 5 g L^{-1} potassium glutamate) with or without anhydrotetracycline (50 ng / mL); plates were incubated for 48 h at 30°C. To test heterologous complementation of IspG/H, single colonies were manually picked and striked on selective LB agar plates with or without anhydrotetracycline (50 ng / mL), and incubated at 30°C for 24-48 h. Subsequently, transformants that were not recovered by ispG/H expression were co-transformed with 'plug adapter' vectors (**Supplementary Table S4.3**) and plated on selective LB agar plates containing 1 mM MVA and 0.25 mM IPTG. After overnight incubation at 37°C, the growth of the resulting strains was tested by plating single colonies on plates containing antibiotics with or without anhydrotetracycline (50 ng / mL) and/or IPTG (0.25 mM). Plates were incubated at 30°C for 24-48 h.

4.4.5 BISABOLENE PRODUCTION PROTOCOL

For bisabolene production experiments, we used *E. coli* BL21(DE3) co-transformed with an isoprenoid biosynthesis vector (constructed MEP pathway vectors or pJBEI-2997) and a bisabolene production vector (all plasmids listed in **Supplementary Table S4.3**). Transformants were grown overnight on selective LB agar plates at 37 °C. Pre-cultures of engineered *E. coli* BL21(DE3) strains were prepared by picking individual colonies into 5 mL selective bisabolene production medium (8.372 g L^{-1} MOPS, 0.717 g L^{-1} tricine, 2.92 g L^{-1} NaCl, 11 mg L^{-1} $MgCl_2$ $7H_2O$, 0.56 μg L^{-1} $CaCl_2$, 1.32 mM K_2HPO_4, 27.6 mM K_2SO_4, 10 μM $FeSO_4$, 200 μL micronutrient stock) supplemented with 10 g L^{-1} D-glucose, 28.5 mM NH_4Cl, and 2 g L^{-1} casamino acids (Sigma-Aldrich

22090). The antibiotics kanamycin (25 μ g/mL), ampicillin (50 μ g/mL) and chloramphenicol (17.5 μ g/mL) were added depending on the appropriate plasmids. From pre-cultures incubated overnight with shaking at 37 °C, bisabolene production cultures were seeded at an initial OD_{600} of 0.08 in 25 mL of selective production medium in 250 mL Erlenmeyer flasks. Cultures were grown at 37 °C with shaking (250 r.p.m.) until reaching OD_{600} = 0.8–1.0; at this point cultures were induced with 0.5 mM IPTG and 50 ng/mL anhydrotetracycline (aTc). Cultures were overlayed with 4 mL dodecane and incubated at 30 °C with shaking (180 r.p.m.). Dodecane overlay samples were collected 24 h after induction and stored at -20 °C until analysis. To confirm the biological production of bisabolene, undiluted samples were analyzed by GC / MS with a Zebron ZB50 column (30 m × 250 μm x 0.25 μm) using the following settings: injector at 225 °C, split 1:15, constant flow at 1.1-1.2 mL/min, transfer line at 250 °C, ion source and detector at 200 °C. Oven conditions: 100 ° C for 1 min, ramp at 5 °C min^{-1} to 220 ° C, hold 2 min. For quantifying bisabolene titers, 20 μL of dodecane overlay were diluted into 80 μL ethyl acetate spiked with caryophyllene, and analysed using GC-214 Gas Chromatograph (Shimadzu) equipped with a FID-Detector, a AOC-20i auto injector and an Agilent CP-Sil 5 CB column (25 m × 0.5 mm ID × 1.2 µm film) using a caryophyllene standard curve and the following conditions: inlet at 225°C; 68 kPa constant; temperature program of 100°C hold 1 min; 20 °C $min^{-1}$to 195 °C hold 2 min, 5°C min^{-1} to 220 °C hold 1 min; Split Ratio 20. Retention times: caryophyllene 12.64 min; bisabolene 14.42 min.

4.4.6 FOSFOMYCIN PRODUCTION VECTORS

Genes encoding the enzymes of the fosfomycin biosynthesis pathway from *S. wedmorensis* (Fom34D12C) were codon optimized for expression in *E. coli*. The biosynthetic was assembled and cloned into the pBbE5k BglBrick backbone using Gibson assembly, to produce pFom34D12C. The plasmid pBtuCEDFB was constructed by amplifying the corresponding genes from the genome of *E. coli* MG1655 (using the primers P1 and P2 listed in **Supplementary Table S4.4**) and cloned into the pBbS2c BglBrick backbone. The operons ScatSufUT, Scat_FdR_SufUT and ScatFdR were placed on pFom34D12C between the BamHI and XhoI sites to generate pFom34D12C_ScatSufUT, pFom34D12C_ Scat_FdR_SufUT and pFom34D12C_ ScatFdR respectively.

4.4.7 FOSFOMYCIN PRODUCTION PROTOCOL

The strain *E. coli* NCM3722 was used for fosfomycin production experiments. We used cells freshly co-transformed with a fosfomycin production vector and the plasmids pBtuCEDFB encoding proteins for cobalamin uptake and transport. Transformants were grown overnight on selective LB agar plates at 37 °C. Antibiotic production cultures were grown in MOPS-based defined

fosfomycin production medium (8.372 g L^{-1} MOPS, 0.717 g L^{-1} tricine, 2.92 g L^{-1} NaCl, 11 mg L^{-1} $MgCl_2$ $7H_2O$, 0.62 μg L^{-1} $CaCl_2$, 1.32 mM K_2HPO_4, 27.6 mM K_2SO_4, 200 μ L micronutrient stock, 162.5 μM $FeCl_3$, 100 μM $MnCl_2$, 0.92 g L^{-1} $MgSO_4$ $7H_2O$) supplemented with 10 g L^{-1} D-glucose and 2.5 g L^{-1} casamino acids (Sigma-Aldrich 22090), 3.7 mg L^{-1} thiamine and 12.6 mg L^{-1} hydroxocobalamin. Pre-cultures of engineered strains were prepared by picking individual colonies into 5 mL selective fosfomycin production medium. From pre-cultures incubated overnight with shaking at 37 °C, fosfomycin production cultures were seeded at an initial OD_{600} of 0.02 in 8 mL of selective production medium in culture tubes. Cultures were grown at 37 °C with shaking (250 r.p.m.) until reaching OD_{600} = 0.08–0.1. Expression of pBtu was induced by addition of 50 ng/mL aTc. The cultures were further incubated until reaching OD_{600} = 0.5, after which fosfomycin production vectors were induced with 0.25 mM IPTG and the medium was supplemented with 150 μM cysteine and 32.5 μM $FeCl_3$. Cultures were then places on a tube rotator at 40 r.p.m. and incubated for 3-4 h at room temperature (20–21 ºC). Subsequently 0.5 mL of culture was sampled and centrifuged at 15,000g for 2 min. The supernatant was carefully discarded and the pellets were resuspended in 1 mL of a solution mixture of methanol, acetonitrile and water at a ratio of 5:3:1 respectively) with 0.1% formic acid. Samples were then dried by vacuum centrifuge, and the pellet resuspended in 100 μL of the same organic solvent mixture before analysis.

4.4.8 LC-MS ANALYSIS

Metabolite levels in fosfomycin producing cultures supernatants were measured using LC–MS. For each sample, 5 μL was injected onto a Merck Millipore peek ZIC-pHILIC column (150x2.1mm, 5μm, PN1504600001) equipped with a precolumn (20x2.1mm PN1504370001). Liquid chromatography separation was conducted at 45 °C using a LC–MS system (Agilent) consisting of a binary pump (G1312B), an autosampler (G7167A), a temperature-controlled column compartment (G1316A) and a triple quadrupole mass spectrometer (G6460C) equipped with a standard ESI source. The mobile phase was composed of 20 mM ammonium carbonate pH 9.2 (solvent A) and 20 mM ammonium carbonate pH 9.2 in 80% acetonitrile (solvent B). After a hold of 2 min at 100% solvent B, samples were separated with a gradient from 100% to 55% of solvent B for 15 min at a flow rate of 0.2 mL/min, which was followed by a gradient from 55% to 100% for 1.5 min at a flow rate of 0.2 mL/min, followed by an increase from 0.2 mL/min to 0.4 mL/min for 1.5 min, held at 100% solvent B for 2 min at 0.4 mL/min, then the flow was reduced to 0.2 mL/min in 2 min. Peaks were analyzed by MS using ESI ionization in MRM mode. The precursor ion analyzed for each compound was determined by mass calculation based on their chemical formula. For each compound, fragmentation spectra and MRM settings are found in **Supplementary Table S4.5**. Antibiotic titers were calculated using a fosfomycin standard curve.

4.4.9 STATISTICS

Unless otherwise noted in the figure legend, bars and lines depict averages of independent cell cultures grown from independent bacterial colonies, by which we define biological replicates. Sample sizes are described in the figure legends. Error bars represent ± 1 s.d. from the mean. Dots in figures report values obtained from independent biological replicates.

4.5 SUPPLEMENTARY INFORMATION

Supplementary Table S4.1 – Origin and amino acid sequence of IspG orthologs used in this study

Organism of origin	Length	Amino-acid sequence
Aeromonas hydrophila hydro. ATCC 7966	371	MSAHESPIIRRKSRQIMVGNVPVGGDAPITVQTMTNTKTTDVAATVEQIQRIERVGADIVRVSVPTMEAAEAFKLI KQQTRIPIVADIHFDYRIALKVAEYGADCLRINPGNIGNEERVRAVVDCARHYNIPIRIGVNGGSLEKDIQEKYGEP TPEALLESAMRHVDYLDRLNFDNFKVSVKASDVFLAVGAYRLLAKQIEQPLHLGITEAGGFRAGAVKSAIGLGML LSEGIGDTLRISLAADPVEEIKVGFDILKSLRIRSRGINFIACPSCSRQEFDVIGTVNALEERLEDIITPMDVSIIGCVV NGPGEALVSDLGLAGAANKSAFYEGGQRVDRLDNKDLIPILERRIRARAAMLDPANQIQVDEG
Anoxybacillus flavithermus WK1	364	MSEIIHRSKTRPVRVGNITIGGNNEVVIQSMTTTKTHDVDATVAQIHRLEEAGCQIVRVACPDERAADAIAEIKKRI NIPLVVDIHFDYRLALKAIENGADKIRINPGNIGKREKVEAVVKAAKERGVPIRIGVNAGSLEKRILDKYGYPTADG MVESALHHIRILEDLDFQDIIVSLKASDVRLAIEAYEKAARAFDYPLHLGITESGTLFAGTVKSAAGLGAILSKGIGN TVRVSLSADPVEEVKVARELLKAFGLAANAATLISCPTCGRIEIDLISIANEIEEYIAQIKAPIKVAVLGCAVNGPGEA READIGIAGARGEGLLFRHGKIVRKVPEETMVEELKKEIDKLAEEYASKGKQK
Azoarcus sp. BH72	415	MVEQAFPFGSLPRRPTRQVRIGGVSVGSAAPVVVQSMTNTDTADVLGTAMQVAELARAGSELVRITVNNEAAA KAVPHIRDRLLALNMDVPLVGDFHYNGHKLLMDNPACAEALAKLRINPGNVGAGAKRDPQFAAIVEMACKYDKP VRIGVNWGSLDQSVLARIMDENATRAEPRDAGAVMREALVVSALESAAKAEEYGLAGDRIILSAKVSSVQDLIAV YRDMARRCDYPLHLGLTEAGMGSKGIVASTAALSVLLQEGIGDTIRISLTPEPNGSRTQEVVVAQEILQTMGLRA FTPMVTACPGCGRTTSTFFQELASGIQGYVREQMPLWREQYDGVETMTLAVMGCVVNGPGESKHANIGISLPG TGESPAAPVFVDGEKTVTLRGDNIAAEFKAIVDNYVATRYQKKAG
Bacillus subtilis subsp. subtilis str. 168	377	MQVSEITHRTKTRPVKVGPLTIGGNNEVVIQSMTTTKTHDVEATVAEINRLAEAGCQIVRVACPDERAANAIADIK KRISIPLVVDIHFDYKLALKAIEGGADKIRINPGNIGRREKVEAVVKAAKDKGIPIRIGVNAGSLEKRILEKYGYPTAD GMVESALHHIKILEDLDFHDIIVSMKASDVNLAIEAYEKAAKAFDYPLHLGITESGTLFAGTVKSAAGLGAILSKGIG NTMRISLSADPVEEVKVARELLKSFGLASNAATLISCPTCGRIEIDLISIANEVEEYISKIKAPIKVAVLGCAVNGPGE AREADIGIAGARGEGLLFRKGKIVRKVPEETMVEELKKEIDILAEEHYAKLEAEKAKLKEETQKA
Blastopirellula marina DSM 3645	381	MQIQRNSTRRVVIGSIAIGDGNPIAVQSMTATKTQDIDATVGQVRALEEAGADVVRIAVDNKKDAEALAEIRRQTS ANLSVDLQENYRLAELVAPHVDKVRYNPGHLYHHERTKPWREKVNYLVGVAQDNDCAIRIGVNCGSVDPDKLA LYDPTDSIAPMLESALEHCEHLDSLGFTRYCVSLKDSDPKKVIEVNQRFAERRADIPLHLGVTEAGMPPDGIIKTR IAFEQLIGRGVGDTIRVSLTVPNPRKPEEIAAGRQILADIAAGRVRSVVDFGLNTLNIISCPSCSRVENEAFVDLAE QVRDMTQYAKEHDITIAVMGCRVNGPGETDDADLGLWCGPNFVNLKRSGEELGAFPYDQILGKLKLELDALIAT RVGAN
Candidatus Pelagibacter ubique HTCC1002	366	MSLRPFRDIHRKKTKVINVGDVKIGGDNPISVQSMTNTLTTDIQATIKQINDIHEEGADLVRVSCPDEESSLALKEI TKHVSVPIIADIHFHYKRAIEAAENGAKCLRINPGNIGDKLKIYDVLSAAKNNDCSIRIGVNAGSLEKDILEKYKEPC PEALVESAQRNIKILEDQDFFNFKISVKSSDIFLSIAAYRQLSKVTDYPLHLGITEAGSFVSGSVKSSIGLGSLLMDG IGDTIRISLSDDPVKEIKIGNEILKSLNLRNRGVKIISCPSCARQAFQVIDTVKILEDKLSHIKTPLTLSIIGCVVNGPG EAAMTDIGITGGGKGNNMLYLSGIQSEKVLSEDIISRVVSEVEKKAAELDKKL
Cellvibrio japonicus Ueda107	374	MHCESPIVRRKSRQIMVGNVPVGGGAPISVQSMTNTETTDVAATVEQIRRIQTAGADIVRVSVPTMEAAEAFGL IRKQVNIPLVADIHFDYRIALRVADLGVDCLRINPGNIGREKRIKAVVDKARDLNIPIRIGVNAGSLEKDLQKKYGE PTPDALVESALRHVEILDGLNFYNFKVSVKASDIFMAVAAYRKLAALIEQPLHLGITEAGGLRAGTVKSAIGLGAL LMDGIGDTIRVSLAADPVEEIKVGWDMLRSLKIRSKGVNFIACPSCSRQNFDVIKTMNDLELRVEDITVPLDVAVI GCVVNGPGEAKEADLGLAGGTPSNLIYIDGEPSQKLTNENLVDNLERLIRAKAAQKQAQLEADAKNIIARA
Chlamydia pneumoniae AR39	613	MTLITPAINSSRRKTHTVRIGNLYIGSDHSIKTQSMTTTLTTDIDSTVEQIYALAEHNCDIVRVTVQGIKEAQACEKI KERLIALGLNIPLVADIHFFPQAAMLVADFADKVRINPGNYIDKRNMFKGTKIYTEASYAQSLLRLEEKFAPLVEK CKRLGKAMRIGVNHGSLSERIMQKYGDTIEGMVASAIEYIAVCEKLNYRDVVFSMKSSNPKIMVTAYRQLAKDL DARGWLYPLHLGVTEAGMGVDGIIKSAVGIGTLLAEGLGDTIRCSLTGCPTTEIPVCDSLLRHTKIYLDLPEKKNP FSLQHSENFVSAAEKPAKTTLWGDVYGVFLKLYPHHLTDFTPEELLEHLGVNPVTKEKAFTTPEGVVVPPELK DAPITDVLREHFLVFHHHQVPCLYEHNEEIWDSPAVHQAPFVHFHASDPFIHTSRDFFEKQGHQGKPTKLVFS RDFDNKEEAAISIATEFGALLLDGLGEAVVLDLPNLPLQDVLKIAFGTLQNAGVRLVKTEYISCPMCGRTLFDLE EVTTRIRKRTQHLPGLKIAIMGCIVNGPGEMADADFGFVGSKTGMIDLYVKHTCVKAHIPMEDAEEELIRLLQEH GVWKDPEETKLTV
Chlamydophila caviae GPIC	606	MVISPSKKQAARRHTHSVKIGNLYVGSEHSIKMQSMTTTPTADVEATVTQICALVEAKCEIARVTVQGIKEAQA CEHIKERLLSMGIAIPLVADIHFFPQAAMHVADFVDKVRINPGNFVDKRNMFTGKTYTDKNYADSLLRLEEKFTP LVEKCKRLGKAMRIGVNHGSLSERVMQRYGDTIEGMVVSALEYIEVCEKLGYRDVVFSMKSSNPKVMVAAYR QLAKDLDARGWHYPLHLGVTEAGIGLDGIIKSAVGIGTLLTEGLGDTIRCSLTGCPTTEIPVCESLLKHTTTYLDL PKKENPFALENSETFVNASKKITKATPWGSVYGVFIKLMKEHLLNNTVEKLLEQLGINPTNGKKDFTAPDGIIVP KSFIGTSVIEKLKEHLLVFHHHEVPCLYNHNEEIWNSEQVLSAPFVHCHATPPFIHSTREFFEKKQSNEQPVKLV FSKDLDDEFEAAVSIATEFGALLLDGLGEAVILDLPNVSLPTVREIAFSTLQSAGVRLVKTEYISCPGCGRTLFDL PEVTTRIREKTKHLVGLKIAVMGCIVNGPGEMADSDFGFVGSKTGMIDLYVKHTCVKAHIPMEDAEEELVRLLQ EHGVWKDPE

Chromobacterium violaceum ATCC 12472	427	MDSVNIARRPTRLVRVGHVLVGSDAPVMVQSMTNTDTADAAGTAEQVYQLAQAGSEVVRITVNSPEAAARVA EIRQRLDDLGCDVPLVGDFHFNGDRLLKEFPDCARALAKYRINPGNVGKGAKGDDKFAFMIRTAMEHDKAVRI GVNWGSLDQALLARMMDANNRAANPLPLPKLMQEALIVSALESAEKAVEIGLSPDNIILSCKVSNVQDLISVYR ELGGRCDYPLHLGLTEAGMGSKGIVASSAALAVLLQEGIGDTIRISLTPQPGEARTKEVVVAQELLQTMGLRSF TPLVTACPGCGRTTSTFFQELADHIQSYLRERMPVWRLQYPGVEDMKVAVMGCVVNGPGESKLADIGISLPGT GEVPVAPVYVDGQKDVTLKGDNIPAEFTAIVDNYVKTRYGEGGAKRREVASRTIPIRPVKA
Clostridium cellulolyticum H10	353	MEDCIKRNQTKKIKVGDIFIGGDSPISVQSMTNTDTRDVKATIHQIKSLEEAGCDIVRLAILDNEAATAIGEIKKHV KIPLVADIHYDYRLAVECMKNGVDKIRLNPGNIGGNDRVRTVAGMAKERGIPIRIGVNSGSVEKRILEKFGGVTA EGMVESALAHVAMLENVDFNDIAISIKASSVPMTIAAYRLLSEKCLYPLHVGVTEAGTVYKGTIKSAVGIGCLLAE GIGDTIRVSLTGDPVEEIKVGKQILKSLDLLKEGIEIVSCPTCGRTQVNLIDIANSIEPLLEKLNKNIKVAIMGCAVN GPGEAKDADIGIAGGVNEVLLFKKGRIIRKIPQENVVEELIKEISEM
Collinsella stercoris DSM 13279	353	MAPCARELTRPVVVGDVQIGGGAPVVVQSMTCTPTTDVASTLAQVRALAEAGCDIVRVSVPTKEALGPFGVVC RESPVPIVADIHFDYRLAIGAVQAGAAKLRINPGNIGDWDRVDAVIDAAGEAGAAIRIGVNAGSLDRTIAERDDLT QPEKLVASSLEFIEHFENRGFADIVLSAKAHSVLTTLDTYRALSREIPHVPLHLGVTEAGTVQQGTIKSSVGLGILL SEGIGDTMRVSLTADPVEEPPVCWGILSALGLRRRGPELVSCPTCGRTQVDLIGLAECVSDRLKTCDKPISVAV MGCVVNGPGEASDADVGVACGRGVGMVFRHGEVIRKVPEAQIVDALMEEIDKL
Colwellia psychrerythraea 34H	376	MFKESPIIRRVSRQIMVGNVPVGGDAPITVQSMTNTLTTDVAATVAQIKALEAVGADIVRVSVPTMDAAEAFREIK KQVNVPLVADIHFDYRIALKVAEYGVDCLRINPGNIGNENRIRSVVECARDNNIPIRIGVNGGSLEKDIQEKYTEPT PEALLESAMRHVDILDRLNFNEFKVSVKASDVFLAVESYKLLAKQIDNPLHLGITEAGGLRSGSVKSSVGLGLLLA QGIGDTIRISLAADPIEEIKVGFDILKSLKLRSRGINLIACPSCSRQEFDVVSTVNALEQRIEDIMTPMDVSIIGCIVNG PGEAMVSDLGLTGSSKKSGYYLDGIRQKERFDNTDLVDQLEQRIRAKARSMSERLDEKNKIDILTKD
Coraliomargarita akajimensis DSM 45221	681	MTDLQHTQLHYCASRFQAQRRLSREINVGGVRVGGNNPIRIQSMTTTDTQDVDATVAQTLALAEAGCEIVRITA PNKKAAEALGEISKKVRAAKCDVPLVADIHFLPAAAMEAAKHVEKVRINPGNYADKKKFAVKEYSDADYDEELE RLHEAFSPIVLRCKELGRAMRIGTNHGSLSDRIMNRYGDSPLGMVESALEFIRIAESHRYQDICLSMKASNPKVMI EAYRLAVARMNQEGMNYPLHLGVTEAGDGEDARVKSAIGIGTLLNDGLGDTIRVSLTEDPIYEVPVARDLADKA MTLWKQESSPQGPIRDAIDPFDFRRRATSTINLSARCAIGAEQLPAVIVKTTHPLRETAAIVQEVCQTQTKQKDS KLEGLIIELNDASELDYFSGLHEALHSVVECIILELNERVDLSDLEAYKWPKGMAGITLLQALPKEDSLYALSLLEF CRQKGFNFALDCTPGDLRDELGPRLQGNAQNLILCCSATPERHHPVGQVRALVDAARREFLPNTPIWIRNTSETT LAPKDYFSDRLLETSVYSGALLCDGIGDAISIETEPLLEKATALAYNILQGARARISKTEFVACPSCGRTLFDLQSV TQRIRSRTDHLKGVTIAIMGCIVNGPGEMADADFGYVGGAPGKINLYVGKECVKYNVPSNEALDRLIDLIKEHGK WVEPS
Crocosphaera watsonii WH 8501	412	MQTLETPKTPKIISQPDFDTTIHRRKTRPVKVGNITIGGGFPVVVQSMINEDTLDIEGSVAAIRRLHEIGCEIVRVTV PSMGHAKALATIKEKLAQVYQPVPLVADVHHNGLNIALEVAKHVDKVRINPGLYVFEKPRATRTEYSQEEFEKIG DKIRQTLEPLVISLRDQGKAMRIGVNHGSLAERMLFTYGDTPEGMVESALEFIHICESLDFYNLIVSLKASRVPVM LAAYRLMVKRMDELGMDYPLHLGVTEAGDGEYGRIKSTAGIGTLLAEGIGDTIRVSLTEAPEKEIPVCYSILQALG LRKTMVEYVACPSCGRTLFNLEEVLHQVREATKHLTGLDIAVMGCIVNGPGEMADADYGYVGKQAGYIALYRG REEIKRVHEEQGVEELINLIKADGRWVDPSSVGS
Cyanothece sp. ATCC 51142	404	MQTLETPQTTSKPEFDTTIHRRKTRPVKVGDITIGGGFPVVVQSMINEDTLDIEGSVSAIRRLHEMGCEIVRVTVP SMAHAKALATIKEKLAQVYQPVPLVADVHHNGLKIALEVAKHVDKVRINPGLYVFEKPSPTRSEYTQAEFEEIGE KIRQTLEPLVISLRDQGKAMRIGVNHGSLAERMLFTYGDTPEGMVESALEFIRICESLDFYNIVISLKASRVPVMLA AYRLMVKRMDELGMDYPLHLGVTEAGDGEYGRIKSTAGIGTLLAEGIGDTIRVSLTEAPEKEIPVCYSILQALGLR KTMVEYVACPSCGRTLFNLEEVLHKVREATKHLTGLDIAVMGCIVNGPGEMADADYGYVGKQAGYISLYRGRE EIKKVPEDQGVEELINLIKADGRWIDP
Deinococcus geothermalis DSM 11300	424	MPPPVRVNWPRMRTTRRQTVTTWVGNVPVGSSHPIVVQSMTNTDTANAEATALQVAQLARAGSEIVRVTVNT REAAAALPEIVARLHDLGLDVPLVGDFHYNGHLLLREYPETARLLAKYRINPGNVGAGQHHDANFATMIEVAKTY GKPVRIGVNWGSLDQQVLARLMDENARRGSPKSGTDVMIDAMVTSALESAAYAEGLGLPHDRIIISVKVSSAPE LWQVYRQLAPLCDYPLHLGLTEAGMGMKGMVASSVALAPLLSEGIGDTIRVSLTPEPGAPRKLEVEVAQQILQS LGLRQFLPQVTSCPGCGRTTSTFFQELARKIQDYIRDAMPEWKAKYPGVEDMQVAVMGCIVNGPGESKHANIGI SLPGTGEDPRAPVYQDGKLLTTLKGPRIAEDFQELLEKYVEERYGHKSAHTTVQE
Desulfitobacterium hafniense DCB-2	364	MERKLTKTVRIGDVIIGGGSPIVVQSMTNTDTRDIPATLAQIQALARAGCEVVRLAVLDREAGYALGEIALKSELPV IADIHFDYQLALLAIEQGVHGLRLNPGNIGARWKVQEVVRACKEREIPIRIGVNAGSLEKEILEKYQGVTPEGMVE SALGHIHLLEEEGYDKIKVSLKASHVPLMLAAYRKMSARVDYPLHIGVTEAGTVRSGVVKSAVGIGSLLAEGIGDT LRVSLTGDPVQEIPVALEILKVLGLRNRGVELISCPTCGRTQVNLAELAEKVEDKLSHLPPLDRPLKVAVMGCAV NGPGEAREADFGIAGGKGMGLLFKKGEIVARLSEEELLPALLAEIENYVKQYGREQHSCV
Escherichia coli str. K-12 substr. MG1655	372	MHNQAPIQRRKSTRIYVGNVPIGDGAPIAVQSMTNTRTTDVEATVNQIKALERVGADIVRVSVPTMDAAEAFKLIK QQVNVPLVADIHFDYRIALKVAEYGVDCLRINPGNIGNEERIRMVVDCARDKNIPIRIGVNAGSLEKDLQEKYGEP TPQALLESAMRHVDHLDRLNFDQFKVSVKASDVFLAVESYRLLAKQIDQPLHLGITEAGGARSGAVKSAIGLGLL LSEGIGDTLRVSLAADPVEEIKVGFDILKSLRIRSRGINFIACPTCSRQEFDVIGTVNALEQRLEDIITPMDVSIIGCV VNGPGEALVSTLGVTGGNKKSGLYEDGVRKDRLDNNDMIDQLEARIRAKASQLDEARRIDVQQVEK
Gemmata obscuriglobus UQM 2246	401	MATSSRFDISRLEAAPRVPGKPRHKTREVKVGTRVFGGSNPVWVQSMTTTDTFDVDATVKQIHALEEAGCELV RVTVPKPEDAGALSAIRARVGIPLICDIHFDYKMALAALDHPVDKIRINPGNINKAGDTTHDRFRQIVRKAKQKGIP MRIGVNAGSLETEVNLKYGFPCPPAMVESALRYIEVAESEGYHDIIVSLKSSDVLVAVEAYRLFAQMCDYPTHIGI TEAGKPPYAVTKSAAGLAPILLDGIGDTIRISLLGDPVPEIAAAFDILQATQRRVRRPELIACPTCGRLAIDLEDIIAK LEARLNGKRLPVKISVLGCIVNGPGEAREADIGIAAGNGQGMIFRNGEMVRRVPEAEIVDALMEELARWESENQ HRIPKTTDAEGLGRRKLPVVAS

Geobacillus thermodenitrificans NG80-2	374	MSEIIHRSKTRPVRVGSLTIGGNNEVIIQSMTTTKTHDVDATVAQIHRLEEAGCQIVRVACPDERAADAIPEIKKRINIPLVADIHFDYKLALKAIEGGVDKIRINPGNIGRREKVEAVVKAAKERGVPIRIGVNAGSLEKRILEKYGYPTAEGMVESALYHIRILEELDFHDIIVSLKASDVRLAIEAYEKAARTFDYPLHVGITEAGTLFSGTIKSAVGLGAILSKGIGNTIRISLSADPVEEVKVAREILKTFGLASNAATLISCPTCGRIEIDLISIANEIEDYIAKIKAPIKVAVLGCAVNGPGEAREADIGIAGGAPGLGLIFRKGEIVRKVSQAEMVGALLAEIEKLPAQ
Heliobacterium modesticaldum Ice1	356	MEIALPRRKTRRIYVGTVPIGDGAPVSVQSMCNTDTRDAAATLDQISRLVAAGCEIIRVAVPDAQAVEALPAIVQGSPVPVIADIHFDHRLAIGALKAGVQGLRLNPGNIGGAVPVREVLAAARERQVPIRIGVNAGSLEKRLLEKYGGVTPEALVESALGHIGLLEDENYPWMKISLKASSVPLMLAAYQSLAEKVDYPLHIGVTEAGTVRSGIIKSAAGIGALLAQGIGDTLRVSLTGDPLPEVRTGWAILKSLGLRKRGPELISCPTCGRCQVNLTAVAEAVEMALEKETRPIKVAVMGCVVNGPGEAREADVGIAGGNDCGLIFRKGEIVRKVSQAEMVGALLAEIEKLPAQ
Herminiimonas arsenicoxydans	425	MSSSDPIATGPFSRRKSRIAVVSYGGREVRIGGDAPVVVQSMTNTDTADVIGTAIQIKELARAGSEMVRITVNTPEAAAAVPAICEQLDKMDVVVPLVGDFHYNGHTLLNDYPDCAKALAKYRINPGNVGKGNKRDTQFAQMIETACRFDKPVRIGVNWGSLDQELLARIMDENAARAHPWTAQAVMYEALVTSAIENAMRAEEVGLAGDRIILSCKVSGVQDLIAVYRELARRCDYPLHLGLTEAGMGSKGIVASTAALSVLLQEGIGDTIRISLTPEPGGDRTREVIVGQEILQTMGLRKFTPMVIACPGCGRTTSTVFQELADGIQTYLRDQMPVWKKQYPGVEAMNVAVMGCIVNGPGESKHANIGISLPGTGESPAAPVFVDGVKTVTLRGDNIAAEFHAIVLDYVKSRYGKENSENSAA
Isosphaera pallida IS1B	390	MAYATTIQRRKTRVVEVGGHGVGGDNPIWVQSMTTTDTFNAAATIEQIHRLEEAGCELVRVTVPKKEDLEAVGYIKSQIKIPLIADIHYDYRMALGCLEAVTPDGKRAIDKVRINPGNIGGEERFKEVIRKAKDKGIPIRIGVNSGSLEKDLLEKYGYPCPEAMVESALRHIETAESLGFDDLIVSVKCSHVPTAVETYTQFSQASDYPTHVGITEAGSREYGSIKSAAGIGAILLKGIGDTIRCSLLGDPVPEIAVCFDILRATGRRVTRPEVVACPTCGRLDIDLERIVGEVEEERMKGLTTPLQISILGCLVNGFGEAREADIGIAAGSGKGIIFKKGVPIRHVKESEMVEALWEEVQRFHEDTPALNAHHEEKRRRKSELPVLNSGS
Jannaschia sp. CCS1	377	MSHNPIRPWRNIDRRKSRQIMVGNVPVGGDAPITVQTMTNTLTTDASATIKQVIAAAEAGADIVRVSVPDADSARAMHEICRESPVPIVADIHFHYKRGIEAAEAGAACLRINPGNIGDAARVKEVVKAARDHNCSIRIGVNAGSLEKHLLEKYGEPCPDAMVESGMDHIKLLEDNDFHEFKISMKASDIFMTAAAYQQLADQTDAPFHMGITEAGGFVGGTVKSAIGLGNLLWAGIGDTMRVSLSADPVEEVKIGFEILKSLGLRHRGVNIISCPSCARQGFDVIKTVEVLEQRLEHIKTPMSLSIIGCVVNGPGEALMTDVGFTGGGAGSGMVYLAGKQSHKMSNEQMVDHIVEQVEKRAEAIEAQAKAADQAAE
Jonesia denitrificans DSM 20603	383	MTVPISLGMPQAPAPVLAPRRKTRKIKVGKVDVGGDAPVSVQSMTTTPTTDINATLQQIAELTASGCDIVRVAVPSADDAAALPAIAKKSQIPVIADIHFQPKYVYAAIDAGCAAVRVNPGNIRKFDDQVKQIAAAAADAGVSIRIGVNAGSLDPRLMKKYGKATPEALVESAVWEASLFEEHGFHDFKISVKHNDPVVMVRAYELLSERGDWPLHLGVTEAGPAFQGTIKSATAFGALLSKGIGDTIRVSLSAPPVEEVKVGNAILESLNLKPRKLEIVSCPSCGRAQVDVYTLAERVTAGLEGLPVPLRVAVMGCVVNGPGEAREADLGVASGNGKGQIFVKGEVIKTVPESQIVETLIEEAMRIADSMEKTGESPSITVG
Methylacidiphilum infernorum V4	606	MRNRGRKRTVRTFVHVFDSRPNMFTDKTHFCSYKRRQAREVAVGKVKLGGGNPVAIQSMLTSDTMDTTACLKETLELVESGCQIVRITAPTVKDASNLEFIRRGLNDRGCDVPLVADIHFKPEAAMEAARWVEKVRINPGNFADRKKFIIRSYTDEEYNQELQRIEQAFLPLVELCRKRKIALRIGTNHGSLSDRIMNRFGDTPQGMVESALEYAAVCRKHDFHDFVFSMKASNPRIMISAYRKLIQTLERLGEDWNYPVHVGVTEAGDGEDGRIKSAIGIGSLLADGIGDTIRVSLTEKSVYEIPVAAKLLSVVDELAQAPSPRLNSFSFSPPLEYNRRPTTKIDLGIAIGGDEPVRVAVPSSHYEYLKSRPPGKDFVAEFPYDPSAIVELDLDDIDSILALRDKPARLVTIKDGCPYPPLYAFRALASLLDPSWPILLKDTFFPLRDQGLGDREKNLIASSLHLGSLLCDGIGDAILLRRDPDPSYGLVLSYAILQAAGNRTVKTEYVACPSCGRTLFDLQTTTARIKKATGHLKGIKIAVMGCIVNGPGEMADADFGYVGGAPGKVNLYVGKKPVRFNIPEAEAVDALIDLIKKEGKWMDSAS
Moorella thermoacetica ATCC 39073	359	MPGRRRPTRRIQVGKVAIGGGAPISVQSMTNTDTRDITATVAQIRRLAAAGCEIVRLAVPDQEAALALAKIKAQVEIPLIADIHFDYRLALAALEAGVDGLRLNPGNIGGPERVKAVVKEAAARRVPIRIGVNAGSLEKEVLAAHGGVTAEAMVASALKHIRLLEDLDFREIKVSLKASEVPLMLAAYRLMAEKVDYPLHLGVTEAGRGLEGAVKSAVGIGILLAEGIGDTIRVSLTGDPVQEVIAGFAILRALGLRQQGIELISCPTCGRCQLDLDAVAARVQEELRGIKQPLKVAIMGCAVNGPGEARQADVGIAGGPGFGLLFRHGRPVRKVKEEDLARALVEEVKRLAAERRREQG
Mycobacterium smegmatis str. MC2 155	388	MTSIGLGMPAPPAPTLAPRRKTRQLNVGGVGIGSEHPIAVQSMCTTKTHDVNSTLQQIAELTASGCDIVRVACPRQEDADALAEIARHSQIPVIADIHFQPKYIFAAIDAGCAAVRVNPGNIKEFDGRVKEVAKAAGDAGIPIRIGVNAGSLDKRFLEKYGKATPEALVESALWEASLFEEHGFGDIKISVKHNDPVIMVAAYEQLAAQCDYPLHLGVTEAGPAFQGTIKSAVAFGALLSKGIGDTIRVSLSAPPAEEVKVGNQILESLNLRPRGLEIVSCPSCGRAQVDVYTLANAVTAGLDGLDVPLRVAVMGCVVNGPGEAREADLGVASGNGKGQIFVKGEVIKTVPEAQIVETLIEEAMRLAEEIGTARGSDDASGSPVVTVS
Nostoc punctiforme	408	MQTLPIVDTSNTASSQHIFDTTIKRRKTRPVKVGNVTIGGGYPVVVQSMINEDTLDIDGSVAGIRRLHEIGCEIVRVTVPSMAHAKALAEIKQKLIKTYQDVPIVADVHHNGLKIALEVSKHIEKVRINPGLYVFEKPNVNRTEYTQSEFDEIGDKIRETLEPLVVSLRDQGKSMRIGVNHGSLAERMLFTYGDTPEGMVESAIEFIRICESLDFRNLVISMKASRVPVMLAAYRLIAKRMDELGMDYPLHLGVTEAGDGEYGRIKSTAGIATLLADGIGDTIRVSLTEAPEKEIPVCYSILQALGLRKTMVEYVACPSCGRTLFNLEEVLHKVREATKHLTGLDIAVMGCIVNGPGEMADADYGYVGKTPGYISLYRGREEIKKVPEDKGVEELINLIKADERWVEP
Novosphingobium aromaticivorans DSM 12444	374	MSSVRPWRDIARRKSRQIMVGTVPVGGDAPITVQTMTNTPTSDAVATIDQIRRCEEAGADLIRVSCPDVESTAAFRQIARAARVPLIADIHFHYKRALEAADAGAACLRINPGNIGSSDRVAEVVRAAKANGCAIRIGVNAGSLEKDLLEKYGEPCPEALVESALDHIKLLQDHDFHEYKVAVKASDVFLAVAAYMGLAEAVDCPLHLGITEAGGLIGGTVKSSVGIGNLLWAGIGDTLRVSLSAEPEEEVRVGFEILKTLGLRTRGVRVVSCPSCARQGFDVIRTVEALEKRLTHIKTPISLSVLGCVVNGPGEARETDIGLTGGGNGKHMVYLSGVTDHHVQSEDMLDHIVSLVEQKAAEMEAAAAEAEAAA

Pantoea ananatis LMG 20103	378	MWRRNMHNQAPIIRRKSKRIYVGQVPIGDGAPIAVQSMTNTRTTDVAATVNQIKALERVGVDIVRVSVPTMDAA EAFKLIKQQVNVPLVADIHFDYRIALKVAEYGVDCLRINPGNIGNNERIRAVVDCARDNNIPIRIGVNAGSLEKDLQ EKYGEPTPQALLESAMRHVDHLDRLNFDQFKVSVKASDVFLAVESYRLLAKQIEQPLHLGITEAGGARAGAVKS AIGLGLLLAEGIGDTLRISLAADPVEEVKVGFDILKSLRIRSRGINFIACPTCSRQEFDVIGTVNALEQRLEDIITPMD VSIIGCVVNGPGEATVSTLGVTGSNKKSGFYEDGVRQRERLDNDDMIDQLEARIRAKAAMLDETRRIDVQQLEK
Pelagibacter ubique HTCC1062	366	MSLRPFRDIHRKKTKVINVGDVKIGGDNPISVQSMTNTLTTDIQATIKQINDIHEEGADLVRVSCPDEESSLALKEI TKHVSVPIIADIHFHYKRAIEAAENGAKCLRINPGNIGDKLKIHDVLSAAKNNDCSIRIGVNAGSLEKDILEKYKEPC PEALVESAQRNIKILEDQDFFNFKISVKSSDIFLSIAAYRQLSKVTDYPLHLGITEAGGFVSGSVKSSIGLGSLLMD GIGDTIRISLSDDPVKEIKIGNEILKSLNLRNRGVKIISCPSCARQAFQVIDTVKILEDKLAHIKTPLTLSIIGCVVNGP GEAAMTDIGITGGGKGNNMLYLSGIQSEKVLSEDIISRVVSEVEKKAAELDKKL
Pirellula staleyi DSM 6068	380	MQIQRNPTRSVRIGSCTIGSGHPIAVQSMTATHTQDIDGTVALVNDLYENGADVVRIAVDSKKDAEALAEIRKQT KANLSVDLQENYRLAELVAPHVDKVRYNPGHLYHHETHKPWQEKVRYLAQVAIDNDCAMRVGVNCGSVDPAK KEMFDPHDSITPMLESAKEHCDLLDSIGFTRYNVSLKDSDPKKVVEVNKRFAEMRPDVPLHLGVTEAGLPPDGII KTRIAFEQLIGRGIGDTIRVSLTVPNDRKKDEISAGRQILADIAAGRVRSVVDYGLKTLNIISCPSCSRVENQAFIEL AQQVKEMTRYAEQHSLTIAVMGCRVNGPGETDDADLGLWCGPNFVNLKRGSTELGQYAYDAILPRLQAELDSL IASRTAS
Planctomyces brasiliensis DSM 5305	397	MSSPLFSLQRQAQNEIMEITRNPTREVRIGSIAIGAGNPIAVQSMTATKTQNVDATVAQIQHLLEANADVIRVAVD SKKDAAALKEIAAQTNANLSVDLQENYRLAELVAPYVQKIRYNPGHLYHHEREKPWQEKVAYIVDVAKENDCAI RVGVNCGSVDPAKLDKYDPEDSISPMLESAFDHCEYLDSLGFTRYCVSLKDSDPQKVIEVNRRFAEKRPDVPLH LGVTEAGMPPDGVIKTRIAFEQLIGKGIGDTIRVSLTVPNDRKREEIEAGRNILADIAAGRVRSVVNFDTSGLNIISC PSCSRVENEAFVELAGQVKEMTAYAKEYDLTIAVMGCRVNGPGETDDADLGLWCAPNFVNLKKGTEALGAYPY DEIIPRLKQELDLLIAEKTVKS
Rhodobacter sphaeroides ATCC 17025	377	MSLNHVRPWRNIHRRKSRQIMVGNVPVGGDAPISVQTMTNTITADAPATIAQVLRAAEAGADIVRVSVPDEAST RALKEIVAASPVPIVADIHFHYKRAIEAAEAGAACLRINPGNIGDASRVREVIKAARDHGCSMRIGVNAGSLEKHLL DKYGEPCPEAMVESGLDHIKILEDNDFHEFKISVKASDVFLAAAAYQALADATDAPIHLGITEAGGLTAGTVKSAV GLGNLLWSGIGDTLRVSLSADPVEEVKVGYEILKSLGLRHRGVTIISCPSCARQGFDVIKTVSELEDRLAHVTTSM SLSIIGCVVNGPGEALMTDIGFTGGGNGSGMVYLAGKQSHKLGNERMIDEIVSMVERRAAEIEAEKSAAAEAAE
Rubrobacter xylanophilus DSM 9941	357	MTETTVRERPVTSRRIMVGDVPIGGGAQIVVQSMTNTPTHDVEATVQQIYDLNAAGAELVRVSVNGSKALKGFR EIVPRSPVPLIADIHFDYRMALGAADAGAACVRINPGNIGSDDRFRKVIEKCQEKGIAMRIGVNSGSVEKRFWHL PKAEAMVASALHKVEIAEEMGFYDFKVSLKASHVPVMVEANKKFREHSDAPLHLGVTEAGTKLPGAIKSAAALG QLLPYGIGDTIRISLAEDPLEELPVAYQILSALELRHRGPNVIACPSCARTLGFDVIGMAARVEERLRDYEDHFTVA VMGCIVNGPGEARDADYGVAGGKDDGVIFAKGRPLRKVHRDEIFDALFEEIEKDGRK
Spirochaeta caldaria H1	372	MKQNQMVRVVKIGGFDHLSSVLVGGAYPVAVQTMWKDRLDETSLQGESGKHIETRIDKLQKLGCSLLRFAVPD LAAADVLGELAKRVTMPLVADIHFDYKIALRCLDFPIAKIRINPGNIGSLDKVKAVAAKAAEKGVPIRIGINAGSLPA DLRKAVDEQQMTRAEALVSTAERELEIFETLNFKNVLLSMKASSIYDTLEANRLIAKRTDVPLHLGVTEAGPLIPGI VRNSIALHTLLAEGIGATIRVSLSDTMENEVIAGREIVSAVADSSGKGVNGQGVIIVSCPRCGRNSFDTHAFTERW RDTLYMMKKDITVAIMGCAVNGPGEARHADIGITGAGNKVLIFRHGNIVRTVAVTEADQAFKEELEKL
Streptomyces cattleya DSM 46488	385	MTQTVSLGMPSVPLKLADRRRSRQIQVGSVAVGGDAPVSVQSMTTTVTADIGATLQQIAELTAAGCQIVRVACP SQDDADALPVIARKSQIPVIADIHFQPKYVFAAIDAGCAAVRVNPGNIRQFDDKVKEIAAAASAAGVPIRIGVNAGS LDKRLLEKYGRATPEALVESALWECSLFEEHGFRDIKISVKHNDPVVMVNAYRLLAQKCDYPLHLGVTEAGPAF QGTVKSAVAFGALLAEGIGDTIRVSLSAPPAEEVKVGIAILESLGLRQRRLEIVSCPSCGRAQVDVYKLADEVTAG LDGLEVPLRVAVMGCVVNGPGEAREADLGVASGNGKGQIFVKGEVIKTVPESKIVETLIEEAMKIAEQMEATGAA QGEPTVIVA
Streptomyces coelicolor A3	385	MTAISLGMPDVPTRLAERRKSRQIQVGPVAVGGDAPVSVQSMTTTRTSDIGATLQQIAELTASGCQIVRVACPT QDDADALPVIARKSQIPVIADIHFQPKYVFAAIEAGCAAVRVNPGNIKQFDDKVKEIAKAAKDHGTPIRIGVNAGSL DRRLLQKYGRATPEALAESALWEASLFEEHDFRDIKISVKHNDPVVMVEAYRQLAAQCDYPLHLGVTEAGPAFQ GTIKSAVAFGALLSQGIGDTIRVSLSAPPVEEIKVGIQILESLNLRQRGLEIVSCPSCGRAQVDVYKLAEEVTAGLE GMEVPLRVAVMGCVVNGPGEAREADLGVASGNGKGQIFVKGEVIKTVPESKIVETLIDEAMKIAEQMEKDGVTS GEPSVSVAG
Synechocystis sp. PCC 6803	403	MVTASLPTPVQPEFDTTIHRRKTRPVPVGAVTVGGGHPVVVQSMINEDTLDVDGSVAGIRRLHEIGCEIVRVTVP SMAHAKALADIKQKLQATYQAVPLVADVHHNGMKIALEVAKHVDKVRINPGLYVFEKPDAQREGYSDQEFAEIG EKIRETLEPLVISLRDQGKSMRIGVNHGSLSERMLFTYGDTPEGMVQSALEFIKICESLDFRNLVVSMKASRVPV MLAAYRLMVKRMDELGMDYPLHLGVTEAGDGEYGRIKSTAGIATLLADGIGDTIRVSLTEAPEKEIPVCYSILQAL GLRKTMVEYVACPSCGRTLFNLEDVLHEVREATKHLTGLDIAVMGCIVNGPGEMADADYGYVGKQAGYIALYR GREEIKRVPETDGVQELINLIKADGRWVDP
Thermosynechococcus elongatus BP-1	402	MQTLPSPVQATPTETAIVRRKTRPVPIGSVVIGGGHPVAVQSMINEDTLDIEGSVAAIRRLHEIGCEIVRVTVPSLA HAKAMEEIRDRLYKTYKPVPLVADVHHNGMKIALEVAKYVDNVRINPGLYVFEKPKPNRTEYTQAEFDEIGAKIR ETLEPLVISLRDQGKSMRIGVNHGSLAERMLFTYGDTPEGMVESALEFIRICESLNFYNLEISLKASRVPVMIAAN RLMVKRMDELGMDYPLHLGVTEAGDGEYGRIKSTAGIATLLAEGIGDTIRVSLTEAPEKEIPVCYGILQALGLRRT MVEYVACPSCGRTLFNLEEVLHKVREATKHLTGLNIAVMGCIVNGPGEMADADYGYVGKQPGYISLYRGREEV KKVPEAEGVAALVELIKADGRWVDP
Thermotoga maritima MSB8	344	MRKSVKVGKVVIGGEAPVSVQSMTTTKTADVEKTVSQIKSLERAGCEIVRVAVQDEEDAKAIRRIKEQIEIPLVAD IQFDYRLAILSIENGADKIRINPGNMSRDRLKDVVAAAKGKGIPIRVGANVGSIKRRTSERWKDLAESALEEVRLLE KEGFYDIIVSVKSSDVLETIKANEYIAEKVEYPIHLGVTEAGVSETAIVKSSIAIGHLLLKNIGDTIRVSISGDPVREVI VGKKILIALGLREGVEVIACPTCGRAEIDVENMAKMIEENFFHVQKRLKIAVMGCVVNGIGEGKDADLGVAGLRD GAVIFVKGEIKERVSKEFVLERLKYYLNELLEEVER

Thermus thermophilus HB27	406	MEGMRRPTPTVYVGRVPIGGAHPIAVQSMTNTPTRDVEATTAQVLELHRAGSEIVRLTVNDEEAAKAVPEIKRR LLAEGVEVPLVGDFHFNGHLLLRKYPKMAEALDKFRINPGTLGRGRHKDEHFAEMIRIAMDLGKPVRIGANWGS LDPALLTELMDRNASRPEPKSAHEVVLEALVESAVRAYEAALEMGLGEDKLVLSAKVSKARDLVWVYRELARRT QAPLHLGLTEAGMGVKGIVASAAALAPLLLEGIGDTIRVSLTPSPKEPRTKEVEVAQEILQALGLRAFAPEVTSCP GCGRTTSTFFQELAEEVSRRLKERLPEWRARYPGVEELKVAVMGCVVNGPGESKHAHIGISLPGAGEEPKAPV YADGKLLTILKGEGIAEEFLRLVEDYVKTRFAPKA
Treponema denticola ATCC 35405	387	MNSIKLPRTIHIGGKGQVKKLTLGGTSPILLQTMWKESLLGADLLSIVKSLNELEQLGCDIVRFAVPDMDSAEQFV KLTRLTEMPLVADIHFDYKLALRCMDGDTAKIRINPGNIGSKEKTEEVIRKAKDTGTAIRIGVNSGSLPSDLKKKIE EANSKRNLSGDKKALDDEISLLRADTLTEAAARELEIFEKADFKDAVVSMKASNVRETVMANEIFAKKFDNPLHL GVTEAGPLIQGIVKSTIAFYRLLEQNIGSTIRVSLSDSCENEVIAGREILTECGKRQGGIRLISCPRCGRKGFDVQA FVKRWQTKLLSEKKDISIAVMGCVVNGPGEGKHADLGITGAEDSVIIFKHGAITKRLDLKKLTEEEKIKAVDKAFIE ELQSL

Supplementary Table S4.2 – List of proteins identified by genome mining and suspected to be involved in the catalytic activation of FeS enzymes

Organism	Type	Name	Description – putative function	Protein Identifier
Bacillus subtilis subsp. subtilis str. 168	Electron carrier	YkuN	Flavodoxin	NP_389298.1
	Electron carrier	YkuP	Flavodoxin	NP_389300.2
	FeS delivery	YutM	Homology to ErpA	NP_391096.1
	FeS assembly	BsubSufCDSUB	Fe/S scaffold and assembly proteins	NP_391150.1, NP_391149.1, NP_391148.1, NP_391147.1, NP_391146.1
Coraliomargarita akajimensis DSM 45221	Electron carrier	CakaFdR1	Ferredoxin	ADE53916.1
	Electron carrier	CakaFdR2	Ferredoxin	ADE55879.1
	Electron carrier	CakaFdR3	Ferredoxin	ADE55140.1
	FeS delivery	CakaATC1	ApbC-type FeS carrier protein	WP_013044713.1
	FeS delivery	CakaATC2	Homology to A-type carrier IscA	WP_013042609.1
	FeS assembly	CakaSufCBDT	Fe/S scaffold and assembly proteins	WP_013042955.1, WP_013042956.1, WP_013042957.1, WP_013042958.1
	FeS assembly	CakaSufE	Relays sulfur from cysteine desulfurase to scaffold.	WP_013044239.1
	FeS assembly	CakaSufS	Cysteine desulfurase	WP_013042855.1
Rhodobacter sphaeroides ATCC 17025	Electron carrier	RsphFld1	Homology to *E. coli* FldA	WP_011910129.1
	Electron carrier	RsphFdR1	NADP reductase	WP_011907396.1
	FeS delivery	RsphATC1	Homology to A-type carrier IscA	WP_011908234.1
	FeS delivery	RsphATC2	Homology to A-type carrier IscA	WP_011907404.1
	FeS delivery	RsphATC3	Homology to A-type carrier ErpA/SufA	WP_011908293.1
	FeS delivery	RsphSufT	DUF59 domain-containing protein	WP_011907405.1
	FeS assembly	RsphSufBCDS	Fe/S scaffold and assembly proteins	WP_011909322.1, WP_011909320.1, WP_011909319.1, WP_011909316.1

	FeS assembly	RsphSufU	SufU-like protein, receives sulfur and iron, builds FeS clusters	WP_011908294.1
Streptomyces cattleya NRRL 8057 = DSM 46488	Electron carrier	ScatFld	Flavodoxin	WP_014143262.1
	Electron carrier	ScatFdR	Flavodoxin	WP_014628745.1
	Electron carrier	ScatFdR1	Ferredoxin	WP_010474859.1
	Electron carrier	ScatFdR2	Ferredoxin	WP_014145872.1
	Electron carrier	ScatFdR3	Ferredoxin	AEW92689.1
	FeS delivery	ScatATC1	Homology to A-type carrier IscA	WP_014142045.1
	FeS delivery	ScatATC2	Homology to FeS carrier NifU	WP_014143687.1
	FeS assembly	ScatSufBDCSU	Fe/S scaffold and assembly proteins	WP_014627519.1, WP_014141844.1, WP_014141842.1, WP_014141841.1, WP_014627518.1
	FeS assembly	ScatSufT	DUF59 domain-containing protein	WP_014141839.1
Synechocystis sp. PCC 6803	Electron carrier	PetF	IspG-interacting Ferredoxin	BAA10197.1
	Electron carrier	IsiB	Flavodoxin	BAA17947.1
	Electron carrier	PetH	Ferredoxin-NADP oxidoreductase	BAA18459.1
	FeS delivery	SyspATC1	Homology to A-type carrier NfuA	BAA18665.1
	FeS delivery	SyspATC2	Homology to A-type carrier SufA	BAA16746.1
	FeS assembly	SyspSufBCDS	Fe/S scaffold and assembly proteins	BAA10542.1, BAA10543.1, BAA10544.1, BAA10545.1
	FeS assembly	SyspSufE	Homology to SufE, Relays sulfur from cysteine desulfurase to scaffold	BAA18628.1
Thermotoga maritima MSB8	Electron carrier	TmarFdR1	Ferredoxin	WP_004080635.1
	Electron carrier	TmarFdR2	Ferredoxin	WP_004079944.1
	FeS delivery	TmarATC	ApbC-type FeS carrier protein	WP_004082033.1
	FeS assembly	TmarSufCBDSU	Fe/S scaffold and	WP_004081564.1

			assembly proteins	WP_004081565.1, WP_004081566.1, WP_004081567.1, WP_010865327.1
Thermus thermophilus HB27	Electron carrier	TtherFdR1	Ferredoxin	WP_011172609.1
	FeS delivery	TtherATC1	ApbC-type FeS carrier protein	WP_011172746.1
	FeS delivery	TtherATC2	Homology to IscA	WP_011173673.1
	FeS assembly	TherSufCBD	Fe/S scaffold and assembly proteins	WP_011173862.1, WP_011173863.1, WP_011173864.1
	FeS assembly	TherSufSU	Homology to SufS and SufU	WP_011173757.1, WP_011173758.1
	FeS assembly	TtherSufE	Relays sulfur from cysteine desulfurase to scaffold	WP_008632164.1

Supplementary Table S4.3 – List of strains and plasmids used in this study

Name	Description	Reference or source	Used in
Strains			
DH5α	*E. coli* strain used for cloning and plasmid amplification	Invitrogen	
BL21(DE3)	*E. coli* strain used for bisabolene production	Invitrogen	
MG1655	*E. coli* strain used to construct knockout strains for complementation studies	ATCC	
MG1655_IMB	*E. coli* strain used to construct *ispG* and *ispH* knockout strains	This work	
E. coli NCM3722	*E. coli* strain used for fosfomycin production		
EC_ΔispG	MG1655_IMB IspG knockout strain	This work	
EC_ΔispH	MG1655_IMB IspH knockout strain	This work	
ΔbioB	*E. coli* MG1655 BioB knockout strain	KEIO collection	
ΔthiC	*E. coli* MG1655 ThiC knockout strain	KEIO collection	
ΔnadA	*E. coli* MG1655 NadA knockout strain	This work	
ΔilvD	*E. coli* MG1655 IlvD knockout strain	This work	
Plasmids for FeS enzyme activation			
'Plug adapter' vectors: pBbA5a backbone containing operon expressing codon optimized putative proteins participating in FeS biogenesis and electron transfer in foreign hosts			
pSysp_ETP	Codon optimized PetF and IsiB from *Synechocystis sp. PCC 6603*	This work	
pSysp_ATC	Codon optimized SyspATC1 and SyspATC2 from *Synechocystis sp. PCC 6603*	This work	
pSysp_ATC_ETP	Codon optimized SyspATC1, SyspATC2, PetF and IsiB from *Synechocystis sp. PCC 6603*	This work	
pPetF	Codon optimized PetF from *Synechocystis sp. PCC 6603*	This work	
pIsiB	Codon optimized IsiB from *Synechocystis sp. PCC 6603*	This work	
pBsub_ETP	YkuNOP from *B. subtilis str.168*	This work	
pBsub_ATC	YutM from *B. subtilis str.168*	This work	
pBsub_ATC_ETP	YkuP, YutM from *B. subtilis str.168*	This work	
pCaka_ETP	Codon optimized CakaFdR1, CakaFdR2, and CakaFdR3 from *Coraliomargarita akajimensis*	This work	
pCaka_ATC	Codon optimized CakaATC1 and CakaATC2 from *Coraliomargarita akajimensis*	This work	
pCaka_ATC_ETP	Codon optimized CakaATC1, CakaATC2, CakaFdR1, CakaFdR2, CakaFdR3 from *Coraliomargarita akajimensis*	This work	

pScat ETP	Codon optimized favodoxins ScatFld1 and ScatFld1 from *S. cattleya*	This work
pScat_ATC	Codon optimized ScatATC1 and ScatATC2 from *S. cattleya*	This work
pScat_ATC_ETP	Codon optimized, ScatFld1, ScatFld1, ScatATC1 and ScatATC2 from *S. cattleya*	This work
pScat_FdR	Codon optimized, ScatFdR1, ScatFdR2, and ScatFdR3 from *S. cattleya*	This work
pScat_SufUT	Codon optimized SufU and SufT from *S. cattleya*	This work
pScat_FdR_SufUT	Codon optimized, ScatFdR1, ScatFdR2, ScatFdR3, SufU and SufT from *S. cattleya*	This work
pRsph_ETP	Codon optimized RsphFld1 and RsphFdR1 from *Rhodobacter sphaeroides ATCC 17025*	This work
pRsph_ATC	Codon optimized RsphATC1, RsphATC2 and RsphATC3 from *Rhodobacter sphaeroides ATCC 17025*	This work
pRsph_ATC_ETP	Codon optimized RsphATC1, RsphATC2, RsphATC3, RsphFld1 and RsphFdR1 from *Rhodobacter sphaeroides ATCC 17025*	This work
pTmar_ETP	Codon optimized TmarFdR1 and TmarFdR2 from *Thermotoga maritima MSB8*	This work
pTmar_ATC	Codon optimized TmarATC from *Thermotoga maritima MSB8*	This work
pTmar_ATC_ETP	Codon optimized TmarATC TmarFdR1 and TmarFdR2 from *Thermotoga maritima MSB8*	This work
pTther_ETP	Codon optimized TtherFdR1 from *Thermus thermophilus HB27*	This work
pTther_ATC	Codon optimized TtherATC1 and TtherATC2 from *Thermus thermophilus HB27*	This work
pTther_ATC_ETP	Codon optimized TtherATC1, TtherATC2 and TtherFdR1 from *Thermus thermophilus HB27*	This work
Plasmids for biofuel production		
pJBEI-2997	Mevalonate pathway: pBbA5c-MevT(CO)-MBIS(CO, ispA)	Addgene
MEP pathway vectors: pBbA5k backbone containing operon expressing Dxs, IspC, IspDF, Idi, ispE, ispH from *E. coli* MG1566 and an ispG variant (except for pMEP_neg)		
pMEP_neg	pBbE5k containing operon expressing Dxs, IspC, IspDF, Idi and ispE from *E. coli* MG1566 (incomplete MEP pathway)	This work
pMEP_EcIspG	IspG from *E. coli* MG1655	This work
pMEP_ BmarIspG	Codon optimized IspG from *B. marina*	This work
pMEP_ BsubIspG	IspG from *B. subtilis str.168* (NC_000964)	This work
pMEP_ CvioIspG	Codon optimized IspG from *C. violaceum*	This work
pMEP_ MinfIspG	Codon optimized IspG from *M. infernorum*	This work
pMEP_PanaIspG	Codon optimized IspG from *P. ananatis*	This work
pMEP_RxylIspG	Codon optimized IspG from *R. xylanophilus*	This work

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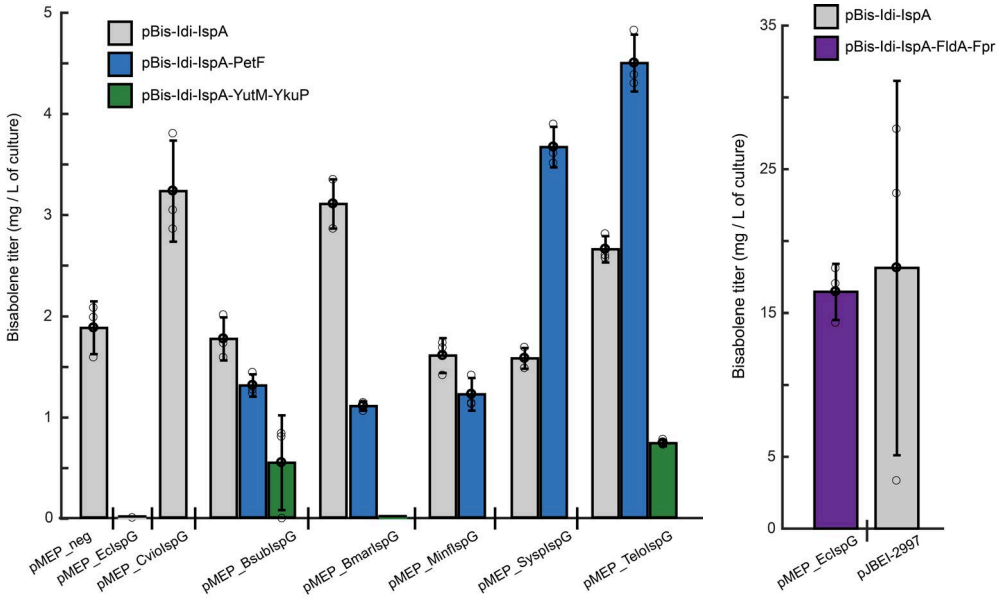
pMEP_SyspIspG	Codon optimized IspG from *Synechocystis sp. PCC 6603*	This work
pMEP_TeloIspG	Codon optimized IspG from *T. elongatus*	This work
Bisabolene production vectors: pBbE2a backbone containing operon expressing codon optimized AgBIS, Idi, ispA, and electron carriers and/or plug adapters linked to IspG activity		
pBIS_IDI_IspA		This work
pBIS_IDI_IspA_FldA_Fpr	FldA and Fpr from *E. coli* BL21(DE3)	This work
pBIS_IDI_IspA_FldA_Fpr_ErpA	FldA, Fpr and ErpA from *E. coli* BL21(DE3)	This work
pBIS_IDI_IspA_YutM_YkuP	YutM and YkuP *B. subtilis str.168* (NC_000964)	This work
pBIS_IDI_IspA_PetF	Codon optimized PetF from *Synechocystis sp. PCC 6803*	This work
pBIS_IDI_IspA_PetFH	Codon optimized PetF and PetH from *Synechocystis sp. PCC 6803*	This work
Plasmids for antibiotic production		
pFom34D12C	Codon optimized Fom34D12C from *S. wedmorensis* in pBbE5k backbone	This work
pBtuCEDFB	Genes btuCEDFB from *E. coli* MG1655 in pBbS2c backbone	This work
pFom34D12C_ScatSufUT	Codon optimized Fom34D12C from *S. wedmorensis*, SufU and SufT from *S. cattleya* in pBbE5k backbone	This work
pFom34D12C_ScatFdR	Codon optimized Fom34D12C from *S. wedmorensis*, with ScatFdR1, ScatFdR2, ScatFdR3 from *S. cattleya* in pBbE5k backbone	This work
pFom34D12C_ScatFdR_SufUT	Codon optimized Fom34D12C from *S. wedmorensis*, with ScatFdR1, ScatFdR2, ScatFdR3, SufU and SufT from *S. cattleya* in pBbE5k backbone	This work

Supplementary Table S4.4 – List of primers used in this study

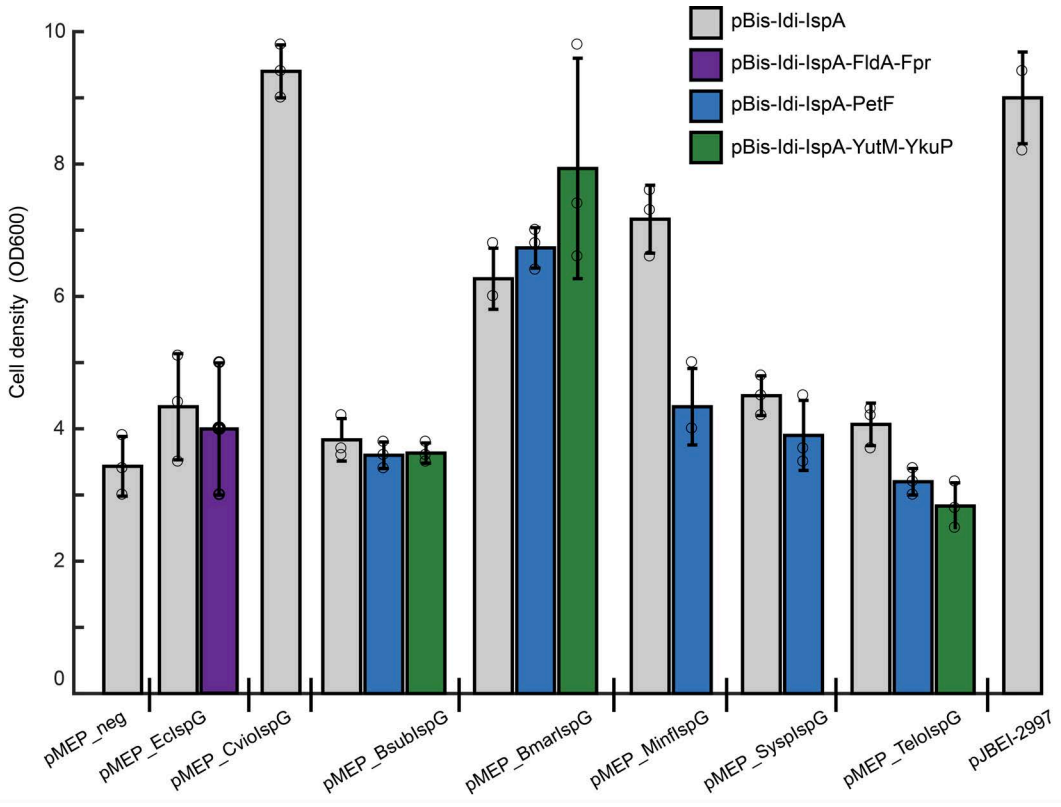
Primer	Sequence
Primers used to construct knockout strains	
P1_intA	TTACTCTGTTAATCAAGTCCAGTGGCAGTAAGCTTTGGCCTTTTCATACTCCCGCCATTCAGAG
P2_intA	GAAGCAGCTCCAGCCTACACCAATGGTGGGCTGATCTCGTAAAAGCAGCAGATAGTGG
P1_ΔispG	GCAGTAACAGACGGGTAACGCGGGAGATTTTTCATTCCGGGGATCCGTCGACC
P2_ΔispG	GAAGCAGCTCCAGCCTACACATGATCGACCAGCTGGAAGCACGCATTCGTGCGAAAG
P1_ΔispH	TTGAAGTGCTGGAAATCGATCCGGCACTGGAGGCGTAACGTCTTGAGCGATTGTGTAG
P2_ΔispH	TAATCGACTTCACGAATATCGACACGCAGCTCTTTCGGCTGTCAAACATGAGAATTAA
P1_ΔnadA	CCGATAACAGCGAATATTACGCTAATGTCGGTTTTAACGTGTCTTGAGCGATTGTGTAG
P2_ΔnadA	TCCACGTAGTGTAGCCGCAAAATCCAGCATACGATTGAGCTGTCAAACATGAGAATTAA
P1_ΔilvD	CGTCCCATTTACGAGACAGACACTGGGAGTAAATAAAGTGTCTTGAGCGATTGTGTAG
P2_ΔilvD	CCAGGCTGGCATAAGCACGCAGGGCAAAGGAGACCTGACCTGTCAAACATGAGAATTAAT
Primers used to construct pMEP_ispG_EC	
P1_dxs	AGAATTCAAAAGATCTAGGAGGGAGATATACATATGAGTTTTGATATTGCCAAATACCCG
P2_dxs	TTATGCCAGCCAGGCCTTGA
P1_ispDF	AGAGGTGATGCGTCTCGCAAGCTGAAGGAGGAAAAAATGGCAACCACTCATTTGG
P2_ ispDF	TCATTTTGTTGCCTTAATGAGTAGC
P1_idi	GCTACTCATTAAGGCAACAAAATGAAGGAGGAAAAAATGCAAACGGAACACGTCATT
P2_ idi	TTATTTAAGCTGGGTAAATGCAGATAATC
P1_ispC	AAAATCAAGGCCTGGCTGGCATAAAGGAGGAAAAAATGAAGCAACTCACCATTCTGG
P2_ ispC	TCAGCTTGCGAGACGCATC
P1_ispE	TCTGCATTTACCCAGCTTAAATAAAGGAGGAAAAAATGCGGACACAGTGGCCCT
P2_ ispE	TTAAAGCATGGCTCTGTGCAAT
P1_ispH	TCCCCATTGCACAGAGCCATGCTTTAAAGGAGGAAAAAATGCAGATCCTGTTGGCCAA
P2_ ispH	GCTAATGACTTAATCGACTTCACG
P1_MEP_ispG_EC	GATATTCGTGAAGTCGATTAAGTCATTAGCCGGCAAGAAGGAGATATACATATG
P2_MEP_ispG_EC	AGCCTTTCGTTTTATTTGATGC
Primers used to construct bisabolene synthesis vectors	
P1_AgBIS	ATCAGTGATAGAGAAAAGAATTCAAGAAGGAGATATACATATGGCGGGTGTTTCTGCG
P2_AgBIS	CGTTTGCATTATCATTACCTCCTAGATCCTTACAGCGGCAGCGGTTC
P1_idi_ispA	GGATCTAGGAGGTAATGATAATGCAA
P2_idi_ispA	AGCCTTTCGTTTTATTTGATGC
P1_btuCED	ATACCATGCTGACACTTGCCC
P2_btuCED	TCAGATGGTCGAAATCAGCA
P1_btuF	TGGCTAAGTCACTGTTCAGGG
P2_btuF	TCAATCTACCTGTGAAAGCGCA
P1_btuB	TGATTAAAAAAGCTTCGCTGCTGA
P2_btuB	TCAGAAGGTGTAGCTGCCAG

Supplementary Table S4.5 – MRM settings for analysis of the fosfomycin pathway metabolites

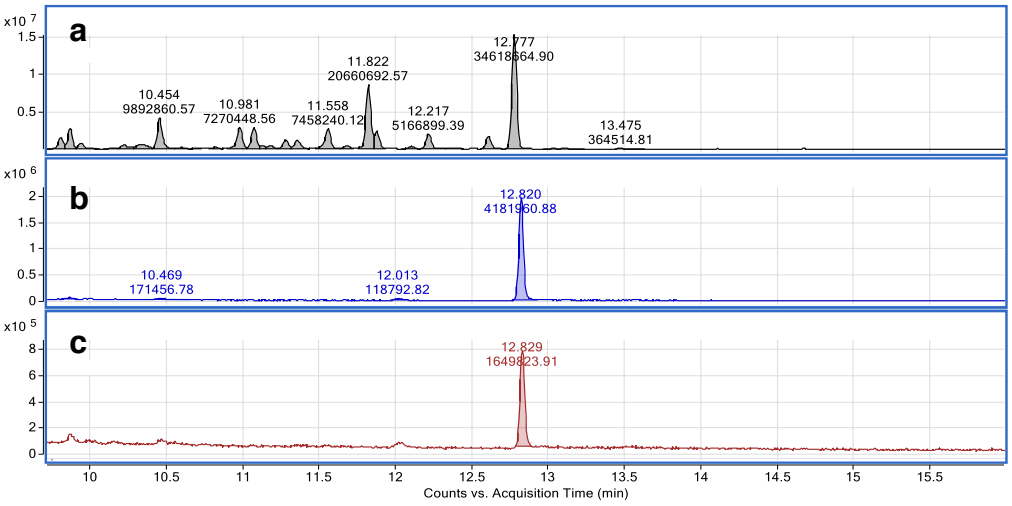
Compound name	Formula	Mass	Precursor ion	Product ions	Dwell	Collision Energy(V)	Polarity
Fosfomycin	$C_3H_7O_4P$	138.06	137	79.1 63.1	120	28 12	Negative
HEP-CMP	$C_{11}H_{17}N_3O_{11}P_2$	431.05	432	324 112.1	100	20 40	Positive
HPP-CMP	$C_{12}H_{19}N_3O_{11}P_2$	445.06	446	112.1 97	100	20 20	Positive

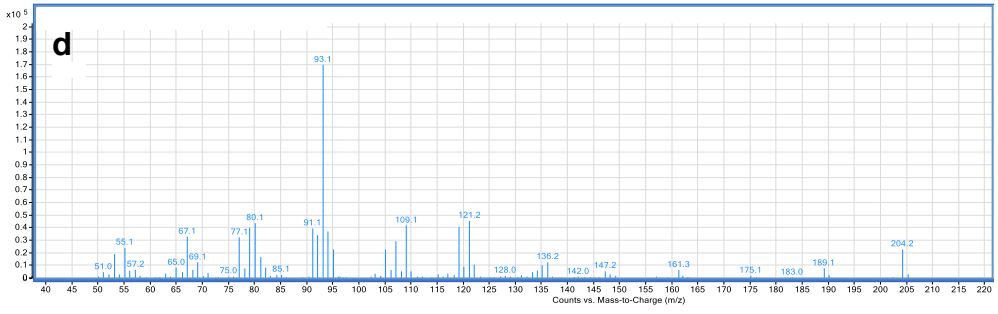


Supplementary Figure S4.1. **Bisabolene titers obtained from cultures expressing MEP pathway vectors.** Titers are calculated from the amount of bisabolene recorded in dodecane overlays 24 h after induction, divided by the volume of culture (25 mL). Data represent the mean ± s.d. of three biological replicates; dots represent each replicate.

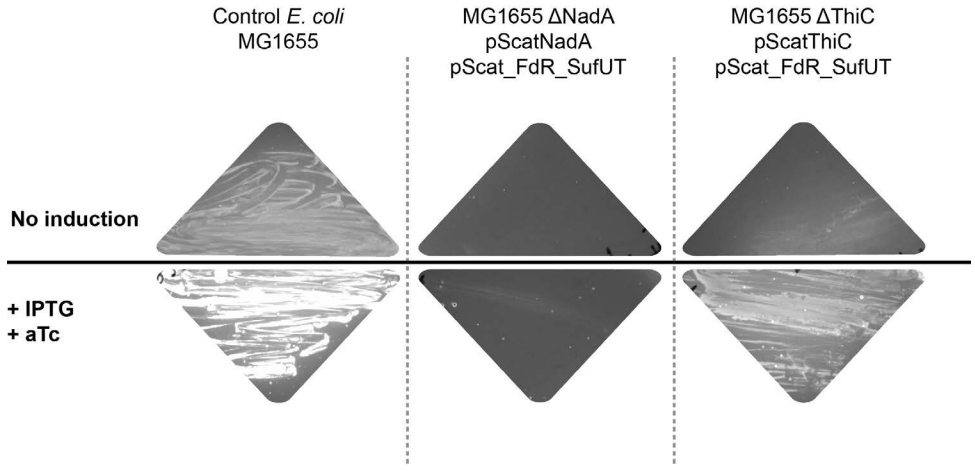


Supplementary Figure S4.2. **OD600 measurements of bisabolene producing cultures 24 h after induction.** Data represent the mean ± s.d. of three biological replicates; dots represent each replicate.

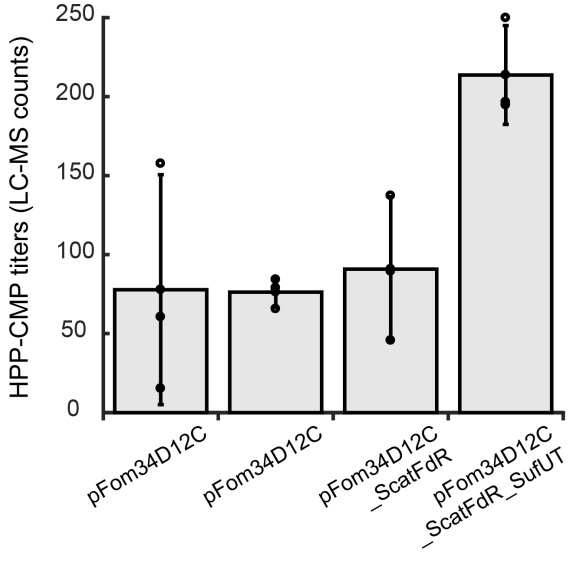




Supplementary Figure S4.3. **Identification of biosynthetic bisabolene produced by engineered *E. coli* cultures. (a)** GC/MS Chromatogram of a synthetic bisabolene standard (mixture of isomers, Alfa Aesar A18724) diluted in dodecane. Representative chromatograms of analyzed dodecane overlays from bisabolene production cultures 24h after induction. **(b)** Strain BL21 pJBEI-2997 pBIS-idi-ispA **(c)** Strain BL21 pMEP_SyspIspG pBIS-idi-ispA_PetF. **(d)** Fragmentation spectrum of biosynthetic bisabolene produced by strain BL21 pJBEI-2997 pBIS-idi-ispA.



Supplementary Figure S4.4. **The co-expression of pScat_FdR_SufUT activates the rSAM enzyme ThiC, but not NadA.** Pictures of complementation assay. Growth of *E. coli* MG1655 strains on selective MOPS minimal medium plates (**top**), and supplemented with 0.25 mM IPTG (for induction of 'plug adapter' vector), and 50 mg/mL aTc (for FeS induction) (**bottom**) after incubation at 30°C for 48 h



Supplementary Figure S4.5 Co-expression of "plug adapter" proteins from *S. cattleya* increases levels of HPP-CMP up to 2.7-fold. HPP-CMP titers (LC-MS counts) recorded in *E. coli* NCM3722 cultures co-transformed with pBtuCEDFB and the indicated FOM production vector. Measurements were performed from samples collected 4 h after induction. Data represent the mean ± s.d. of three biological replicates; dots represent each replicate.

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5

CONCLUDING REMARKS AND PERSPECTIVES

"Biology today is little more than an engineering discipline.
[…] Society today perceives biology as here to solve its problems, to change the living world. Both physics and biology are primary windows on the world; they see the same gem but different facets thereof (and so inform one another). Knowing this, society will come to see that biology is here to understand the world, not primarily to change it. Biology's primary job is to teach us. In that realization lies our hope of learning to live in harmony with our planet." [1]

C. R. Woese

[1] Text from: Woese, C. R. A New Biology for a New Century. Microbiol. Mol. Biol. Rev. 68, 173–186 (2004).

1.1 CONCLUDING REMARKS ABOUT THE WORK IN THIS THESIS

In this thesis, we presented research efforts to develop new synthetic biology and metabolic engineering tools for the microbial production of relevant industrial compounds. In particular, our work focused on engineering novel strategies to address some of the major current challenges in the generation of proof-of-principle microbial cell factories. Here we discuss the successes and recommendations for future research resulting from our work.

Bacteria can be harnessed as antibiotic production hosts

The use of bacteria as hosts for the production of bacteriocidal antibiotics (such as carbapenems) remained unexplored due to the obvious toxicity of the antibiotic product. In this thesis, we investigated the performance of *E. coli* strains to produce potent carbapenems, and demonstrated that a few targeted metabolic engineering interventions resulted in higher antibiotic titers compared to natural producers. Moreover, the work presented in Chapter 2, goes beyond previous examples of engineering production of bacteriostatic antibiotics (e.g. erythromycin[1]) to pioneer methods for ameliorating toxicity of a bactericidal and lysogenic antibiotic to bacterial production hosts. Our engineered *E. coli* strains provide a useful testbed for developing novel approaches for bactericide production in bacterial hosts. Indeed, our strategy to increase tolerance via engineered growth-arrest is broadly applicable to antibiotics that target cell wall biosynthesis (i.e. β-lactams or fosfomycin). We hope this work will encourage the development of large-scale bacterial bioprocesses that will contribute to the fight against antimicrobial resistance.

In addition to the synthesis of toxic antimicrobials, we demonstrate that metabolic engineering of antibiotic pathways in *E. coli* provides a platform for the production of pathway intermediates and precursors that can be combined with chemical synthesis to develop novel semi-synthetic industrial processes.

However, to truly evaluate the industrial and economic potential of our engineered *E. coli* strains, it is necessary to translate our results from laboratory-scale fermentations into industrial-scale fermenters. Indeed, in this thesis we have not carried out experiments to scale-up carbapanem production, and have not studied potential issues that might affect or hinder this process. Additional engineering interventions are likely to be required during the scale-up to fully optimize viable industrial bioprocesses. Therefore it is critical to test the performance of our engineered strains using laboratory or pilot-scale fermenters as a first step to scale-up production, and to efficiently identify bottlenecks to their industrial implementation[2]. In addition, downstream techniques for product purification and/or extraction will be required.

Moreover, as discussed in this thesis, characterizing relevant antibiotic pathways is necessary to fully exploit the clinical potential of novel antimicrobials and to stimulate their commercialization. However, the

characterization of new antibiotic pathways can be a difficult and laborious task, since the vast majority is naturally found in the genome of unculturable and/or genetically untractable organisms. In order to accelerate pathway characterization, we strongly encourage further efforts to (re)construct relevant pathways within common microbial chassis like *E. coli.* Indeed, As DNA synthesis and assembly technologies are increasingly faster and cheaper, the heterologous expression and modification of codon optimized biosynthetic clusters is becoming a viable strategy to advance their complete elucidation. Beyond the characterization of the Car biosynthesis cluster, we hope the combination of our engineered strains with FeS 'plug adapters' will contribute to the full characterization of the thienamycin pathway from *S. cattleya*.

While *E. coli* is a convenient chassis for characterizing and testing engineered pathways, we believe it is important to further develop alternative chassis for optimizing antibiotic production. Indeed, the genetic and metabolic modifications that are found to be effective in *E. coli* could be implemented to improve production in other hosts. For instance, the overexpression of a feedback inhibition mutant of the enzyme ProB, which increases the levels of a carbapenem precursor, could be engineered in natural carbapenem producers. Other chassis with better tolerance to the antibiotic product (such as yeast species) should also be considered. Eventually these efforts might enable the sustainable large-scale synthesis of complex antibiotics (i.e. thienamycin and derivatives) within industrial chassis.

New tools to study and enable heterologous (metallo)enzyme activity are required

Synthetic biology endeavors to exert control over biological processes in a predictable and programmable manner are known to be greatly limited by the context-dependency of biological parts. In particular, the activity of many enzymes is considerably altered when expressed in foreign organisms, often resulting in poor or no activity. In Chapter 3 we discussed engineering tools previously developed to activate and enhance metalloenzymes for metabolic engineering applications, notably within heterologous hosts. In Chapter 4 we go beyond pathway-specific strategies, to present a novel experimental approach for the systematic identification of 'plug adapter' proteins to activate FeS enzymes in *E. coli*. We show that the transferability of FeS enzymes across species is unpredictable, and highly dependent on the presence of compatible maturation and electron transfer pathways within the host. Our work demonstrates that inactive foreign FeS enzymes can be activated in *E. coli* by the co-expression of compatible maturation of electron transfer proteins from heterologous organisms. Although the underlying molecular criteria that determine the functional compatibility of FeS enzymes remain largely unknown, our results establish a novel proof-of-concept for how to activate these enzymes within industrial hosts. Our complementation assays and genome mining tools could be directly scaled-up to identify effective 'plug adapters' for a

wide variety of FeS enzymes (e.g. by using bioinformatics algorithms for genome, generating libraries of expression vectors via DNA synthesis, or using automation to perform assays). This will likely enable the use of unexplored FeS enzymes for metabolic engineering applications. We hope that these tools, in combination with further biochemical studies, will help developing custom strategies to import foreign FeS enzymes involved in the production of high-value chemicals.

Encouraged by the results obtained from our small-scale experiments, we strongly recommend the further development of experimental assays to characterize the barriers to enzyme transferability and to develop strategies that circumvent them. Indeed, similar experimental approaches could be implemented to advance the activation and optimization of other relevant metalloenzymes (i.e. methane monoxygenases, P450s or hydrogenases) within heterologous hosts. In addition, novel *in vivo* assays to quantify the efficiency of electron supply[3] to given metalloenzymes and/or their maturation, could accelerate their functional characterization and catalytic activity.

Developing reliable high-throughput analytical tools is critical

A major bottleneck in the development of the microbial strains described in this thesis was undoubtedly the initial lack of straightforward analytical tools to evaluate pathway performance. The necessary development of a reliable LC/MS method to analyze in parallel the levels of the Car pathway metabolites was a very laborious task that took 2.5 years to realize. This was largely due to the lack of commercial chemical standards and the intrinsic molecular properties of these compounds (i.e. low mass, high polarity and hydrophilicity), which encumbered their identification and separation in complex culture media. Moreover, these factors were a significant barrier to the estimation of Car titers, which was a particularly difficult task. For several months, we attempted to obtain purified forms the antibiotic from broth cultures using various techniques such as ion-pair extraction, compound derivatization and HPLC. The known instability of Car and its specific properties in aqueous solution obstructed its purification from supernatant samples. In addition, we contacted a commercial custom synthesis team, which attempted for months to synthetize standards for Car pathway metabolites, unfortunately in vain. These issues exemplify the difficulties that can be associated with the optimization of metabolic pathways that produce compounds that are poorly characterized, unstable and/or difficult to synthetize/extract with chemical methods.

The rapid development of novel analytical methods to enable the high-throughput, rapid and cheap characterization of engineered strains (i.e biosensors, colorimetric assays, high-throughput MS analysis) is an urgent necessity in the field of metabolic engineering. In order to advance the understanding of metabolic pathways, additional methods to characterize modified organisms over a range of systems scales (e.g. DNA, RNA, proteins, and metabolome) should be implemented. For instance, the availability of

methods to quantify pathway intermediates and enzyme levels in our lab would have greatly improved our understanding of how the overexpression of foreign FeS enzymes and 'plug adapters' alter the MEP pathway flux. Moreover, proteomics tools to reliably quantify Car pathway enzymes could allow generating kinetic models of carbapenem production in *E. coli*, and reveal new opportunities for metabolic engineering. Therefore, beyond the tools required to construct microbial strains, metabolic engineering research groups should ensure that they have sufficient technical and monetary means to provide the necessary analytical tools required for each bioprocess. This can be achieved either by developing new methods, implementing existing ones in-house, or outsourcing sample analysis.

1.2 REMAINING CHALLENGES IN INDUSTRIAL-SCALE BIOMANUFACTURING

Although the promise of microbial cell factories is significant, their use in the development of bioprocesses for manufacturing commercial products is still in its infancy[4]. As discussed in this thesis, the advantages of using engineered microbes for manufacturing purposes are eminent, but only a few cost-competitive large-scale bioprocesses have reached the market in some specific sectors.

In contrast to microbial biomanufacturing, chemical synthesis technologies have been long established and widely used for the commercial production of a tremendous amount of valuable natural and unnatural industrial molecules. Chemical engineering has enabled the industry to harness a vast chemical space for commercialization (more than 70,000 products[5]), a capacity that cannot yet be realized with biotechnology. Despite recent advances in artificial biocatalysts to synthetize unnatural products, most scalable bioprocesses are still restricted to the production of compounds that are naturally produced by living organisms. Further mining efforts for identifying and characterizing natural and engineered enzymes are needed to broaden the capacity of microbial processes.

Moreover, chemical engineering has allowed many industrial sectors to design and implement robust, predictable, scalable and cost-effective manufacturing methods. Compared to these traditional chemical methods, the capacity, performance and efficiency of microbial bioprocesses remain limited. Therefore, the field of microbial biosynthesis must overcome a number of technical challenges before it can fully transform industrial manufacturing[2], which include: (i) bioprocesses are slow (take days instead of hours) and usually discontinuous (ii) the conversion efficiency of substrate to products is low; (iii) biological systems can behave unpredictably, and the quality of the process is often difficult to control; (iv) substrates/feedstocks often rely on agricultural products; (v) downstream processing (i.e. product extraction and

treatment) can be expensive and laborious; (vi) each engineered strain is typically restricted to the production of one specific molecule. Moreover, developing industrially viable bioprocesses takes more time (5 to 10 years) and costs more money compared to traditional synthetic methods[5].

The biomanufacturing field has still many hurtles left to overcome in order to enable a sustainable bioeconomy. Here, we discuss some of these major challenges, grouped in 3 vast categories: accelerating organism engineering, expanding feedstock use, scaling-up technologies.

Accelerating organism engineering

In spite of the significant progress in the fields of synthetic biology and metabolic engineering, developing high-performing microbial strains for commercial bioprocesses remains an expensive and challenging endeavor, which generally requires over $50 million and 6-8 years of research[6]. Most initial natural or synthetic strains produce target molecules at very low yields, and their optimization often involves several “debugging” steps and iterations of the DBTL cycle. Due to the unpredictability (and often irreproducibility) of biological processes, many strain and fermentation modifications are still required to achieve commercial viability. Developing our ability to engineer novel microbial cell factories will contribute to expand the chemical space and feedstock repertoire of biomanufacturing, as well as improving the stability and reproducibility of industrial bioprocesses, which are critical factors previously discussed in this thesis. Continuous technical and scientific advances are needed in accelerating and improving each component of the design-build-test-learn cycle for developing microbial cell factories.

Currently, the turnover rate of the DBTL cycle for engineering microbial cell factories is greatly limited by the speed, cost, reproducibility and precision of each module. For instance, the cycle still strongly depends on the artisanal laboratory work of highly specialized scientists, which remains low throughput, difficult to reproduce, and prone to human factors and errors. Consequently, increasing efforts have been focused on developing automated biofoundries[7] to conduct experiments and integrate data in a faster, standardized, reproducible, cheaper, and more scalable manner. In particular, cost effective automation technologies to accelerate the construction of large libraries of organisms and to rapidly test their performance are needed in the field. The success of organism engineering companies such as Ginkgo Bioworks and Zymergen are strongly related to the efficiency and performance of their automated biofoundries and prototyping capabilities. Expanding these capabilities are key to accelerate the development of commercially viable microbial cell factories both in academic and industrial settings. Moreover, it will likely allow scientists to

In addition to the use of microbial chassis, biomanufacturing approaches relying on synthetic cell-free systems have been proposed to bypass with the complexity and constraints (i.e. growth conditions, product

toxicity) of whole-cell biocatalysts[8]. These systems have the potential to provide a greater control, modularity and flexibility of biosynthetic pathways, which could enable the development of new commercial bioprocesses. Moreover, cell-free systems can be employed to speed up the DBTL cycle by enabling rapid and cheap prototyping platform for metabolic engineering[9].

Beyond technical improvements, it is necessary to further develop generalized and standardized communication platforms for research groups to share designs, protocols and generated data.

Improving scaling-up technologies

While the impressive progress in biotechnologies has successfully allowed engineering diverse microbial processes for the production of many relevant chemicals in the laboratory, most have not yet been translated to industrial-scale fermentations[10]. Unlike with traditional chemical methods, scaling-up engineered bioprocesses to meet commercial needs remains a critical challenge in the field[2]. This is largely due to the fact that engineered microbial strains often lose their performance and productivity when cultivated in industrial fermentation conditions[4]. Indeed, many factors can drastically affect the overall productivity and efficiency of a bioprocess, causing discrepancies between laboratory-scale and large-scale fermentations. Some of these factors include differences in medium composition and cultivation conditions (i.e. pH and temperature variations, cell density, agitation, accumulation of toxic byproducts, nutrient and oxygen transfer). For instance, aerobic fermentations processes are very sensitive to aeration-agitation conditions, which define the oxygen transfer rate and directly affect cellular metabolism. Moreover, the loss of performance of engineered strains can also be attributed to genetic instability that can arise in industrial fermentation processes (i.e. mutations, plasmid loss), and which results in the loss of the desired high-performing phenotype. New genetic engineering tools to construct strains with more robust biosynthetic and regulatory programs (i.e. chromosomal modifications or integrations) can be employed to tune and maintain microbial performance at large-scales[4]. Pilot-scale fermentations are increasingly performed at early stages of bioprocess design in order to identify and predict the critical parameters that can potentially affect performance during the scale-up[11]. These experimental approaches can be combined with predictive computational models in order to predict and improve the performance of microbial cell factories at industrial scales. Reducing the time and monetary costs of scaling-up fermentations is key in determining the economic attractiveness of biomanufacturing processes.

Expanding biomanufacturing feedstocks

The microbial production of industrial chemicals relies on renewable plant-derived carbon feedstocks, which are primarily sugars (i.e. sucrose and starch). The nature of the feedstock is a key factor in defining the cost and economics

of a given biological process. Currently, sugar feedstocks are one of the main contributors to the total costs of biomanufacturing processes, representing up to 65% of the total production cost of large-volume chemicals such as biofuels[12]. The dependence of the large majority bioprocesses on sugars is not optimal for a sustainable bioeconomy. Indeed, biomanufacturing would compete for food resources and land use, and their costs would highly depend on regional availability and fluctuating agricultural markets. Therefore, improving the sustainability, availability, and reliability of the feedstocks employed in biomanufacturing processes is crucial in accelerating their industrial application and economic impact.

Increasing efforts are been directed to develop closed loop fermentation processes from lignocellulosic biomass feedstocks[12]. Indeed, the macromolecules that compose lignocellulose – lignin (15–25%), cellulose (30–45%), and hemicellulose (25–40%) – from agricultural waste (i.e. corn stover, wheat and rice straw) can be used as carbon source for biological processes. However, the utilization of lignocellulose first requires the action of complex chemical and/or biochemical processes to hydrolyze the macromolecules into simple fermentable sugars. Strategies to improve the efficiency and viability are required to further expand the full use of lignocellulosic waste as input for industrial bioprocesses, which could reduce the costs and environmental impact of biomanufacturing[12].

Additionally, the development of bioprocesses that depend on other alternative feedstocks, such as carbon dioxide, methane gas (and derivatives), or formate has gained considerable interest[13]. The use of C1 gases, in particular methane, as next-generation carbon feedstocks is an attractive solution due to their abundance, low cost and huge availability[14]. Moreover, methane and carbon dioxide bioconversion would contribute to the reduction of greenhouse gas emissions[15]. Further improvements in the use of more economically and environmentally sustainable feedstocks will continue to advance the industrial impact of biomanufacturing technologies.

1.3 BIOMANUFACTURING AND SOCIETY

Bioengineers will undoubtedly continue to expand their capability to transform/control life and its processes. Biology today, including in this thesis, is often presented as an emerging engineering discipline with enormous potential to provide solutions to society's biggest challenges: ensuring global health and agricultural production, enabling a sustainable economy, or solving the acute environmental crisis. However, the impact that microbial cell factories will have on society will not exclusively depend on technical advances; it will be proportionately defined and mediated by economic, cultural and political factors. Indeed, science and technology cannot be decoupled from the individuals and communities that drive, support and employ it, and which are

continuously influenced by their socio-economic and cultural context. Therefore, the success of biomanufacturing technologies is directly linked to their economic viability, real-world applicability, risk-benefit ratio and public acceptance.

Efforts to develop efficient governance and responsibility frameworks in bioengineering communities have been made in the past decades to anticipate and assess related risks/benefits. These frameworks aim to define the standards, regulations and values required for promoting responsible innovation[16–18]. Such efforts are necessary given the unpredictable and uncertain nature of the potential social issues and harms that may arise from the application of new biotechnologies in general. In particular, the engineering approach of synthetic biology is continuously generating a broad range of applications and products with unexplored repercussions. Consequently, it has been proposed that a case-by-case approach should be most appropriate to study, anticipate and manage in depth the risks/benefits of a given bioengineered product/technology[17]. Indeed, each product is associated to specific accidental or deliberate threats to public health, the environment, ethics or socio-economic security. For instance, the regulations that apply to food products containing isolated ingredients produced with engineered microbes (i.e. flavors, vitamins, colorants, pharmaceuticals, etc.), are very different to those that involve the ingestion or environmental release of genetically modified organisms (GMOs). In depth evaluation of the sustainability, safety, accountability, benefits and containment should be an immediate priority in the development of synthetic biology and metabolic engineering projects, in order to efficiently prevent misuse and undesired/harmful consequences.

Another important challenge to the commercialization of biomanufactured products is consumer rejection. The product end-use and specific application have been shown to be major factors determining public acceptance and consumers' risk perception[19], particularly regarding health-related hazards: non-food applications (i.e. renewable materials, bioenergy) are typically more accepted due to the strong negative perception of GM food. However, public acceptance does not exclusively depend on the technology and its application; the levels of trust in the industry and the prevailing policies are equally important. Therefore, effective and targeted communication strategies with the wider public should be implemented, not only to promote outreach and education, but also to engage in constructive conversations about the social implications of biotechnology. To ensure that synthetic biology and biomanufacturing work for society, I believe bioengineers bear the responsibility of clearly communicating and exposing the inner workings of the research they conduct, and the transformative power of the technologies they develop.

Because biotechnologies rely on society's full acceptance to thrive, both researchers and decision makers should provide effective environments and tools to promote communication and education, as well as to foster responsibility, trust, and critical thinking. From early years, the synthetic biology

community has been involved in diverse outreach and education endeavors, from which the most significant and influential has been the creation of the International Genetically Engineered Machine (iGEM) Foundation[20], an independent NGO dedicated to promote the responsible practice and advancement of synthetic biology. The annual iGEM student competition gives the opportunity to young practitioners around the world to learn, create and harness synthetic biology tools to develop biotechnologies for tackling real issues facing our world and society. The competition, which gathers nearly 6000 students each year, has offered a novel effective framework to collectively explore, assess and discuss how specific biotechnologies could interact with society. Other efforts from academic institutions to develop new educational tools for the general public have been made. For instance, in 2013, I have designed and kick-started an educational video game funded by an initiative of the European Commission to develop new forms of public participation in research (e.g. Citizen Cyberlab). The open-source video game 'Hero.coli' was designed to offer an engaging and contextualized space for people to combine Biobricks and build intuition for genetic design while playing online[21].

However, although education/outreach initiatives are crucial, it is important for bioengineering communities to understand that public rejection and mistrust do not exclusively depend on public ignorance[22]. Beyond technical factors, acknowledging the complex considerations and heterologous dynamics that exist between science, technology and society is vital to ensuring the responsible, acceptable, viable and effective development of biotechnologies. Academic and industrial researchers should openly recognize that their practice cannot be detached from economic, political, ethical and cultural considerations, and that their activities are not immune to contextual biases. It is important that the synthetic biology community exposes how these considerations influence their organization, values and goals in a transparent and participatory manner. Since biotechnologies and society mutually shape one another, such conversations and interactions are not meant to be one-sided: society should be informed and educated about how science and technology are evolving; scientists should be educated about society and understand its dynamic evolution. Since the capacity, scale and speed of biotechnologies continue to expand, a positive feedback loop between science and society is key to properly assess their global impact. Dialogues and decisions that take into account all stakeholders are necessary to build mutual understanding and trust between the industry, scientists, policy makers, founding agencies, NGOs and the general public. To this purpose, it is essential to establish inclusive, transparent, stable, fair and effective dialogues among stakeholders. Such conversations are crucial to define an efficient governance framework and the ways science and technology intend to benefit society in the long term. As Christina Agapakis proposes[22]:

> *For engineers […] it is important to understand and specify who is affected by the problem we are solving as well as who might benefit from a potential solution. Moreover, nuanced discussions of problems and their multifactorial causes and effects can help engineers better understand where a technological approach might or might not be appropriate, as well as what might count as a problem in the first place.*

Today, bioengineers are often not trained or accustomed to include external parties to critically engage with their research/technology, neither in properly assessing the potential consequences of their work[22,23]. This has led for example to recent controversial research resulting from unilateral decisions, which were kept confidential until their publication and that violated regulatory guidelines (i.e. genetic modification of human embryos[24], or the resurrection of the horsepox virus[23]). The scientific community necessitates the counsel and involvement of professionals from other disciplines in order to build meaningful, useful and ethical technologies: sociologists, designers, artists, community representatives, economists, ethicists, hobbyist scientists etc. By collaborating with professionals from the humanities and creative disciplines, bioengineers can for example develop methods to facilitate critical engagement with their activities, as well as to use speculation and fiction to envision the potential futures of technology thought different times, scales and conditions[22,25]. By interacting with DIY (do-it-yourself) synthetic biology communities, academic researchers and companies can learn about the socially significant problems their research is not tackling, as well as identify emerging risks and misuses[26]. Some specialized biomanufacturing companies have made efforts to implement collaborations and partnerships to promote deeper conversations about the social implications of their products through narratives and practices that transcend the technical aspects of their activities. For instance, the company Perfect Day Foods, which produces animal-free dairy food products using engineered microbes, works in closed partnership with marketers, food developers and chefs to properly integrate their products into the market, while transparently communicating about their production process and technology[27]. Ginkgo Bioworks has developed a creative residency program to engage practitioners from creative disciplines to frame the future potential and social implications of the company's products and activities[28].

In addition to enabling critical conversations about the utility and application of synthetic biology, art and design offer a framework, beyond the confines of scientific methods, to challenge and question established mindsets, philosophies, intentions and concepts that drive biological engineering today. As we develop our capacity to harness the living world as a medium for human exploitation, society needs to explore and (re)think its relation to life and Nature at large. Some bio-art pioneers, such as Oron Catts and Joe Davis, have been interested in challenging what they perceive as a single-minded reductionist

approach of synthetic biology, which regards life as a matter for commercial purposes. According to Catts[29]:

> *On a macro scale, when we marry the type of business model that creates contemporary economies with the power of those new technologies of biology – and especially the mindsets that assume power over biological systems – we'll need to figure out ways in which our understanding of biological systems can also help us understand that we need to change the way we think about our place in the world, and how we think about controlling it and who's got the power to control it.*

Through their work, artists and designers attempt to counterbalance researchers approach, which has increasingly emphasized the utility and control of living systems. As a result, biological engineering has been integrated into their practice, and harnessed to use life as raw material to offer alternative frames of thought that serve non-commercial purposes[30,31]: artistic, philosophical, political, cultural and ontological scrutiny. Such conversations and explorations could "help science understand itself"[30], and assist synthetic biology – a discipline in the making – to (re)define its guiding vision for the future.

For the microbiologist and biophysicist Carl Richard Woese, if biologists do not seek to collectively build a new and inspiring vision that goes beyond serving contextual societal needs, their discipline will be primarily concerned with temporal practical problems, and eventually fail in its primary purpose of understanding the world[32]. Indeed, he urges contemporary biologists/bioengineers to not let the engineering approach monopolize their discipline[32]:

> *A society that permits biology to become an engineering discipline, that allows that science to slip into the role of changing the living world without trying to understand it, is a danger to itself.*

As biotechnologies hold a tremendous power to alter and transform the world, it is our responsibility to fully engage in new conversations about the kind of society we want to build, and the kind of relation to the living world humans should foster.

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SUMMARY

For millennia, humans have used microbes to produce industrial products of social and economical value through fermentation processes. In recent years, the application of engineering principles to microbiology have dramatically expanded our ability to modify and optimize microbes for the production of a wide variety of commercial products from renewable feedstocks: food and commodity chemicals, to biofuels and fine chemicals such as pharmaceuticals, fragrances, cosmetics or dyes. The use of microbial bioprocesses for the production of natural products represents an attractive and sustainable alternative to current industrial production methods, which mainly rely on chemical synthesis and/ or extraction from native producers. Advanced biomanufacturing technologies would not only provide sustainable economic benefits (by reducing the monetary cost of production of useful chemicals), but also offer social and environmental benefits.

Synthetic biology has allowed engineering the production of many industrial compounds within microbes that do not naturally produce them – this is called "heterologous microbial biosynthesis". In addition to replacing current manufacturing processes, heterologous microbial biosynthesis likely offers the only viable platform to produce certain natural products at industrial scales. Indeed, many relevant compounds cannot be viably manufactured through chemical synthesis, and/or are produced at undetectable/insufficient levels in native organisms. However, many heterologous bioprocesses remain in their infancy to fully enable an economically viable delivery of relevant natural products to the market. In order to build and sustain the promise of a bioeconomy for the 21st century, metabolic engineering is under pressure to continue to provide largescale, sustainable and cost-competitive bioprocesses that meet global needs.

In this thesis, we focus on the development of microbial strains to accelerate the microbial production of 2 different families of high-value compounds of prominent biotechnological relevance within the established microbial chassis *Escherichia coli*: antibiotics and isoprenoids. The fight against antimicrobial resistance is considered one of the greatest public health challenges of the 21st century. Recent technologies have uncovered new antibiotics that, if harnessed, might help alleviate this crisis. However, most of these new antibiotic compounds are far too complex for economical chemical synthesis, and are naturally produced by unculturable and/or genetically intractable microbes. Developing new heterologous microbial platforms for antibiotic production may be an efficient solution for harnessing the clinical potential of these molecules and their commercialization. Isoprenoids represent one of the largest families of natural compounds (over 50,000 molecules) with an incredible number of

practical uses, and of great commercial value: from high-value compounds such as many pharmaceuticals, fragrances and flavors, to commodity chemicals such as solvents, rubber or advanced biofuels. We focus in particular on relevant obstacles associated with the development of proof-of-principle strains for the laboratory-scale production of these high-value chemicals.

In **Chapter 2**, we explore how bacteria could be engineered to produce an antibiotic. We engineered *E. coli* to produce Car, a small antibiotic from the carbapenem family. Carbapenems are considered last-resort weapons against multi-drug resistant infections, and their production relies on costly and polluting chemical methods. Metabolic engineering of the Car pathway in *E. coli* improved production rates of Car by 60-fold over our initial design. We further increased antibiotic productivity by engineering *E. coli* growth-arrested cells with improved tolerance against carbapenem-induced lysis. The combination of our approaches surpasses reported titers obtained from natural carbapenem-producing strains. Our results demonstrate how *E. coli* can be harnessed as a platform for biosynthesis of bactericidal antibiotics.

Just as complex carbapenem pathways, many other industrially-relevant metabolic pathways contain biocatalysts that employ metal cofactors to perform remarkable biochemical reactions, referred to as metalloenzymes. The functional expression of metalloenzymes relies on post-translational modifications (PTMs) mediated by helper proteins. These modifications often involve multi-protein maturation and/or electron transfer pathways. The dependence of metalloenzymes on PTMs often limits their functional expression and catalytic activity in heterologous organisms. This has greatly hindered their application to the development of novel microbial cell factories. **Chapter 3**, is a comprehensive literature review that highlights the biotechnological relevance of metalloenzymes in natural product biosynthesis, and discusses recent biological engineering strategies developed to enable/improve their activity in native and foreign bacterial hosts.

In Chapter 4, we describe our efforts to develop novel synthetic biology strategies to enable the functional heterologous expression of a specific family of important metalloenzymes: iron-sulfur (FeS) cluster enzymes. These enzymes hold a tremendous untapped potential for biomanufacturing. Indeed, many FeS enzymes are involved in the biosynthesis of a huge variety of valuable natural products, such as biofuels, fragrances and flavors, or pharmaceuticals (including carbapenems and other antibiotics). Here, we describe a simple complementation assay that quickly reports the functionality of foreign FeS enzymes within *E. coli*, including 44 orthologs of the enzyme IspG, involved in isoprenoid biosynthesis. Our experimental data shows that FeS enzymes are typically inactive when expressed within evolutionarily-distant species. We demonstrate that inactive FeS enzymes can be recovered by co-

expression of foreign FeS maturation and electron transfer proteins. We next show that these proteins can act as efficient “plug adapters” to activate foreign FeS within *E. coli*. In addition, we explore the application of activated FeS enzymes for the biosynthesis of the biofuel precursor compound bisabolene, and the antibiotic fosfomycin. The further development of such “plug adapters” will enable and enhance the *in vivo* activities of relevant FeS enzymes, and expand the microbial synthesis of high-value chemicals.

Finally **Chapter 5** provides concluding remarks regarding the work presented in this thesis and discusses perspectives on the future of industrial biomanufacturing technologies.

SAMENVATTING

Millennia lang maken mensen al gebruik van microben tijdens fermentatieprocessen voor de productie van industriële producten van sociale en economische waarde. Recente biotechnologische ontwikkelingen hebben het mogelijk gemaakt om gemodificeerde microben te optimaliseren voor de productie van verscheidende producten, variërend van hernieuwbare grondstoffen voor levensmiddelen en chemicaliën tot biobrandstoffen en fijnere chemicaliën zoals geneesmiddelen, geurstoffen, cosmetica en kleurstoffen. Het gebruik van microbiologische processen ter productie van natuurlijke producten is een attractief en duurzaam alternatief vergeleken huidige industriële productiemethoden gebaseerd op chemische synthese en/ of het vergaren van het product vanuit het organisme dat het van nature aanmaakt. De ontwikkeling van geavanceerde bio-productie technologieën heeft economische voordelen (zinvolle chemicaliën kunnen dan goedkoper worden geproduceerd). Deze ontwikkelingen zijn tevens beter voor het milieu en brengen sociale voordelen met zich mee.

Synthetische biologie maakt het mogelijk om microben chemicaliën te laten produceren die van nature door hetzelfde organisme niet worden aangemaakt – de zogeheten 'heterologe microbiële biosynthese'. Naast de mogelijkheid om bestaande productieprocessen te vervangen, is heterologe microbiële biosynthese voor sommige natuurlijke producten de enige manier die rendabele productie op grote schaal mogelijk maakt.
Veel relevante chemicaliën kunnen niet synthetisch worden gemaakt of worden door het originele organisme in onmeetbare/ onvoldoende hoeveelheden aangemaakt. Echter, het gebruik van heterologe microbiële biosynthese in de industrie staat vooralsnog in de kinderschoenen, waardoor het nog niet mogelijk is om op rendabele wijze relevante producten op de markt te brengen. Om de bio-economie van de 21^{e} eeuw tot stand te brengen is er een blijvende vraag naar de ontwikkeling van 'metabolic engineering' (het aanpassen van het microbiële metabolisme) strategieën om op grote schaal, duurzame en op kost-efficiënte wijze aan de wereldbehoefte te voldoen.

In dit proefschrift hebben wij, gebruikmakend van *Escherichia coli* als chassis, microbiële stammen ontwikkeld ter bevordering van de productie van twee klassen van hoogwaardige chemische verbindingen die beide een prominente rol spelen in de biotechnologie: antibiotica en isoprenoïden. De strijd tegen antibioticaresistente bacteriën wordt door de gezondheidszorg gezien als een van de grootse uitdagingen van de 21^{e} eeuw. Recente technologische ontwikkelingen hebben nieuwe antibiotica ontdekt, een mogelijke remedie mits

deze geoogst kunnen worden. Helaas zijn deze antibiotica te complex om op goedkope wijze te synthetiseren en worden van nature geproduceerd door niet cultiveerbare dan wel genetisch onhandelbare organismen. Het ontwikkelen van heterologe microbiële systemen voor het produceren van antibiotica biedt mogelijk een efficiënte oplossing waarmee ook deze nieuwe antibiotica klinisch kunnen worden toegepast en op de markt kunnen worden gebracht. Isoprenoïden behoren tot een van de grootste klassen van natuurlijke chemische verbindingen (meer dan 50,000 moleculen) met vele toepassingen die van groots commercieel belang zijn: van hoogwaardige chemische verbindingen zoals als geneesmiddelen, geur- en smaakstoffen tot chemisch minder complexe producten als oplosmiddelen, rubber en geavanceerde biobrandstoffen. Wij hebben vooral de nadruk gelegd op de relevante obstakels die de ontwikkeling van stammen waarmee voor het eerst deze waardevolle chemicaliën in het lab kunnen worden geproduceerd in de weg staan.

In hoofdstuk 2 onderzoeken wij hoe bacteriën kunnen worden ingezet om een antibioticum te produceren. Wij hebben *E. coli* stammen ontworpen die Car produceren, een klein antibioticum behorend tot de carbapenem familie. Carbapenems worden als laatste redmiddel gebruikt tegen meervoudig resistente bacteriële infecties. Bovendien is de productie hiervan duur en milieuonvriendelijk. Onze uiteindelijk ontworpen *E .coli* stammen produceren Car meer dan 60 maal sneller vergeleken ons eerste ontwerp. Door het tot stand brengen van koloniën die niet meer groeien en een verhoogde resistentie hebben tegen door de carbapenem veroorzaakte lyse wordt de productie van antibiotica verder verhoogt. Onze resultaten tonen aan dat bacteriën kunnen worden ingezet om bacteriedodende antibiotica te maken.

Net als de complexe synthese van carbapenem, maken vele andere technologisch relevante metabolische processen gebruik van biokatalyse waarin metalen opmerkelijke chemische reacties tot stand brengen. Om functionele vormen van deze zogeheten ‘metallo-enzymen’ tot expressie te brengen worden vele modificaties na translatie uitgevoerd, waarvoor vaak meerdere proteïnen nodig zijn en electron-overdrachtreacties worden uitgevoerd. De afhankelijkheid van metallo-enzymen van deze verdere modificaties limiteert over het algemeen de expressie ervan in heterologe organismes. Hierdoor wordt de toepassen van metallo-enzymen tot het ontwikkelen van nieuwe microbiële cel fabrieken grotendeels verhinderd. **Hoofdstuk 3** biedt een uitgebreid overzicht van de huidige literatuur met een nadruk op de biotechnologisch relevante rol die metallo-enzymen spelen tijdens natuurlijke biosynthese. Verder biedt het een beschouwing van strategieën waarmee de productie van metallo-enzymen kan worden verbeterd, of überhaupt mogelijk worden gemaakt, in zowel de natuurlijke als een heterologe bacterie.

In hoofdstuk 4 beschrijven wij onze pogingen om nieuwe strategieën in de synthetische biologie te ontwikkelen waarmee een specifieke familie van belangrijke metallo-enzymen in functionele vorm tot heterologe expressie gebracht kunnen worden: ijzer-zwavel (FeS) cluster enzymen. Deze enzymen hebben groot nog onbenut potentieel voor de bioproductie. FeS enzymen zijn betrokken in de biosynthese van een grote diversiteit aan waardevolle natuurlijke producten, zoals biobrandstoffen, geur- en smaakstoffen en geneesmiddelen (o.a. carbapenems en andere antibiotica).
Wij omschrijven een eenvoudig uitvoerbaar protocol waarmee snel de functionaliteit van vreemde FeS enzymen binnen *E. coli* kan worden getoetst, onder andere 44 orthologen van het IspG enzym dat een rol speelt in de biosynthese van isoprenoïde. Onze experimentele data toont aan dat FeS enzymen typisch inactief zijn als deze tot expressie worden gebracht in een evolutionair gezien niet gerelateerd organisme. Wij demonstreren dat inactieve FeS enzymen kunnen worden hersteld wanneer deze samen met vreemde FeS 'maturation en electron transfer' proteïnen tot expressie worden gebracht. Vervolgens laten wij zien dat deze proteïnen op efficiënte wijze als het ware als 'adapters' fungeren om FeS enzymen binnen *E. coli* te activeren. Tevens onderzoeken wij de toepassing om deze geactiveerde FeS enzymen in te zetten voor de biosynthese van bisabolene, een voorloper voor biobrandstof, en het antibioticum fosfomycin. Met de verdere ontwikkeling van deze 'adapters' zal de *in vivo* activiteit van relevante FeS enzymen worden mogelijk gemaakt en/of verbeterd. Hiermee zullen de mogelijkheden voor het gebruik van microbiële biosynthese van waardevolle chemicaliën worden uitgebreid.

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The BN Department is special in many ways; the open, vibrant and social atmosphere is one of its biggest assets. I'd like to thank all the people with whom I had shared many great moments over the past years, at work and after work. **Eve**, working in the lab wouldn't have been the same without you! You gave me massive moral support and always took care of me. I'm thankful for all the jokes, NSFW conversations, dancing, cocktails, cakes, and for helping me survive this PhD. You're a strong and competent woman; embrace the future! / **Esengül**, I'll miss you dear… You're a wonderful person and I thank you for your love, for always cheering me up, and the girly chitchat. I hope we'll always be in touch! / BN is lucky to have so many strong, smart, beautiful and badass women. I want to thank: **Nicole**, **Becca**, **Mathia** (we had fun! There's so much joy, strength and determination in you. I wish you all the happiness in the world), **Laura** (my latina mate!, no one dances reggaeton like you. And thanks for the locobeads)**, Alicia** (you crazy chica… good luck with your new projects), **Paola** (thanks for the good chats and enjoy your time in Delft!), **Michel** (the sassiest girl in town; we should compete on the dance floor), **Viktorjia** (Enjoy the motherhood adventure!), **Jorine**, **Lisa**, and **Stephanie**. / **Misha**, you're awesome! Your enthusiasm, kindness, and talent (both scientific and musical) have always impressed me. Thanks for the songs we played together, for laughing at my stupid jokes, for the many great

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Helena
Rotterdam, February 2019

LIST OF PUBLICATIONS

3. **H. Shomar**, E. Fernandez-Fueyo, G. Bokinsky, *Expression of small electron and iron-sulfur cluster carriers activate heterologous iron-sulfur enzymes in Escherichia coli*. Manuscript in preparation

2. **H. Shomar**, G. Bokinsky, *Synthetic biology strategies to enhance metalloenzyme activity in bacteria*. Manuscript in preparation

1. **H. Shomar,** S. Gontier, N.J.F. van den Broek , H. Tejeda Mora, M.J. Noga, P.L. Hagedoorn, G. Bokinsky, *Metabolic engineering of a carbapenem antibiotic synthesis pathway in Escherichia coli*. Nature Chemical Biology (2018).

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