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Calero Valdayo, Patricia Maria; Nikel, Pablo Ivan

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Minireview

Chasing bacterial *chassis* for metabolic engineering: a perspective review from classical to non-traditional microorganisms

Patricia Calero and Pablo I. Nikel*
The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2800 Kongens Lyngby, Denmark.

Summary

The last few years have witnessed an unprecedented increase in the number of novel bacterial species that hold potential to be used for metabolic engineering. Historically, however, only a handful of bacteria have attained the acceptance and widespread use that are needed to fulfil the needs of industrial bioproduction – and only for the synthesis of very few, structurally simple compounds. One of the reasons for this unfortunate circumstance has been the dearth of tools for targeted genome engineering of bacterial *chassis*, and, nowadays, synthetic biology is significantly helping to bridge such knowledge gap. Against this background, in this review, we discuss the state of the art in the rational design and construction of robust bacterial *chassis* for metabolic engineering, presenting key examples of bacterial species that have secured a place in industrial bioproduction. The emergence of novel bacterial *chassis* is also considered at the light of the unique properties of their physiology and metabolism, and the practical applications in which they are expected to outperform other microbial platforms. Emerging opportunities, essential strategies to enable successful development of industrial phenotypes, and major challenges in the field of bacterial *chassis* development are also discussed, outlining the solutions that contemporary synthetic biology-guided metabolic engineering offers to tackle these issues.

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*For correspondence. E-mail pabnik@biosustain.dtu.dk; Tel. (+45 93) 51 19 18; Fax (+45 45) 25 80 00.
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Introduction

Decades of research considerably expanded the repertoire of biological functions that microbial cells can incorporate into their physiological and metabolic agendas. Nowadays, *designer cells* can be constructed by adopting a combination of genome editing tools, chemical DNA synthesis and DNA assembly technologies – thereby fulfilling the practical goal of synthetic biology, that is, the construction of living cells from individual parts, which are purposefully assembled to yield a functional entity (Jullesson *et al*., 2015). Some important hurdles, however, have been just started to be taken into consideration. Cells naturally control the gene expression flow using a sophisticated variety of RNA, protein and DNA-modifying layers of regulation, and these regulatory networks enable them to effectively respond to their environments and external cues. Therefore, adding novel, genetically encoded biological functions to these complex networks is expected to have different consequences depending on the host in which they are introduced into, as these regulatory layers would be likewise different across species. Several bacterial hosts have been adopted for plugging-in and plugging-out genetic circuits for specific purposes and, in most cases, the selection of the host cell was merely dictated by its availability and/or by historical tradition. However, synthetic biology ultimately aims at programming cells that can execute the implanted functions in a predictable fashion, and such objective thus calls for the adoption of specific, *formatted* hosts that will necessarily have different properties depending on the application envisioned. In the broadest sense of the term, a biological *chassis* can be defined as the physical, metabolic and regulatory containment for plugging-in and plugging-out dedicated genetic circuits and regulatory devices. This wide definition incorporates a clear engineering standpoint, in which

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a set of predefined parts are assembled together in a rational, standardized way leading to the final object (Endy, 2005). Moreover, and as proposed by Danchin and Sekowska (2013), the concept of *chassis* encompasses the notion that there is a clear distinction between a program or *software* that encodes the target function(s), and a machine or *hardware* (i.e. the *chassis* itself) that expresses and executes the program.

Apart from their role in the context of synthetic biology, the adoption of suitable bacterial *chassis* has a considerable impact in the field of metabolic engineering (Stephanopoulos, 2012; Nielsen and Keasling, 2016; Smanski *et al*., 2016). The integration of synthetic biology tools and strategies into advanced metabolic engineering has enabled the incorporation of a number of non-traditional microorganisms as hosts for developing efficient microbial cell factories. The (already extensive) list of the microbial hosts that can be adopted for such purposes continues to expand as more tools for precise gene and genome manipulation become available. Yet, the implementation of a given host for practical applications seems to be still governed by some degree of randomness, and a remarkably small number of microbial cell factories have achieved full commercial exploitation. This situation contrasts with the unparalleled momentum that the development of industrial microbial processes is gaining nowadays, driven by increased concerns about environmental issues and the prospect of dwindling petroleum resources worldwide that has increasingly shifted the industrial focus towards the use of microorganisms as biocatalysts. Yet again, the production of only a limited number of compounds has successfully reached commercial scale, which indicates how difficult the whole process of bringing a product into the market is. Improving yields, titres and productivity of microbial processes to enable commercialization requires rational manipulation of the microbial physiology, stress responses, and, even more importantly, engineering the core metabolism in the selected *chassis*. An often overlooked aspect is that the *chassis* has to be adapted to specific substrate (s) and its impurities in an industrial setup, which ultimately dictates the needs and characteristics of downstream processing. Consequently, a major topic in the field of metabolic engineering is the selection of an optimal *chassis* not only able to execute the functions needed for efficient bioproduction, but also hefty enough to tolerate the harsh operating conditions characteristic of industrial processes, which are of course different from the mild, controlled conditions that prevail in the laboratory.

In general, the physical and spatial shape of a microbial cell, its genomic complement, its default gene expression machinery and the complement of metabolic functions are not enough to automatically meet the requirements of a suitable *chassis*, and further manipulations are needed in order to fulfil the characteristics desirable for specific applications. Building on the wealth of information generated over the years, in this review we discuss the state of the art in bacterial *chassis* development, presenting the main advantages and limitations of traditional hosts (including a summary of recent efforts on development of dedicated tools), also bringing under the spotlight novel bacterial species that hold promise for future developments. For the sake of the present discussion, we will restrict the scope of this review to bacterial *chassis*, without forgetting that the development of other microbial platforms (e.g. yeast and filamentous fungi) continues to gain considerable attention for both fundamental and practical applications. Finally, we also present and discuss what we perceive as being the main challenges that need to be overcome in the design and construction of bacterial *chassis* in order to reach a level of maturity compatible with commercial exploitation.

Desirable properties in the ideal bacterial *chassis*: bridging the knowledge-to-application gap through synthetic biology

Over the past two decades, and together with the explosive expansion of the fields of metabolic engineering and synthetic biology, the development of suitable *chassis* that could host newly-designed biological functions and processes has achieved substantial relevance, as it becomes apparent by inspecting the exponential increase in the number of publications on the topic over the last few years (Fig. 1). Only during the last year, almost 100 articles had the word '*chassis*' listed as a keyword – a citation trend that mirrors the general increase in the number of publications on synthetic biology and metabolic engineering. This observation indicates how intimate the connection between new engineering endeavours and the adoption of specific hosts is. Yet, what would be the starting point in the process of selection and development of a bacterial *chassis* for this purpose?

From 'built-in' properties to emergent bacterial phenotypes

Ideally, a microbial *chassis* should possess certain features to meet the practical necessities of metabolic engineers and to ensure an easy and fast construction workflow of reliable production strains. These features include (i) enough basic knowledge on the microorganism, setting the basis for the design of culture media and bioprocesses, (ii) simple nutritional requirements, including readily accessible carbon and nitrogen

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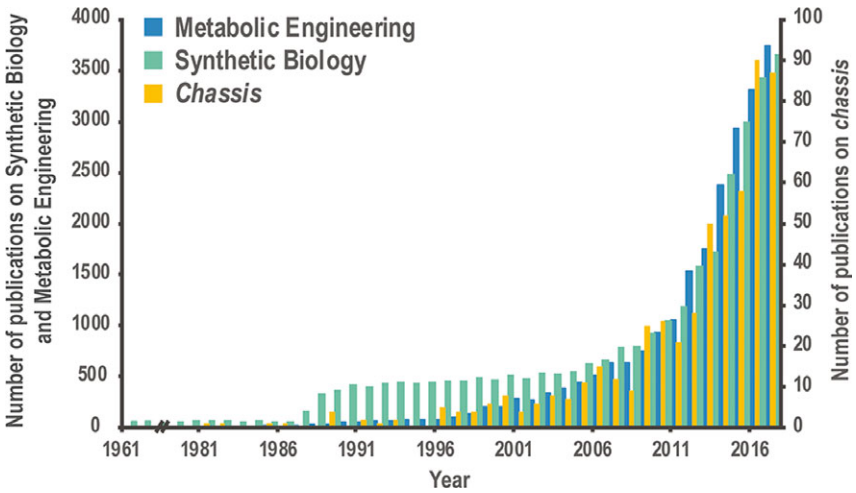

Fig. 1. Intersection between the adoption of microbial *chassis* and the fields of metabolic engineering and synthetic biology, as reflected in the relevant literature since 1961 up to date. The diagram indicates the number of times that the words 'Metabolic Engineering' (*blue*), 'Synthetic Biology' (*green*) and '*chassis*' (*yellow*) have been used as keywords in research and review articles in the field literature over the years (source: PubMed, accessed in May 2018). Note that the scale is different for '*chassis*' (indicated to the right of the diagram) and both 'Metabolic Engineering' and 'Synthetic Biology' (indicated to the left of the diagram).

sources, (iii) 'built-in' high resistance to physicochemical stress, (iii) fast and efficient growth, (iv) availability of (and possibility of developing novel) tools for targeted genome manipulations, (v) efficient secretion systems (either natural or amenable for engineering) to facilitate downstream purification steps, especially when heterologous protein production is the target, and (vi) tolerance to extreme conditions, for example high temperature. Needless to say, there are very few microorganisms that would naturally fulfil all these six traits (Beites and Mendes, 2015). Traditionally, only well-characterized bacteria that have been studied over the years in a laboratory setup have been adopted as *chassis* for metabolic engineering. This is also the case of microorganisms with a long tradition in the fields of microbiology and biotechnology that have managed to reach an industrial scale of production. The (somewhat short) list of bacterial hosts falling into this category includes *Escherichia coli* (Pontrelli *et al.*, 2018), *Bacillus subtilis* (Gu *et al.*, 2018), *Streptomyces* sp. (Spasic *et al.*, 2018), *Pseudomonas putida* (Nikel and de Lorenzo, 2018), and *Corynebacterium glutamicum* (Wendisch *et al.*, 2016), for which extensive background fundamental knowledge has been amassed. The wide use of these well-established *chassis* notwithstanding, there has been a renewed interest for bringing up-and-coming host alternatives to the metabolic engineering community, thus broadening the repertoire of *chassis* available. Nevertheless, establishing a new *chassis* encompasses challenges that need to be tackled and for which we propose a streamlined process as disclosed below.

The very starting point in this process is frequently a not-so-well-known microorganism (most usually, a natural microbial isolate), which might possess some of the traits of interest for the field (e.g. pre-endowed high tolerance to physicochemical stress and sufficiently rapid growth on a simple culture medium). As recently proposed for the development of the so-called *model organisms* for biotechnology (Liu and Deutschbauer, 2018), a given wild-type bacterial strain proposed as a potential *chassis* has to be subjected to extensive studies to fully exploit its potential before its wide adoption becomes possible. The necessary steps to be taken into this direction are summarized in Fig. 2, and they include key approaches such as (i) detailed sequencing of the genome, followed by expert, well-curated annotation of genes to evaluate *in silico* the metabolic potential (and potentially negative traits, as pathogenicity) of the host, (ii) development of genetic tools, including both replicative and suicide plasmids, characterized promoters covering a wide range of gene expression levels, and genome engineering tools, for example CRISPR/Cas9 devices, for precise genetic manipulations, (iii) experimental enrichment of the knowledge on metabolism and physiology, through the implementation of *omics* technologies and genome-wide metabolic reconstructions (this is a crucial aspect of the whole process, as feeding *in silico* metabolic models with actual experimental data provides the basis for further refinement of the predictions), (iv) construction and testing of libraries of knock-out mutants in non-essential genes, and (v) continuous curation and updating of all the data gathered into publically available databases to reach the whole scientific community.

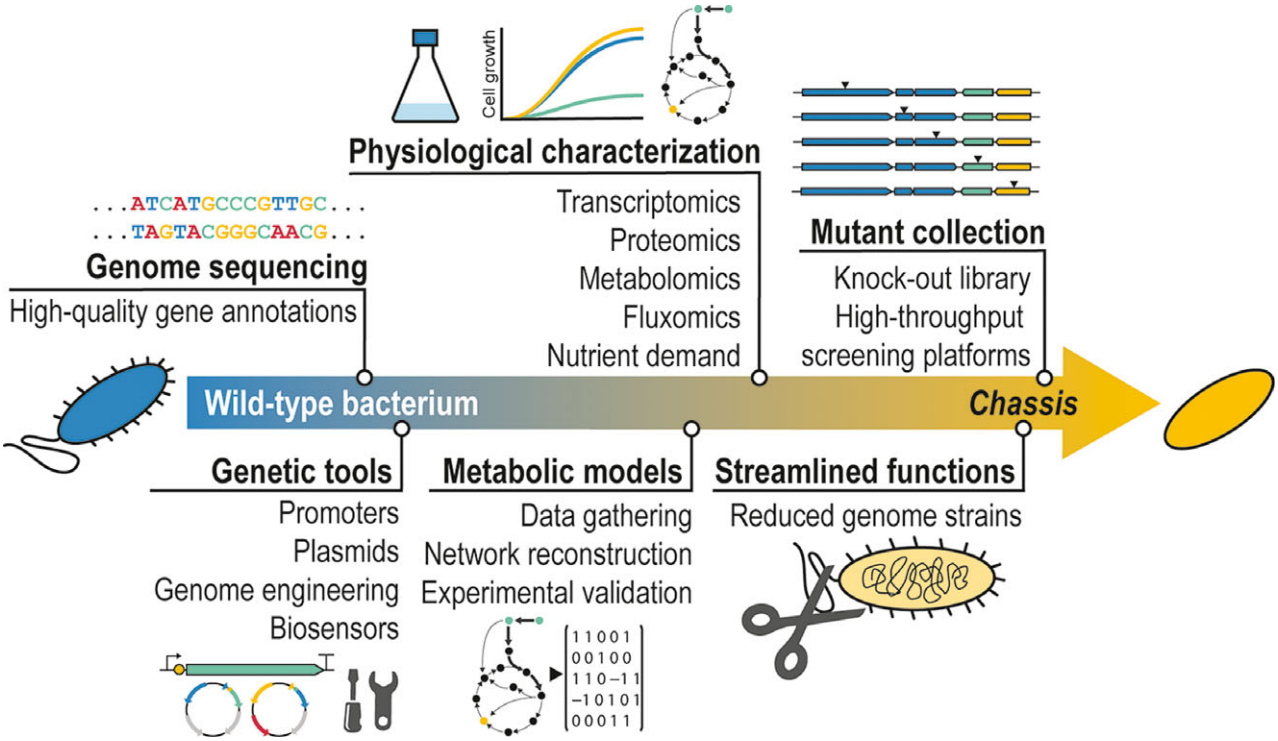

Fig. 2. Proposed chart for the development of a bacterial *chassis* for metabolic engineering, indicating the key steps required for domestication of a potentially interesting wild-type strain. The entire process builds upon six main interconnected aspects, which cover the whole range between gaining fundamental insight into functional genomics and physiology of the strain at stake and the design and adoption of dedicated synthetic biology tools. Although there is a structure to be followed along the process (that usually starts with the sequencing and expert annotation of the genome and a thorough physiological characterization), the steps indicated in the chart are not necessarily sequential in nature.

In the quest of reduced- and minimal-genome bacterial chassis by trimming out unnecessary functions

Minimal interference of the endogenous biochemistry with any given heterologous pathway plugged-in into the metabolism of a target host is a desirable trait for metabolic engineering (de Lorenzo *et al.*, 2015). However, this is not often the case as metabolic intermediates or final products of the pathway may (i) cause toxicity issues by themselves, (ii) become targets of non-predicted enzymatic reactions (resulting in misrouting or, again, toxicity), and/or (iii) act as inhibitors of other functions. In all these possible scenarios, the consequence is a decrease in the metabolic efficiency of the target pathway(s) that could severely affect final titres and yields. The construction of cell factories based on a reduced or, ideally, minimal-genome *chassis* has been proposed as a potential solution to avoid these issues (Martínez-García and de Lorenzo, 2016). The premise here is that such *minimal cell factories* are designed for *specific* production purposes, containing the minimal information and functions needed to grow and execute heterologous pathway(s) for efficient production with little to no interference arising from native functions. The behaviour of these minimal cells factories is expected to be more predictable due to their decreased complexity, and they should also have a higher catalytic efficiency as they do not waste energy in transcribing and translating unnecessary genes that would otherwise give rise to likewise unnecessary functions (Choe *et al.*, 2016). A major challenge here is cataloguing such 'unnecessary' genes and functions, which would strictly depend on environmental (e.g. industrial operation) conditions. From a fundamental perspective, the quest for the elusive minimal genome has been proposed as the way forward to get access to all necessary components defining a living cell – and then using this information for the construction of efficient biocatalysts. One possibility to tackle this challenge would be to start off by trying to identify all the functions that are ubiquitously present in extant bacterial genomes. This approach, however, takes for granted that genomic analysis offers access to ubiquitous cell structures (or, rather, gene sequences, which are seldom sufficient to predict structures) and not functions. A more heuristic approach to overcome this problem is the identification of *persistent genes*, that is, genes that tend to be present in a *quorum* of genomes with a preset conservation percentage threshold (Acevedo-Rocha *et al.*, 2013). This classification divides the (bacterial) genome into two components, the *paleome*, which encodes all

the functions needed to reproduce the cell in its progeny, while replicating its genome, and the *cenome*, which allows the cell to belong to a specific environmental niche. Such classification would in principle enable the user to decide on the set of (dispensable) cell functions that can be erased in any given bacterial *chassis* for specific applications. Transcriptional data is also a very important information to be exploited in the selection of dispensable cell functions, as the expression of many genes (including many of those making up for the paleome) is, as indicated above, highly dependent on the specific culture conditions (Kim *et al*., 2013).

As mentioned above, it is important to stress out that the set of genes necessary to sustain life is not the same across different environmental conditions, and it will vary significantly depending on whether the cells are growing in a rich or a simple, minimal medium and/or if there are toxic elements and compounds present. Consequently, the construction of a minimal bacterial genome might not lead to robust growth across all conditions. Pathways that are crucial for optimal growth under specific stress conditions or environmental fluctuations, for instance, may not be deemed essential under homoeostatic, balanced growth conditions (Nogales *et al*., 2012). This aspect is especially relevant when these cells are meant to be used for bioproduction, as each target product may require special operating conditions. As a more general principle, a reduced set of genes should not be taken as a synonym of robustness or fast growth, as shown for bacteria *naturally* endowed with very small genomes, for example *Mycoplasma pneumoniae* (Yus *et al*., 2009).

Top-down approaches have been traditionally employed in engineering endeavours for obtaining minimal-genome bacterial *chassis*, in which the genome of wild-type laboratory strains has been reduced as much as possible without impairing growth (at least under certain growth conditions). Not surprisingly, *E. coli* has been the main subject of dedicated genome reduction projects (Hashimoto *et al*., 2005; Pósfai *et al*., 2006; Hirokawa *et al*., 2013; Park *et al*., 2014), as well as other bacterial strains of industrial relevance, for example *B. subtilis* (Ara *et al*., 2007; Morimoto *et al*., 2008), *Streptomyces avermitilis* (Komatsu *et al*., 2010) and *Pseudomonas putida* (Leprince *et al*., 2012; Martínez-García *et al*., 2014c; Lieder *et al*., 2015). Further information about other reduced-genome bacterial strains constructed *via* top-down approaches can be found in recent reviews (Xavier *et al*., 2014; Choe *et al*., 2016). Other strategies have been recently adopted for the construction of minimal-genome bacterial cells. The significant advancement in fast and cost-effective techniques for DNA synthesis, assembly and efficient sequencing has contributed to the *de novo* construction of synthetic *Mycoplasma mycoides* cells as part of the *Minimal Genome Project* running at the J. Craig Venter Institute (Hutchison *et al*., 2016). In this case, the authors have adopted a bottom-up genome minimization approach (Gibson *et al*., 2010) that, apart from enabling the construction of such a minimal-genome bacterial *chassis*, has shed light on the complexity of bacterial genome structures. The first proof-of-principle of the project consisted of a *de novo* synthesized, modified version of the 1,000,000-bp long genome of *M. mycoides* that was implanted into a DNA-free *Mycoplasma capricolum* 'envelope', resulting in strain *JCVI-syn1.0*. Building on this first version of the minimal bacterial cell, an even smaller genome was synthesized (*JCVI-syn3.0*), spanning 531,560 bp and 473 genes. A comparison of these two synthetic genomes revealed a common set of 256 genes, which probably represent the *authentic* minimal set of genes needed for (limited) cell viability. Surprisingly, almost one-third of these genes in this synthetic construct encode unknown functions. Recent efforts by Danchin and Fang (2016) lead to the assignment of a significant number of functions to these unknown elements, belonging to core cellular processes such as DNA replication and cell division (e.g. a membrane protease involved in bacterial division), DNA metabolism (e.g. deoxyribonucloside kinases and phosphatases), RNA metabolism (e.g. ribonucleases, nanoRNAses, and helicases), translation-related functions (e.g. methyltransferases) and functions within general metabolism (e.g. enzymes involved in redox balance, peroxiredoxines and ATP-dependent Fe^{2+} transporters).

In all, the studies above pinpoint, again, the difficulties in *a priori* deciding which cell functions might be dispensable, and constant developments in the field are expected to further clarify this issue in the near future. While most of the available information on reduced- and minimal-genome bacterial *chassis* is restricted to just a handful of species, other strains will be surely added to the metabolic engineering agenda for specific applications soon. The general physiological and metabolic properties, as well as the advantages and the potential shortcomings, of some of the most representative bacterial strains that could constitute relevant *chassis* are described in the next section.

Bacterial species adopted as a *chassis*: from historical examples to up-and-coming additions

Several wild-type bacteria have been chosen as *chassis* for bioproduction, from laboratory-derived *E. coli* to (more recently) microbial species isolated because of specifically interesting phenotypic properties. In an attempt to describe the main advantages of these hosts in particular applications, we start off by presenting the

state of the art of one of the most used Gram-negative bacterial *chassis*, *E. coli*, and one Gram-positive bacterium that has been likewise used extensively, *Bacillus subtilis*. Building on their applications, other microorganisms that have gained relevance as a *chassis* for metabolic engineering (e.g. *P. putida*) are discussed at the light of their unique physiological and metabolic properties. For the sake of simplicity, we will focus our discussion mostly on the examples listed above. Other relevant bacterial species that have found important industrial applications, for example *Corynebacterium*, *Clostridium*, and *Streptomyces*, are collected in Table 1, highlighting some of their unique physiological properties along with key examples from the literature that the reader could access if interested on such platforms. In the sections below, we discuss the most relevant features and advantages of *E. coli*, *B. subtilis* and *P. putida* in relation to metabolic engineering efforts and bioproduction of biochemicals, as well as different examples of industrial and/or laboratory-scale processes using them as platforms. Figure 3 summarizes the main properties of these bacterial *chassis*, listing the practical applications in which they are known to excel.

Escherichia coli

More than 50 years of intensive research on *E. coli* have positioned this bacterium as the best-studied example among all prokaryotes, and as an obvious first-choice as a *chassis* for development of cell factories. *E. coli* is a Gram-negative, rod-shaped, facultative anaerobic bacterium (although a more proper classification of its lifestyle would be as a *facultative aerobe*), which can be found in mammalian intestines, some natural environments, and, in some cases, contaminated foods. Its role as a host in biotechnology can be tracked back to its use for L-threonine production in the 60s (Huang, 1961), and its application for insulin production (Riggs, 1981). It is characterized by (i) rapid growth rates, (ii) low nutrient requirements (which lead to likewise reduced production costs), (iii) the possibility of stablishing high-cell-density cultures through fed-batch fermentation (Shiloach and Fass, 2005), (iv) a versatile metabolism that has been thoroughly investigated and (v) a variety of tools for genetic manipulations and strain development. Although some strains are known to be pathogenic, many of them are safe to use for bioproduction. *E. coli* strains most commonly used nowadays are based on the K-12 and B ancestor strains, isolated in 1922 and 1918 respectively (Blount, 2015). From these two original isolates, a wide variety of improved strains has been tailored for specific purposes, for example *E. coli* BL21 Rosetta, designed for production of heterologous proteins using tRNAs that recognize rare codons in mRNA; *E. coli* BL21(DE3), containing a copy of the gene encoding the RNA polymerase from phage T7 integrated in the chromosome; or *E. coli* C41(DE3) and C43(DE3), characterized by a high tolerance to (usually toxic) membrane protein expression (Miroux and Walker, 1996). A comprehensive list of *E. coli* strains adapted for other purposes has been listed by Baeshen *et al.* (2015). In addition, a number of reduced-genome strains have also been constructed starting from wild-type *E. coli* strains. Some of them show significant physiological advantages when compared to the parental, K-12 strain MG1655. This is the case for the strain set dubbed MDS41, 42 and 43 (15% of the bacterial genome deleted), which have high electroporation efficiency and increased stability of foreign, difficult-to-clone DNA due to the removal of all insertion sequences (Pósfai *et al.*, 2006). Another set of reduced-genome *E. coli* strains, derived from W3110, were constructed in the context of the *Minimum Genome Factory* project launched in Japan in 2001 (Mizoguchi *et al.*, 2007). One such strain, termed MGF-01 and having

Table 1. Examples of bacterial platforms endowed with unique physiological and metabolic properties for bioproduction.[a]

Microorganism	Advantages	Main type of products obtained	References
Clostridium acetobutylicum	Suitable for anoxic bioprocesses, good solvent tolerance, grows on several feedstocks	Acetone, butanol, and butanol, and other solvents in engineered strains	(Ni and Sun, 2009; Jang *et al.*, 2012)
Streptomyces sp.	Wide variety of secondary metabolites and cognate pathways	Antibiotics	(Li and Townsend, 2006; Komatsu *et al.*, 2013; Hiltner *et al.*, 2015; Palazzotto and Weber, 2018)
Corynebacterium glutamicum	Used for industrial production of amino acids for over 60 years	L-amino acids (e.g. glutamate and lysine), organic acids, diamides	(Becker and Wittmann, 2012; Wendisch, 2014; Heider and Wendisch, 2015; Unthan *et al.*, 2015)
Rhodococcus sp.	Lignin degradation and high tolerance to toxic compounds	Acrylamide, triacylglycerols	(McLeod *et al.*, 2006; Kosa and Ragauskas, 2013; Röttig *et al.*, 2016; Sun *et al.*, 2016)
Mycobacterium sp.	Natural capability of synthesizing and metabolizing sterols	Steroid intermediates	(Fernández-Cabezón *et al.*,2018)

a. Selected examples of bioproduction are presented based on the main industrial applications of these bacterial species.

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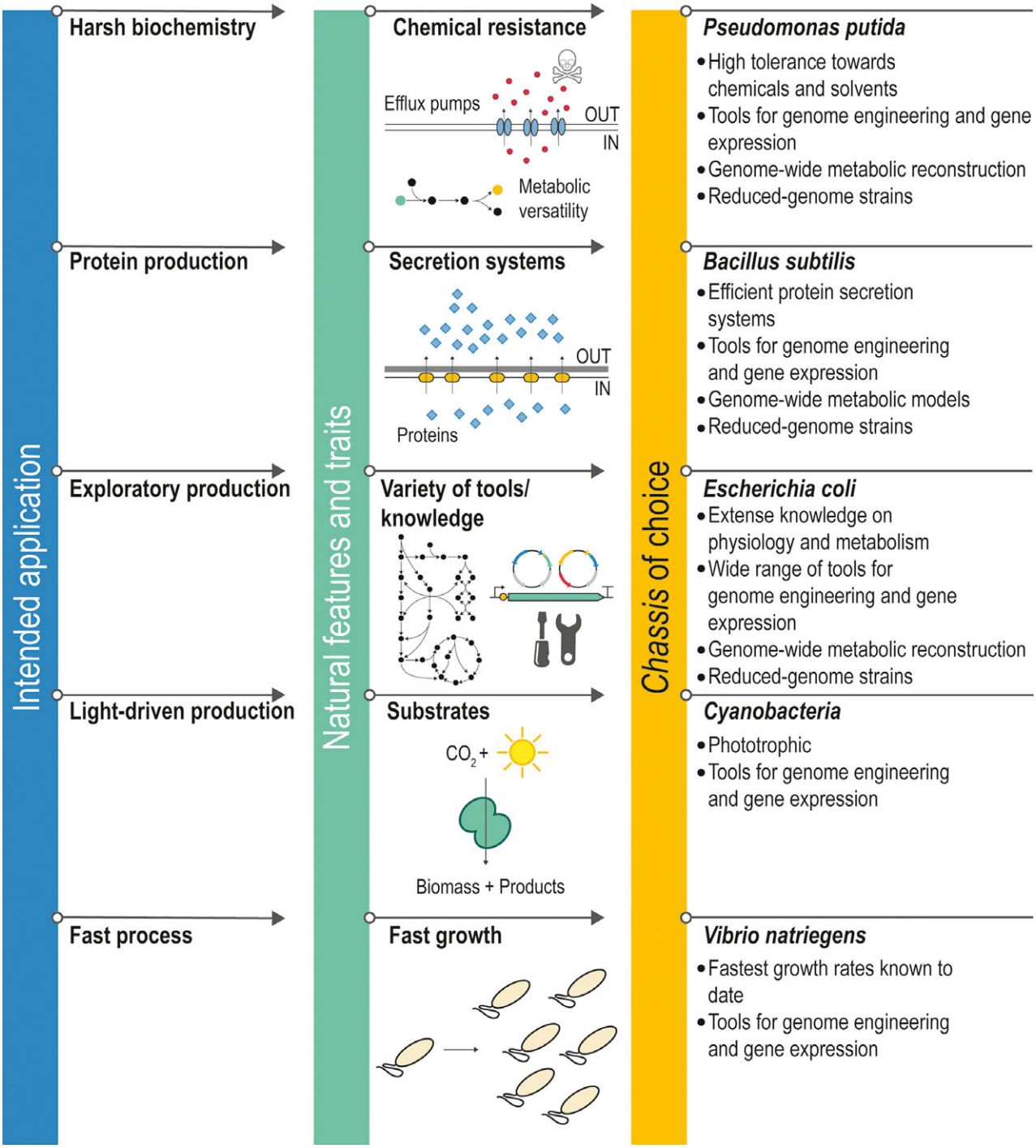

Fig. 3. Functional relationship between intended industrially relevant practical applications and different bacterial *chassis* of choice, depending on some key physiological and metabolic features they present. For the sake of simplicity, only some selected applications are shown along with the bacterial species that could be adopted as the starting point for robust *chassis* design and construction. Note that, given the characteristics of some of these bacterial species, they could potentially fulfil more than one application.

22% of its genome deleted, reached a final cell density significantly higher than the parental strain and it produced twice as much (i.e. 10 g l^{-1}) L-threonine when grown under the same culture conditions.

A vast number of plasmid vectors and gene expression systems can be found among the tools specifically developed for *E. coli*. This (ever expanding) toolbox includes an extensive catalogue of optimized natural and

synthetic promoters, libraries of ribosome binding sites and other regulatory elements, plasmidial origins of replication, resistance markers, affinity tags for protein purification and efficient transcriptional terminators (Terpe, 2006; Rosano and Ceccarelli, 2014; Dvořák *et al*., 2015; Marschall *et al*., 2017; Segall-Shapiro *et al*., 2018). Following the very spirit of synthetic biology, some of these tools have adopted specific standards and protocols followed suit, such as the *Registry of Standard Biological Parts* (Peccoud *et al*., 2008) or the *BioBricks* repository (Røkke *et al*., 2014). Moreover, numerous genome edition tools have been developed, that have enabled fast and easy strain manipulation through insertion and deletion of genetic circuits into the *E. coli* chromosome (Datsenko and Wanner, 2000; Bloor and Cranenburgh, 2006). More recently, the adoption of techniques based on clustered regulatory interspaced short palindromic repeats (CRISPR) in association with Cas9-based counter selection systems for genome editing (Jiang *et al*., 2013, 2015; Doudna and Charpentier, 2014), deactivated Cas9 protein for targeted gene regulation (*CRISPRi*, CRISPR interference; Qi *et al*., 2013) and multiplex automated genome editing (*MAGE*; Wang *et al*., 2009) have enormously helped to boost our ability to construct engineered *E. coli* strains in a straightforward, streamlined fashion – thus increasing the diversity of strains that can be used for specific bioproduction purposes. In addition to these technologies, the discovery, understanding and application of gene expression devices (e.g. riboswitches and aptamers) allow for the fine-tune regulation of gene expression in complex pathways (Kushwaha *et al*., 2016). Likewise, the development of biosensors has helped in screening and improving the synthesis of both bulk and fine chemicals. Metabolite biosensors, which respond to the presence of a certain molecule with an easily detectable output, provides an easy way of screening for efficient-producing strains from complex libraries (Zhang and Keasling, 2011; Liu *et al*., 2015). Moreover, a collection of knock-out mutants (i.e. the *Keio collection*) is available, providing a simple and high-throughput way for testing phenotypes and functions of single, non-essential genes (Baba *et al*., 2006). Data integration from deep genome sequencing (including strains from the Keio collection), as well as elaborated *omics* techniques, has led to the development of robust computational models that predict the metabolic behaviour of an organism under certain simulated conditions. Not surprisingly, such *genome-scale metabolic models* were firstly reconstructed for *E. coli* (Orth *et al*., 2011). All *E. coli* metabolic models available up to date, as well as their potential practical applications, have been reviewed by McCloskey *et al.* (2013) and, more recently, by O'Brien *et al.* (2015). The combination of this broad range of dedicated tools, both for *in silico* predictions and wet-laboratory manipulations, has made it possible to adopt *E. coli* as a model bacterial *chassis* for metabolic engineering and synthetic biology – being a veritable workhorse for developing and testing designer metabolic pathways.

The development of even more robust production strains using *adaptive laboratory evolution* (ALE, also known as *evolutionary engineering*) has enabled the production of high amounts of toxic compounds (Shepelin *et al*., 2018). An ALE application studied in great detail is increasing growth efficiency on specific carbon sources, for example glycerol (Herring *et al*., 2006) or glucose (Notley-McRobb and Ferenci, 1999; LaCroix *et al*., 2015). Moreover, other *E. coli* strains have been evolved to utilize alternative carbon sources, for example citrate (Blount *et al*., 2008), which are not naturally consumed by the parental strain. ALE-mediated adaptation of bacterial *chassis* to industrially relevant toxic compounds as well as by-products generated by the extant metabolism, which may affect bacterial growth during production, have been likewise demonstrated. Some examples of adaptation to organic solvents yielded *E. coli* strains with high tolerance towards ethanol (Goodarzi *et al*., 2010), *iso*- and *n*-butanol (Atsumi *et al*., 2010; Dragosits and Mattanovich, 2013), and ionic liquids (Mohamed *et al*., 2017). Recently, ALE has been applied together with flux balance analysis to generate an *E. coli* strain with increased succinate production from glycerol (Tokuyama *et al*., 2018), and L-serine, an amino acid usually considered to be toxic (Mundhada *et al*., 2017). The potential of ALE for evolving industrially relevant production phenotypes has been mostly limited to *E. coli*, and this technique will surely help to establish other bacterial *chassis* for metabolic engineering by generating useful phenotypic traits.

Different *E. coli* platform strains have been developed for boosting the formation of key metabolites of central carbon metabolism that can be then used as key precursors for target compounds. Typical examples of this sort of manipulations include manipulations leading to increased pyruvate or acetyl-coenzyme A (CoA) levels. These central metabolites can be used as building blocks in recombinant *E. coli* for small molecules such as butanol (Shen *et al*., 2011), or more complex chemical species such as polyhydroxyalkanoates [PHAs (Anjum *et al*., 2016; Chen and Jiang, 2017), specially the simplest form of these polymers, poly(3-hydroxybutyrate), PHB (Gomez *et al*., 2012)] and fatty acids (Sarria *et al*., 2017). The strategies used for enhancing the formation of such metabolic precursors usually involve deleting reactions that deplete pyruvate or acetyl-CoA or boosting carbon fluxes through glycolytic pathways (Sánchez-Pascuala *et al*., 2017). Such metabolic engineering strategies, recently reviewed by Matsumoto

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et al. (2017), not only encompass the manipulation of target structural genes but also global regulators of metabolism, such as the ArcAB two-component system (Bidart *et al.*, 2012). These platform *E. coli chassis* characterized by an increased availability of key metabolites can be further used to express a wide variety of heterologous pathways tailored to produce heterologous compounds that are not part of the extant biochemical network. The production of aromatic compounds constitutes a traditional example of this type of metabolic manipulations (Martínez *et al.*, 2015). Structural precursors for aromatic compounds are produced in bacteria through the shikimate pathway, which starts with the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P). The shikimate pathway is an essential part of the extant metabolism, serving as the precursor of the aromatic amino acids tyrosine, tryptophan and phenylalanine. Apart from their intrinsic value as food additives, these amino acids can be converted into relevant added-value compounds, for example vanillin or *p*-hydroxystyrene (Lee and Wendisch, 2017), or can be used as drug precursors. Other intermediates within the shikimate pathway can redirected to the synthesis of phenol and *cis,cis*-muconic acid. The formation of the key metabolites PEP and E4P has been manipulated in order to increase the overall flux through the shikimate route, and, in a recent example, these manipulations lead to a significant increase in salicylic acid formation (Noda *et al.*, 2016). Another strategy adopted for this purpose has been to relieve the feedback regulation that occurs in one of the first enzymes of the pathway, AroF. The *aroF* gene encodes a 3-deoxy-D-arabino-heptulonase (DHAP) synthase, and a single mutation, leading to an amino acid change in position 148 of the protein ($AroF^{P148L}$), generated the feedback-resistant $aroF^{fbr}$ variant (Weaver and Herrmann, 1990). This specific AroF variant has been used in many studies to boost the shikimate pathway for production of aromatic compounds (Sengupta *et al.*, 2015). Targeted deletion of some native genes involved in amino acid synthesis to remove competition with the heterologous enzymes has also been reported, although these manipulations often result in altered growth or significant increases in the process cost, as some amino acids and vitamins have to be supplemented to the culture medium (Noda *et al.*, 2016). Another approach, recently implemented to overcome nutritional deficiencies in engineered *E. coli* strains, includes transcriptional down-regulation of *aroK* (shikimate kinase, which consumes shikimate) using growth-phase dependent promoters (Lee and Wendisch, 2017).

Another family of products of great interest in the field of metabolic engineering are those derived from the mevalonate (MVA) pathway. MVA is the main precursor of a range of added-value products such as terpenoids and isoprenoids, through the key intermediates isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP). These compounds found wide applications in the fields of therapeutics, cosmetics, biofuels, and as colour and fragrance (food) additives. Although IPP and DMAPP can be synthesized endogenously by *E. coli* through the methyleythritol-4-phosphate (MEP) pathway without generating MVA; this route has been shown to have a very limited flux (Ajikumar *et al.*, 2010). Even though the endogenous MEP pathway is less efficient than expressing the heterologous MVA pathway (Morrone *et al.*, 2010), quite some work has been devoted to optimize its performance and further understand its wiring (Kim and Keasling, 2001; Zhou *et al.*, 2012; Bongers *et al.*, 2015). The emerging picture indicates that further optimization is needed when the MVA pathway is used to release metabolic bottlenecks and reduce toxicity of some metabolic intermediates therein (Martin *et al.*, 2003; Ajikumar *et al.*, 2010; Ma *et al.*, 2011; Wang *et al.*, 2016a). The adoption of alternative bacterial *chassis* could certainly be a way forward to overcome some of these limitations.

Industrially, *E. coli* has also been used for the production of 1,3-propanediol by DuPont and 1,4-butanediol by Genomatica (Sabra *et al.*, 2016). 1,3-Propanediol, a monomer for the synthesis of industrial polymers such as polytrimethylene terephthalate, can be naturally produced from glycerol by some microorganisms. In order to construct a high-yield *E. coli* producer, the genes encoding all the functions needed for 1,3-propanediol synthesis were cloned from *Klebsiella pneumoniae* (Gatenby *et al.*, 1998), followed by several metabolic engineering strategies on the resulting strain to ensure sufficient precursor availability. 1,4-Butanediol, a structurally similar diol, is another bulk chemical used as precursor of industrially-important plastics and spandex fibres. A library of heterologous genes needed for 1,4-butanediol formation from succinyl-CoA, intermediate of the tricarboxylic acid cycle, has been tested in *E. coli* (Yim *et al.*, 2011). This early attempt was soon combined with sophisticated metabolic engineering approaches, resulting in a set of *E. coli* strains that achieved yields of 1,4-butanediol – high enough to warrant commercialization. The sequential process for construction and optimization of these engineered *E. coli* strains has been recently reviewed in Burgard *et al.* (2016).

All our extensive knowledge and range of tools for engineering *E. coli* make this enteric bacterium a suitable choice as a *chassis* for bulk product formation as well as using it as a proof-of-concept for designer (synthetic) pathways at their initial stages of development. There is, however, a limit in the potential uses of *E. coli*,

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which is not a suitable platform for all bioprocesses and chemicals in the currently expanding bioproduction agenda. Some examples of challenging processes are the production of secreted proteins at high yields or the production of highly toxic compounds, which negatively affect the growth and catalytic efficiency of *E. coli*. Moreover, new and cheaper (raw) alternatives to traditional substrates are required in order to minimize production costs. The utilization of different bacterial *chassis*, described below, attempts to overcome these challenges.

Bacillus subtilis

Bacillus subtilis is an aerobic, rod-shaped, Gram-positive bacterium found in soils and in the plant rhizosphere. It is one of the best known and characterized Gram-positive microorganisms, due to a number of early studies on its natural competence for DNA transformation and the formation of spores as resistant, non-reproductive structures (Spizizen, 1958). The genome of *B. subtilis* strain 168 was one of the first to be sequenced (Kunst *et al*., 1997), and a number of tools for genome manipulation have been developed ever since, as well as a genome-scale reconstruction of its metabolic network 10 years after the genome sequence became available (Oh *et al*., 2007). More recently, a full collection of knock-out mutants of *B. subtillis* has been developed (Koo *et al*., 2017). Importantly, *B. subtilis* is free of endotoxins and has the generally recognized as safe (*GRAS*) status as a microorganism for protein production. The availability of numerous genetic tools, such as vectors and gene expression systems, makes it an easy-to-handle organism (Liu *et al*., 2017a). As mentioned in the preceding sections, there has been extensive work in constructing reduced-genome versions of *B. subtilis.* Among them, the reduced-genome strain MGB874 has been shown to produce higher concentrations of alkaline cellulose and protease as heterologous proteins (Morimoto *et al*., 2008; Manabe *et al*., 2013).

Recombinant proteins and natural enzymes and proteases constitute the main portfolio of industrially relevant molecules produced in *B. subtilis chassis* – essentially because its efficient secretory machinery allows for the transport of proteins into the culture medium to reach concentrations in the range of grams per litre, thus reducing purification and recovery costs (van Dijl and Hecker, 2013). The adequate signal peptides are required in the target proteins to ensure a correct and efficient secretion. Such signal peptides are composed by three domains, known as N-, H- and C- regions, recognized by different secretory machineries for the proper translocation of proteins from the cytoplasm through the membrane and to the external medium. The best-studied protein secretory systems in *B. subtilis* are the Sec and Tat pathways (Hohmann *et al*., 2017). However, *B. subtilis* naturally produces a number of proteases and, although some of them might have an interest for the industry on their own, their activity usually limits the overall efficiency of heterologous protein production. For this reason, the construction of proteases-defective mutants has been crucial for the development of *B. subtilis* as a *chassis* for protein production. Some of the most used mutants of this sort are the type strains WB600 (Wu *et al*., 1991) and WB800 (Wu *et al*., 2002), in which six and eight proteases, respectively, have been eliminated.

Furthermore, the development of *B. subtilis* as a *chassis* for heterologous protein production has included the design of a wide variety of gene expression systems. Different types of strong, constitutive as well as inducible promoters used in *B. subtilis* have been reviewed by Song *et al.* (2015). The *Bacillus* Genetic Stock Centre (BGSC) was created for maintenance and distribution of the (ever increasing) catalogue of characterized *Bacillus* strains and knock-out mutants. Apart from the strains, the BGSC collection also contains cloning vectors and expression plasmids that can be used in *B. subtilis*. However, some of these plasmids tend to be unstable and, due to the natural ability of *B. subtilis* of DNA uptake and integration into the chromosome through double crossover, homologous recombination, single-copy DNA integration is the most used method for heterologous gene expression (Kunst and Rapoport, 1995), although novel and easy ways for DNA transformation and mobilization are being developed (Miyano *et al*., 2018). Several genomic regions in the *B. subtilis* chromosome have been characterized for heterologous gene expression; such as *amyE* (encoding an α-amylase) which provides a coloured-colony phenotype by performing an α-amylase test to check for successful gene integration. This strategy has been adopted by Commichau *et al.* (2014) for engineering efficient vitamin B6 production in *B. subtilis* strain 168.

As described before, *B. subtilis* is a very attractive organism for enzyme production, as their easy secretion outside the cell simplifies the downstream processing as well as their (re)folding when needed (Westers *et al*., 2004). The global industrial production of enzymes market is expected to reach $ 6.2 billion in 2020, and these enzymes have applications as varied as production of detergents, treatment of textiles, additives for the food industry, cosmetics and waste degradation (Singh *et al*., 2016). Some examples of industrially relevant enzymes produced in *B. subtilis chassis* are subtilisin (an alkaline serine protease), α- and β-amylases, β-glucanases and laccases (Schallmey *et al*., 2004). Numerous examples on the optimization of heterologous enzyme production

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in *B. subtilis* have been published – which attempt to improve the overall process efficiency, finding new interesting enzymes and to develop more suitable *chassis* for the industry (Chen *et al*., 2016; Feng *et al*., 2017).

Apart from its prominent role in protein production, *B. subtilis* is also used for industrial processes aimed at the synthesis of nucleotides, vitamins, surfactants and antibiotics, for example bacitracin and subtilin. Nucleotides, for example inosine monophosphate (IMP) and guanosine monophosphate (GMP), are extensively used in a wide range of processed foods as flavour boosters in combination with monosodium L-glutamate. Industrial production of these nucleotides has traditionally relied in Gram-positive microorganisms and, among them, *B. subtilis* and other *Bacillus* species have a prominent role due to a large accumulation of inosine in the culture medium (Chen *et al*., 2005). Building on this natural occurrence, multiple studies have been performed to improve nucleotide accumulation, using strategies such as classical random mutagenesis (Matsui *et al*., 1982), culture optimization (Chen *et al*., 2005), target mutagenesis to avoid IMP degradation (Asahara and Mori, 2010) or introduction of nucleotidases that remove the phosphate group from IMP and GMP (Terakawa *et al*., 2016). A number of vitamins are also being produced industrially in *B. subtilis* as the *chassis*, mainly riboflavin, cobalamin and biotin. Riboflavin, a component of the vitamin B2 complex, is a major nutrient in human diet, which can be naturally found in vegetables and raw milk. Nowadays, riboflavin is produced by fermentation for its use as an additive in the food industry, and *B. subtilis* is one of the microorganisms most often used in such bioprocess. A limitation in the availability of precursor nucleotides (i.e. GTP), however, is known to hinder riboflavin production. For this reason, a deregulation of purine synthesis has been implemented to ensure efficient accumulation of this vitamin (Stahmann *et al*., 2000; Shi *et al*., 2014).

Poly-γ-glutamic acid is a homopolyamide naturally produced by *B. subtilis* and related bacteria. A relevant microbial polymer used in food, medical, cosmetic and waste treatment industries (Shih and Van, 2001; Bajaj and Singhal, 2011), it is composed of D- and L-glutamic acid units interconnected through the amide linkage of their α-amino and γ-carboxylic groups. The cost-effective production of poly-γ-glutamic acid has been achieved using natural-producer *Bacillus* strains (Tanaka *et al*., 1997), as well as by improved strains using genome manipulation techniques such as genome shuffling (Zeng *et al*., 2016). *B. subtilis* has also been used to produce pure chiral stereoisomers, for example D-(—)-2,3-butanediol, another promising diol used as a biofuel and bulk chemical for chemical synthesis (Fu *et al*., 2016). Other molecules, for example hyaluronic acid (a high-molecular-weight glycosaminoglycan used in the pharmaceutical and cosmetic industry), have been produced in *B. subtilis* up to the range of grams per litre (Jin *et al*., 2016). *Bacillus* has been also applied as a functional *chassis* to produce and secrete a synthetic cellulosome for further use in the degradation of raw cellulosic substrates (Lin *et al*., 2017). The production of other important pharmaceutical molecules, for example *N*-acetylglucosamine, has been attempted as well, and the bioprocess has been optimized through pathway modulation and successful colocalization of pathway enzymes using scaffold proteins (Liu *et al*., 2017b,c).

All the examples above accredit the value of *B. subtilis* as a robust *chassis*, well established in the biotechnology field for production of endogenous enzymes – and also for the production of heterologous proteins, although the titres are usually not comparable to those of endogenously synthesized proteins. For these reasons, more research needs to be performed in this bacterial strain, including genome reduction, identification and optimization of signal peptides, development of novel, more efficient secretion and expression systems, and further optimization of both culture settings and resistance to stressful conditions (Tjalsma *et al*., 2004; Song *et al*., 2015; Oztürk *et al*., 2016). In bioprocesses for which *built-in* resistance to harsh operating conditions is needed, the focus shifts to a different type of bacterial *chassis*, as discussed in the next section.

Pseudomonas putida

Chemical stress, for example under the form of either endogenously produced or exogenously added chemicals, is one of the main hurdles encountered in industrial bioprocesses. Either the desired product or some of the substrate feedstocks (e.g. some compounds found in biomass hydrolysates) can inhibit bacterial growth or even cause cell death (Keasling, 2010). In both cases, the yields and titres of the bioprocess are negatively affected, leading to sub-optimal production performance. Moreover, two-phase fermentations, where a second organic phase is used to extract the product from the aqueous phase [e.g. during production of *p*-vinylphenol or 1,3-propanediol (Rujananon *et al*., 2014; Salgado *et al*., 2014)], require the use of microorganisms able to tolerate the solvent(s) used as second phase. Specifically, due to toxic effects, the choice of solvents for whole-cell biotransformations in two-phase solvent-water systems is usually very limited. Only low-toxicity solvents with high hydrophobicity coefficients can be applied for this purpose, limiting the scope of possible bioprocesses that can be carried out under these conditions. For these reasons, the adoption of bacterial *chassis* with an enhanced tolerance to chemical stresses is needed (Nicolaou *et al*., 2010; Kusumawardhani *et al*., 2018).

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The ubiquitous saprophytic, soil-colonizer *P. putida*, a Gram-negative, rod-shaped bacterium is increasingly being used as a *chassis* for applications characterized by harsh operating conditions. *P. putida* KT2440, which has been certified as a GRAS platform for recombinant protein production, is the most studied and used strain within the genus, and it is considered a safe host for cloning and expressing heterologous genes (Poblete-Castro *et al*., 2017). *P. putida* KT2440 possesses many of the desired features in an ideal bacterial *chassis*, such as rapid growth, low nutritional requirements and availability of sophisticated tools for genome and genetic manipulation (Nikel and de Lorenzo, 2018). A collection of mutant strains obtained by random integration of mini-Tn*5* elements, known as the *Pseudomonas* Reference Culture Collection, is available for strain KT2440 (Duque *et al*., 2007). Moreover, this soil bacterium is endowed with built-in, advantageous evolutionary traits, for example a remarkably versatile metabolism that serves as a treasure trove for enzymatic activities, and increased tolerance towards oxidative stress. *P. putida* can also sustain high rates of NADPH regeneration when growing on hexoses, due to the fact that glucose is converted into glyceraldehyde-3-phosphate and pyruvate *via* the Entner-Doudoroff (ED) pathway (Martins dos Santos *et al*., 2004; del Castillo *et al*., 2007; Nikel *et al*., 2015a), and part of these trioses-phosphate are recycled back into hexoses-phosphate, generating one molecule of NADPH *via* a combination of activities of the ED pathway, the pentose phosphate pathway, and an incomplete Embden-Meyerhof-Parnas route, collectively termed *EDEMP cycle* (Nikel *et al*., 2016). This particular metabolic architecture has been shown to enable a better expression of heterologous enzymatic pathways and serves as an efficient source of reducing power for maintaining a high tolerance to stressful conditions (Blank *et al*., 2008; Chavarría *et al*., 2013; Nikel *et al*., 2014b). Furthermore, *P. putida* KT2440 is able to use a wide range of compounds as carbon sources, such as succinate, citrate and other intermediates of the tricarboxylic acid cycle. More importantly, the original soil environment of *P. putida* KT2440 and its ability to thrive in the rhizosphere are connected with its ability to degrade aromatic compounds derived from lignin degradation, for example benzoate, *p*-coumarate, caffeate and vanillate (Jiménez *et al*., 2002; Dvořák *et al*., 2017). This fact makes *P. putida* KT2440 an important candidate for its use to grow in lignin-derived feedstocks (Linger *et al*., 2014; Ragauskas *et al*., 2014; Beckham *et al*., 2016; Ravi *et al*., 2017).

Remarkably, *P. putida* has a number of mechanisms for tolerance towards high concentrations of aromatic chemicals, for example toluene, xylenes and styrene (note that these toxic compounds can also be used by some *P. putida* strains), including a wide repertoire of efflux pumps (Inoue and Horikoshi, 1989; Ramos *et al*., 2002; Santos *et al*., 2004; Domínguez-Cuevas *et al*., 2006; Calero *et al*., 2018). Toluene, for instance, is an aromatic and highly toxic solvent that kills most microorganisms at concentrations as low as 0.1% (v/v). Being an industrial feedstock, it kept accumulating in the environment since its very discovery back in the 19th century. Several efforts were therefore aimed at finding microorganisms able to degrade toluene, converting it into less-harmful compounds. Even when some *Achromobacter* and *Nocardia* species were known to tolerate moderate concentrations of toluene, a true robust and resistant strain chasing was largely missing in the picture for many years. The seminal work by Prof. Horikoshi's group in the late 80s in this regard showed that an isolate of *Pseudomonas* sp. could thrive in the presence of very high concentrations of toluene. Soon after the publication of this article, Prof. de Bont's group in The Netherlands and that of Prof. Ramos in Spain reported the isolation of two other *Pseudomonas* strains that were also able to grow in the presence of saturating concentrations of toluene. A most surprising finding was that all three strains isolated in the three countries and different niches happened to be the same microorganism, *P. putida*. The discovery of solvent tolerance opened a new research avenue and aroused great interest in the use of this kind of bacterial *chassis* in bioremediation and biotransformations in biphasic systems, as well as in the development of biosensors for environmental contaminants. How do *Pseudomonas* cells thrive under these harsh conditions? As indicated above, the most relevant of the many mechanisms for solvent tolerance in *Pseudomonas* seems to be related to the action of a series of efflux pumps that extrude toluene (and, likely, other solvents) from the cell membranes to the outer medium, a high energy-demanding process whose price bacteria are forced to pay to survive under such extreme conditions. Another important mechanism that contributes to solvent tolerance involves changes in phospholipid composition, that is isomerization of *cis*-unsaturated fatty acids to *trans*-isomers, and changes in head group composition in membrane phospholipids. All these changes influence membrane fluidity and consequently have an effect on resistance to the membrane chaos brought about by solvents. Apart from lipid disorganization, protein unfolding occurs when toluene and other solvents dissolve in cell membranes. This situation brings forth a general stress response on bacteria that, in most cases, is accounted for by expressing several molecular chaperones and other enzymes related to stress resistance.

Tolerance to solvents, and the ability of using them as carbon sources, is a very oxygen-demanding cellular

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process. *P. putida* KT2440 is a strict aerobe due to the absence of fermentative pathways and the inability of using alternative electron acceptors other than oxygen – leading to a somewhat limited number of naturally synthesized by-products under industrially relevant conditions (Martínez-García *et al*., 2014b; Tiso *et al*., 2014). The lack of fermentation pathways also leads to a very high oxygen requirements for optimal bacterial growth, which can be a problem when culturing bacteria in large bioreactors giving the non-homogenous distribution of nutrients (Davis *et al*., 2015), as well as impairing the practical use of *P. putida* KT2440 to carry out anoxic, oxygen-sensitive reactions. This problem was assessed by introducing synthetic fermentation pathways that, when expressed in strain KT2440, lead to higher survival under anoxic conditions (Nikel and de Lorenzo, 2013). Schmitz *et al.* (2015) and Lai *et al.* (2016) also tackled this problem by employing bioelectrochemical systems. This is an area of intense research currently under development, which aims at modifying the lifestyle of environmental bacteria, and holds the promise of further multiplying the uses of *P. putida* as a functional *chassis* under a range of operating conditions.

A number of tools for genetic and genome engineering of *P. putida* have been developed, including the complete set of modular vectors of the *Standard European Vector Architecture* (SEVA) platform (Silva-Rocha *et al*., 2013; Martínez-García *et al*., 2015), transposons (Martínez-García *et al*., 2014a) and a wide range of promoters, both natural and synthetic, which have been characterized in strain KT2440 (Zobel *et al*., 2015; Calero *et al*., 2016). Homologous recombination-based techniques (e.g. using the homing endonuclease I-*Sce*I from *Saccharomyces cerevisiae* that recognizes an 18-bp DNA sequence, not present in bacterial chromosomes) have been implemented for deleting large genomic fragments (Martínez-García and de Lorenzo, 2011). The procedure is based on forcing homologous recombination by the appearance of a double strand break in the target genome upon cleavage *in vivo* by I-*Sce*I, the intracellular expression of which is driven by the 3-methylbenzoate-inducible promoter *Pm* in a broad host range expression plasmid. Using this system, 69 genes involved in synthesis and functioning of the flagellar machinery were successfully deleted from strain KT2440 (Martínez-García *et al*., 2014c), as well as a number of genes related to genetic instability, such as insertion sequences and transposons (in total, 4.3% of the genome DNA was eliminated). These operations generated a reduced-genome *P. putida chassis*, EM42, which showed a significant increase in ATP and NAD(P)H availability. The growth of the resulting *chassis* showed improvements in both rich and minimal medium with different carbon sources, with enhanced resistance to reactive oxygen species, which in turn led to an increased heterologous GFP and luciferase production (Martínez-García *et al*., 2014b). In further tests conducted in bioreactors, the reduced-genome *chassis* had significantly improved plasmid stability and heterologous protein production, among other traits, as compared to the parental strain (Lieder *et al*., 2015). Novel tools for advanced genome edition, based on the expression of specific DNA recombinases, are constantly being developed for *P. putida* KT2440 (Martínez-García and de Lorenzo, 2017), for example for increasing the efficiency of DNA recombination. The development of these techniques will allow for the use of precise MAGE and CRISPR/Cas9-based technologies in the near future, speeding-up genomic manipulations in *Pseudomonas chassis*.

In addition to the genetic and genome engineering tools, a number of bioinformatic tools have been developed to facilitate the rational design of metabolically engineered strains based on *P. putida* KT2440. Soon after the sequencing of its entire genome in 2002 by Nelson *et al.* (2002), three genome-scale metabolic models were developed for strain KT2440 (Nogales *et al*., 2008; Puchałka *et al*., 2008; Sohn *et al*., 2010) – with a recent update on the occasion of the genome re-sequencing and re-annotation (Belda *et al*., 2016), and the most comprehensive genome-wide metabolic reconstruction build to date (Nogales *et al*., 2017). The integration of *in silico* model predictions and experimental data from *omics* (e.g. deep RNA sequencing) has provided further insights into the metabolism of *P. putida* KT2440 (Kim *et al*., 2013; Nikel *et al*., 2014a). Finally, a number of databases are currently available, with continuously updated genetic information (e.g. the *Pseudomonas* Genome Database; Winsor *et al*., 2016) and a protein–protein interaction database (*PutidaNET*; Park *et al*., 2009). A platform integrating all the *omics* information available for *P. putida* would be highly desirable, facilitating further manipulations of an already attractive bacterial *chassis*.

Although the seminal works on the practical biotechnological applications of *P. putida* have been focused on xenobiotics degradation, the use of this *chassis* as a bacterial cell factory for bioproduction increased exponentially over the last decades (Poblete-Castro *et al*., 2012; Loeschcke and Thies, 2015; Nikel *et al*., 2016). One of the most well-known products synthesized using *Pseudomonas* species is the family of PHAs polyesters (Prieto *et al*., 2016). PHAs are naturally synthesized by *P. putida* under specific conditions, for example nitrogen-limited conditions in the presence of sufficient amounts of a suitable carbon source (Hoffmann and Rehm, 2004), and these polyesters are used as carbon and energy storage. The biodegradability of PHAs as

well as their material properties, such as thermoplasticity, insolubility and lack of toxicity, make them good alternatives for fuel-based plastics for ecofriendly packaging and other industrial purposes (Steinbüchel and Lütke-Eversloh, 2003; Ouyang *et al.*, 2007). In addition to the biosynthesis of PHA biopolymers (for which strain Gpo1 has been used at the industrial scale), *P. putida* is gaining importance as a cell factory for *de novo* biosynthesis of heterologous, often difficult-to-produce chemical compounds. For many of the target chemicals in this family, the natural tolerance of *P. putida* towards aromatic molecules and its ability to convert these chemical structures *via* mono- and di-oxygenases (Pérez-Pantoja *et al.*, 2013) have been exploited for strain engineering. Relevant cases of this sort include the synthesis of 3-methylcatechol (Hüsken *et al.*, 2001), *o*-cresol (Faizal *et al.*, 2005), *cis,cis*-muconate (van Duuren *et al.*, 2011, 2012) and styrene (Blank *et al.*, 2008).

Other compounds produced in *P. putida* are rhamnolipids, low toxic, biodegradable bacterial biosurfactants, *via* the heterologous expression of the *rhl* genes from the *Pseudomonas aeruginosa* biosynthesis pathway (Wittgens *et al.*, 2011; Tiso *et al.*, 2017). *P. putida* has also been used as a functional *chassis* for the production of terpenoids, taking advantage of the natural stress resistance of this species to toxic chemicals. Production of (*S*)-perillyl alcohol, for instance, has been achieved by expressing a cytochrome P450 (van Beilen *et al.*, 2005); and *de novo* production of geranic acid has been engineered by coexpressing a geraniol synthase from *Ocimum basilicum* together with genes encoding the MVA pathway for isoprenoid synthesis (Mi *et al.*, 2014). Zeaxanthin is another class of terpenoid that has been efficiently produced in *P. putida* (Beuttler *et al.*, 2011; Loeschcke *et al.*, 2013). Moreover, a number of aromatic compounds have been produced in *P. putida* using the amino acids derived from the shikimate pathway as precursors. For the production of such molecules, a small number of genes were introduced into the engineered strains, and further metabolic engineering to increase the availability of amino acid precursors was performed in order to improve yields. Interestingly enough, the regulation of these pathways in *P. putida* KT2440 and S12 (a solvent tolerant strain) seems to differ from that in *E. coli*, which requires the adoption of specific metabolic engineering strategies. Examples of production of added-value aromatic molecules in *P. putida* are illustrated by cinnamic acid (Nijkamp *et al.*, 2005) and phenol (Wierckx *et al.*, 2005; Wynands *et al.*, 2018) – achieved, among other manipulations, by introducing a phenylalanine-ammonia lyase from *Rhodosporidium toruloides* and the tyrosine phenol lyase from *Pantoea agglomerans*. Other strains producing derivatives of these aromatic compounds were developed, producing, for example *p*-hydroxystyrene (Verhoef *et al.*, 2009), *p*-hydroxybenzoate (Yu *et al.*, 2016), anthranilate (Kuepper *et al.*, 2015), vanillate (Graf and Altenbuchner, 2014) and *p*-coumaric acid (Calero *et al.*, 2016). Furthermore, a number of molecules are being synthesized in industrial-scale fermentations using engineered *P. putida* strains. Such examples include the synthesis of 5-cyanopentanamide by DuPont (USA) or 5-methylpyrazine-2-carboxylic acid by Lonza (Switzerland) (Kiener, 1992; Tiso *et al.*, 2014).

In all, *P. putida* is considered as one of the most promising *chassis* for handling the synthesis of difficult-to-produce chemicals, involving harsh reactions and complex biochemistries that impose a high level of chemical stress to the host cells. The broad, rich metabolism of *P. putida* also makes it a suitable candidate for the use of cheap feedstock substrates with high levels of impurities, for example lignocellulosic biomass hydrolysates, although more research is still required to increase growth rates and product yields and titres under these operating conditions. While all these issues are actively being investigated nowadays, the next relevant question pertains to the adoption of novel bacterial species that could also serve as the starting point for the construction of robust *chassis*.

Emergent bacterial *chassis*

Microbial diversity provides a phenomenal source of solutions to the practical problems encountered in metabolic engineering. Screening through the natural biological repertoire of solutions and exploit them for bioproduction must be part of the engineering agenda (Price *et al.*, 2018). Some of the current challenges are (i) limitations in growth rates and product yields in engineered bacteria, (ii) minimizing production costs (i.e. feedstock prices) and (iii) simplifying downstream processing. Against this background, in this section, we discuss up-and-coming bacterial *chassis* that have exceptionally attractive features to develop them as hosts for bioproduction. The selected examples include *Vibrio natriegens* (endowed with a remarkably fast growth), cyanobacteria (due to their photosynthetic capabilities) and *Roseobacter* and *Halomonas* as marine bacterial species with an unique tolerance to saline stress. Other relevant, emerging bacterial species that could be potentially developed as *chassis* for specific applications include both *Shewanella* and *Geobacter* sp., which can be used to engineer *microbial fuel cells* due to their electron-accepting capabilities; *Klebsiella* sp., which can produce a variety of low-molecular-weight bulk products using glycerol as carbon source; and *Deinococcus*, due to its high resistance to DNA damage and broad range of feedstock utilization (Fredrickson *et al.*, 2008; Dantas

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et al., 2015; Gerber *et al.*, 2015; Kumar and Park, 2018). Although significant research efforts have been invested on alternative bacterial *chassis* in recent years, much work is still lying ahead to place them at a similar state of acceptance and widespread use as *E. coli* or *B. subtilis*. These challenges notwithstanding, in this section we discuss the state of the art and developments to move the field of metabolic engineering forward by adopting non-traditional bacterial *chassis*.

Vibrio natriegens

One of the desired traits of the ideal *chassis* to be used as a cell factory for the production of biomass-associated products is the fastest growth possible with the lowest nutrients need. *V. natriegens* is a Gram-negative, facultative anaerobic bacterium. Formerly known as *Pseudomonas natriegens* and *Beneckea natriegens,* it was firstly isolated and described in 1961 (Eagon, 1961; Payne *et al.*, 1961). This bacterium has the shortest doubling time known to date: 15 min in brain heart infusion medium supplemented with KCl, $MgCl_2$ and additional NaCl at 37°C, and 9.8 min in the same culture conditions but with a culture medium supplemented with sea salt. These doubling times are around two times shorter than those of a fast-growing *E. coli* strain (e.g. NEB Turbo™) grown in the same culture conditions. The genome of *V. natriegens* has been recently sequenced (Maida *et al.*, 2013; Lee *et al.*, 2016) and, in an attempt to develop this host as a functional *chassis* for bioproduction, a number of techniques and tools have been tested and developed, including expression vectors, protocols for DNA transformation, synthetic promoters covering a range of expression strengths and tools for manipulating gene expression levels *via* CRISPRi (Lee *et al.*, 2016; Weinstock *et al.*, 2016). The fast growth of this bacterium allows for a significant speed-up of laboratory cloning procedures, as colonies are already visible on a plate after a mere six-h post-transformation incubation. *V. natriegens* was also found to have a secretory system able to export proteins to the culture medium (Weinstock *et al.*, 2016). Moreover, natural transformation and multiplex genome editing was achieved by overexpressing the *tfoX* gene from *V. cholera* and *V. natriegens*, achieving up to four simultaneous scarless genome edits (Dalia *et al.*, 2017). Further research on the central carbon metabolism of *V. natriegens* has been performed in glucose-grown cells using ^{13}C-based metabolic flux analysis, showing a flux distribution in central carbon metabolism similar to that of *E. coli* (Long *et al.*, 2017). Its suitability to be grown in large bioreactor cultures using different carbon sources has been also tested, achieving cell densities around 20 g cell dry weight l^{-1}, and rates of substrate consumption faster than those of *E. coli* and *S. cerevisiae* have been reported under these growth conditions (Hoffart *et al.*, 2017). *V. natriegens* has been successfully applied for the production of industrially relevant chemical compounds such as PHAs and L-alanine, hinting a great potential for biotechnological applications in the near future (Dalia *et al.*, 2017; Hoffart *et al.*, 2017).

Cyanobacteria

Cyanobacteria are increasingly becoming attractive *chassis* to sustain production of biofuels and chemicals due to their ability to use sunlight through photosynthesis, which provides a renewable, cheap, and almost unlimited energy source. Since CO_2 is fixed during the process, a significant reduction of production costs related to feedstocks is also expected. In contrast to other photosynthetic organisms, such as plants, cyanobacteria are characterized by a relatively fast growth (Carroll *et al.*, 2018). Several strains of cyanobacteria have been used so far for metabolic engineering, for example *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942, and *Synechococcus* sp. PCC 7002, the genomes of which have been sequenced (Kaneko *et al.*, 1995). A number of tools for genome engineering have been developed for these cyanobacterial strains (in particular, for *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942), and a suite of transformation techniques have been likewise tested, for example *via* natural transformation or electroporation (Wang *et al.*, 2012; Yu *et al.*, 2013). Neutral insertion genome regions in the chromosome and fluorescent reporters have been studied (Ruffing *et al.*, 2016). Moreover, genetic engineering of a *Synechococcus* strain has been achieved using the CRISPR/Cas9 system, although expression of the gene encoding Cas9 brought about severe growth impairment (Wendt *et al.*, 2016). Nonetheless, efficient gene repression of several genes has been implemented using CRISPRi in *Synechocystis* sp. PCC 6803 (Kaczmarzyk *et al.*, 2018). Cyanobacteria have attracted a lot of attention for the production of biofuels as an alternative to other photosynthetic systems like plants. Apart from the obvious differences in replication rates, the use of cyanobacteria has been proposed as an alternative to overcome the problem of competition with croplands for food, while still avoiding the need of using sugars as feedstock (Ducat *et al.*, 2011; Nozzi *et al.*, 2013; Savakis and Hellingwerf, 2015). Although cyanobacteria have found a somewhat limited use in industry thus far, several added-value compounds have been produced using this *chassis*. A classic example of this sort is represented by several biofuels, for example ethanol, bisabolene, farnesene, 1-butanol, isoprene and isopropanol

(Dexter and Fu, 2009; Lindberg *et al*., 2010; Lan and Liao, 2011; Kusakabe *et al*., 2013; Davies *et al*., 2014; Halfmann *et al*., 2014) – which even led to the creation of the spin-off company Photanol in the Netherlands for cost-efficient production of biochemicals using cyanobacteria. Bioproduction of other compounds, for example 1,3-propanediol using CO_2 as the only carbon substrate, has been engineered through the use of specific promoters and optimization of culture conditions (Hirokawa *et al*., 2017). Furthermore, a wide range of cyanobacteria strains are able to produce PHAs. 3-Hydroxybutyrate, the key metabolic precursor of PHB, has also been produced in *Synechocystis* sp. PCC 6803 in an attempt to reduce the high biopolymer production costs associated with traditional, sugar-based fermentation approaches (Wang *et al*., 2013). Although sufficiently high PHB accumulation has been reached, up to 85.1% of the cell dry weight (Samantaray and Mallick, 2012), the volumetric productivities are still far from being economically competitive (Singh and Mallick, 2018). One of the main reasons for this low productivity is the lack of methods and efficient processes for biomass production at a large, industrial scale and the somewhat limited set of tools available for targeted and fast genome manipulations. Sophisticated metabolic engineering approaches for facilitating CO_2 and formate fixation have been developed over the last few years (Yishai *et al*., 2017; Cotton *et al*., 2018), and will surely help launching cyanobacteria as functional *chassis* for novel metabolic functions towards sustainable bioproduction.

Roseobacter *and* Halomonas

Besides bioproduction, alternative engineering applications call for an alternative *chassis* as well. As proposed by Borg *et al.* (2016), this is the case for the bioremediation of plastic waste in marine environments, a specific process for which marine bacteria would be the most suitable *chassis*. One of the most important clades in marine habitats is *Roseobacter*. These bacteria have been described previously as source of secondary metabolites, for example antibiotics (Martens *et al*., 2007; Brock *et al*., 2014; Wang *et al*., 2016b), and techniques for genome manipulation have been developed (Borg *et al*., 2016; Tang *et al*., 2016). Another case of an alternative, marine *chassis* microorganism is *Halomonas*, which can grow in the presence of high salt concentrations under non-sterile conditions (Tan *et al*., 2011) – significantly reducing costs associated with sterilization in the fermentation process. Although the toolbox for genetic manipulation of this bacterial species is somewhat limited, advances have been made to overcome this problem, for example the development of constitutive and inducible promoters for targeted gene expression (Yin *et al*., 2014; Zhao *et al*., 2017), construction of knock-out mutants through homologous recombination stimulated by double-strand breaks in the bacterial genome (Fu *et al*., 2014), and the implementation of the CRISPRi technology (Tao *et al*., 2017). In terms of practical applications, and by combining all these engineering strategies, *Halomonas* strains have been successfully used for bioproduction of PHB and PHAs copolymers containing 3-hydroxyvalerate (Fu *et al*., 2014), and the osmocompatible solute ectoine (Chen *et al*., 2017).

Outlook and the challenges ahead

The preceding sections indicate that, although there is a reasonably sizeable number of bacterial *chassis* available for specific applications that have been developed (and continue to be refined) over the years, further developments are needed as a decisive step towards the adoption of a universal *chassis*. Having discussed the main phenotypic properties and metabolic characteristics of a handful of bacterial species that serve as functional *chassis* for bioproduction, in this section we present the challenges that we perceive as a significant hurdle to overcome in the near future – and we propose that tackling them should be an integral part of the whole process that starts from selecting a promising bacterial species until the establishment of a given bioprocess at the industrial scale (Fig. 4). Importantly, these issues are known to be important for several bacterial *chassis*, even when the phenomena described below have been mostly described in *E*. coli-derived *chassis*.

Unsolved aspects of microbial metabolism still pose considerable challenges when selecting a given bacterial *chassis* for executing specific (biochemical) functions. One of these problems is the *loss of catalytic efficiency* when the production process is scaled-up in large bioreactors, compatible with industrial fermentations – which serves as a reminder that metabolic efficiency at the laboratory scale hardly means high fitness in an industrial bioreactor. As recently shown for a recombinant *E. coli* strain carrying genes needed for MVA synthesis cultured in a rich medium, cells engineered for bioproduction usually lose the implanted catalytic functions in several generations due to an evolutionary process in which enhanced fitness is selected for, which usually implies blocking or elimination of heterologous pathways (Rugbjerg *et al*., 2018). One of the main reasons behind this general effect is the presence of heterogeneities in the metabolism across the entire cell population (Nikel *et al*., 2015b), that could be either *endogenous* (i.e. arising from the native genomic and metabolic properties of the cells) or *exogenous* (i.e. due to the burden that producing a chemical compound at high yields usually imposes on the engineered cells) (Akkaya *et al*., 2018). Tackling

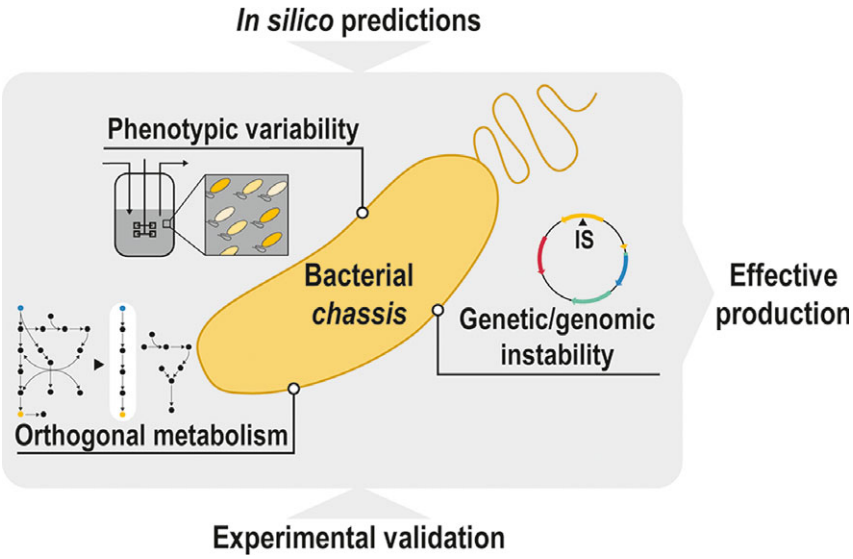

Fig. 4. Some of the challenges ahead for developing functional bacterial *chassis* for metabolic engineering. Effective bioproduction could only be achieved by overcoming the current hurdles of genetic and genomic instability (e.g. leading to alteration or loss of production phenotypes), phenotypic variability across individual cells in the microbial population, and the inevitable interactions between metabolic implants and the extant biochemistry of the bacterial *chassis*. The way forward to tackle these issues requires the combination of robust *in silico* predictions and in-depth experimental validation, if possible under conditions compatible with industrial production.

this problem is therefore one of the major challenges in bringing bacterial *chassis* to actual industrial-scale bioproduction. In connection with this aspect, the evolution of alternative metabolic architectures in the engineered biochemical network (as opposed to changes operated merely at the genetic level) during bioproduction will help designing more efficient bioprocesses (Nikel and de Lorenzo, 2018).

Metabolic orthogonality is a major objective (as well as a significant challenge) in the field of metabolic engineering. The design of authentic orthogonal metabolic pathways, a concept referring to the minimization (and, ideally, suppression) of potential interactions between the synthetic metabolic pathway to be implanted and the extant metabolism of the bacterial *chassis*, has become a holy grail to optimize bioproduction (Pandit *et al*., 2017). A much needed further step into this direction would be to rationally combine the different tools so far developed for genome engineering and experimentally curated computational prediction [e.g. *retrosynthesis approaches* (Delépine *et al*., 2018)] for designing entirely novel (i.e. synthetic) metabolic architectures that can be plugged-in into any given bacterial *chassis*. The emerging field of designing and implementing orthogonal metabolisms is being boosted by applying sophisticated strategies for enzyme engineering and *de novo* protein design (Erb *et al*., 2017; Arnold, 2018). Such tailor-made enzymes, potentially able to catalyze novel, non-natural biochemical reactions, hold the promise of paving the way for the design of *portable* synthetic metabolisms that can be integrated in any given *chassis*. Automation and standardization, two core tenets of synthetic biology, should be incorporated in the workflow of designing orthogonal metabolisms as well. The combination of these approaches would result in the efficient design of *trans*-metabolisms for bioproduction of virtually any chemical structure in a bacterial *chassis* of choice.

In close connection with engineering completely novel *trans*-metabolisms, the field of metabolic engineering is currently primed for the expansion of products in the biotechnological agenda – as industrial-scale bioproduction is still largely limited to a handful of chemicals that exist only naturally in biological systems. Accessing new-to-Nature products through metabolic engineering is not only desirable but an actual necessity in a rapidly changing world in which access to natural, fossil-based resources is becoming critical. *Redesigning the biochemical palate* of bacterial *chassis* is the way forward to access these novel products, and rewriting the metabolic blueprint to broaden the chemical repertoire achievable by engineered cells is a condition to access novel products (Hossain *et al*., 2018). Note that such ambitious objective calls for deepening our current understanding of the very core metabolic wiring in bacteria, a subject that has recently gained much attention after quite some time of (undeserved) ostracism (Aslan *et al*., 2017). Including rare chemical elements into the biochemical agenda of bacterial *chassis*, for example halogens and boron (Kan *et al*., 2017), will revolutionize bioproduction by multiplying the catalytic power of bacterial cell

factories. Yet again, these operations would be enabled by an in-depth knowledge of microbial metabolism, and standardized workflows should be developed to access these novel biochemistries.

Finally, and in addition to all these scientific and technical aspects, legislative issues will play a decisive role in the practical implementation of novel bacterial *chassis* for bioproduction. The development of suitable regulatory frameworks will be needed to bring superior biocatalysts into the biotechnological arena by tackling and overcoming legal restrictions. In any case, and irrespective of the current hurdles, the whole field of metabolic engineering is witnessing an exceptional increase in the number of bacterial *chassis* adopted for bioproduction, especially considering that the revolution brought about by the implementation of sophisticated tools for genome engineering (e.g. CRISPR/Cas9-based approaches) has eliminated many of the common barriers that used to hinder the *à la carte* construction of bacterial *chassis*. The time has definitely come to capitalize on the knowledge amassed over the years for the smart design and construction of robust bacterial cell factories by combining *in silico* and wet-laboratory approaches.

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Conflict of interest

None declared.

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