Bacillus subtilis Chassis in Biomanufacturing 4.0

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Abstract

Synthetic biology, an emerging research field, can promote biomanufacturing by offering various efficient chassis. Engineering *Bacillus subtilis*, an important workhorse in industrial biotechnology, through synthetic biology approaches may be a disruptive innovation. Advancements in chassis engineering, a synthetic biology strategy for genome-reduced cell factories, cell-free systems, and synthetic microbial consortia would be a driving force facilitating microbial production. We discussed chassis engineering categories and applications for *B. subtilis*. Prospects and challenges for chassis engineering in *B. subtilis* were also analyzed in this review article.

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Abstract : Synthetic biology, an emerging research field, can promote biomanufacturing by offering various efficient chassis. Engineering *Bacillus subtilis*, an important workhorse in industrial biotechnology, through synthetic biology approaches may be a disruptive innovation. Advancements in chassis engineering, a synthetic biology strategy for genome-reduced cell factories, cell-free systems, and synthetic microbial consortia would be a driving force facilitating microbial production. We discussed chassis engineering categories and applications for *B. subtilis*. Prospects and challenges for chassis engineering in *B. subtilis* were also analyzed in this review article.

Keywords : *Bacillus subtilis* ; Biomanufacturing; Cell-free system; Chassis; Genome reduction; Synthetic microbial consortium

Introduction

Biomanufacturing can crucially contribute to sustainable human development by utilizing biological systems to convert simple molecules, naturally occurring materials and even waste to value-added chemicals and biopolymers. Of note, Synthetic Biology (SynBio) offers disruptive technology for biomanufacturing, denoted as biomanufacturing 4.0 (BM 4.0) (Fig. 1) [1]. Recent developments in smart biomanufacturing and Biological Design Automation usher BM 4.0 into a new era (Table 1) [2-4].

The application of chassis in biomanufacturing 4.0 and the emergence of enabling technologies would drive the translation of the SynBio chassis from laboratory to industrial applications by minimizing regulating

complexity and enhancing biological entity performance predictability, although synthetic biologists originally expected to study chassis, also referred to as the “living machine”, as a way to decipher the nature of life [5].

Bacillus subtilis, a robust, fieldable and well-characterized host is likely one of the most promising chassis for biomanufacturing 4.0 [6, 7], considering that *B. subtilis* can biosynthesize value-added products such as single-chain antibodies, nucleotides, vitamins, antibiotics, biosurfactants, carbohydrates, and biodegradable polymers [8-13].

To offer an efficient, compatible and robust expression environment for aimed biosynthesis modules, *B. subtilis*, the host cell should be modified with SynBio approaches according to certain engineering principles, which can be termed as chassis engineering.

Some excellent reviews summarized synthetic biology toolbox and strategies for improving *B. subtilis* chassis performances [7, 14, 15]. However, they did not explain why different forms of chassis are essential for biomanufacturing 4.0. This mini-review will initially explain the definition, selection and categories of chassis. Then, we discussed the reasons why *B. subtilis* is suitable for biomanufacturing 4.0. Next, we highlighted progress in cell-free systems and synthetic microbial consortia, which are promising for biomanufacturing 4.0 as alternative forms of chassis. Finally, the challenges in the industrial applications of chassis and the next-generation chassis were analyzed, which may also be applicable for engineering chassis of other species like *B. licheniformis* for biomanufacturing [16].

Biomanufacturing requires “better” chassis

A chassis, a machine metaphor of a host cell, is an engineered life system that provides resources such as gene expression, protein translation and metabolic precursors, and it is also the carrier of biological components and devices, providing a predictable environment according to the principles in engineering practice, such as abstraction, modularity, orthogonality, robustness and compatibility to artificial components [17].

In a laboratory setting, chassis engineering refers to an engineering process that transforms existing natural biological systems or synthesizes artificial life systems from scratch, with the goal of providing an environment to enhance the robustness of biological parts (components) and devices (Table 1).

The basic strategy for current chassis engineering is to initially confirm the essentiality of each gene and then streamline the genome to build a “minimal cell”. Successful construction of the minimal genomic JCVI-syn1.0 and JCVI-syn3.0 indicated the feasibility of the current research roadmap [18]. However, we speculate that chassis should not be limited to “minimal genomic cells”, and chassis engineering should not be confined to “minimal genomic cells”.

First, the definition of essentiality is context-dependent because it is related to cell culture conditions. Hence, the degree of genome reduction needs to be determined according to the defined living environments. Thus, the genome reduced cell or “minimal cell” is a relative concept. Second, the identification of “essential genes” can be influenced by research techniques. For example, not all essential genes belong to the paleome, the so-called persistent genes identified by sequence alignments reflecting bacterial original niches, causing inaccuracy in predicting essential genes by *in silico* methods [19, 20]. In addition, chassis in industrial environments and laboratory settings must meet different requirements. “Industrial chassis” require excellent robustness for adverse industrial environments. Therefore, chassis engineering for industry will be a process of installing artificial metabolic pathways or functional modules with the aid of enabling tools for designing, debugging and optimizing the expected functions of microbial manufacturing into genome-reduced chassis. In other words, industrial application of chassis engineering will be a process of not only genome reduction but also addition of certain functional modules. Accordingly, the degree of genome minimization needs to be tested and validated by wet experiments, not by computer prediction alone. Furthermore, the so-called minimal cells have not been widely exemplified in large-scale industrial applications; many challenges remain in the translation of genome-reduced chassis from laboratory to industry. Therefore, it is necessary to select acceptable chassis, appropriate forms and a right engineering roadmap, which would be explicitly explained

as follows.

More chassis options for biomanufacturing

Selecting suitable platform cells or chassis is the initial step for biomanufacturing 4.0. The ideal platform cells that can be used for scale-up production should have the advantages of fast growth rate, high energy conversion efficiency, simple culture conditions, well-documented genetic background, desired tolerance to substrates and target products, as well as broad environmental adaptability [21]. Laboratory chassis, for example *Escherichia coli* and *Saccharomyces cerevisiae*, often fall short of these requirements to some extent, but *B. subtilis* is expected to be developed as an industrial chassis. Several advantages of *B. subtilis* chassis are summarized hereinafter from an industrial perspective. The reported minimal genomic chassis (MGC) is the current representative of single-celled chassis (Fig. 2). However, we speculate that chassis should not be limited to unicellular MGC. Other forms of chassis, such as cell-free systems (CFSs) and synthetic microbial consortia (SMC), may also have some basic characteristics of chassis.

Bacillus subtilis: promising chassis

To select and develop an industrial microbial workhorse as a cell factory, several factors should be considered, for instance, metabolic resources (e.g., precursors and cofactors), cell secretion capacity, product toxicity, genome manipulation toolset, cultural conditions, feasibility of large-scale cultivation, cost, and proper protein fold [17, 22, 23]. Suitable chassis can also be screened through bioinformatics tools following engineering principles such as compatibility between parts and chassis, availability of parts and cell growth rate [24, 25]. Comparing *B. subtilis* with other industrial workhorses, such as *Pseudomonas putida*, *Geobacillus* sp., *Deinococcus* sp., *Corynebacterium glutamicum*, *Streptomyces* sp., *Clostridium acetobutylicum*, *Rhodococcus opacus* and cyanobacteria, we speculate that *B. subtilis* has some unique advantages in exploiting synthetic biology techniques in biomanufacturing [6, 17, 26].

B. subtilis is a Gram-positive sporiferous model microorganism with a long history of application in industry [7, 8, 27-29]. Some unique merits of *B. subtilis* make it appropriate for microbial manufacturing [30-32]. For example, several bulk industrial enzymes were produced by *B. subtilis* [9, 33]. Engineered *B. subtilis* cells can also be used as biosensors, probiotics or vaccine delivery systems [8, 10, 34-36]. Additionally, *B. subtilis* has some benefits not found in laboratory chassis. Although *B. subtilis* has a simple cell envelope, it is highly resistant to omnipresent stress. This bacterium can assimilate cheap substrates, secrete a high titer of target proteins, induce competent or super-competent state, and accommodate large fragments of foreign DNA [6, 17, 37]. In contrast to *E. coli*, *B. subtilis* has no endotoxin and avoids the step of removing endotoxin in biopharmaceutical processes. Therefore, it is easier to produce extracellular products using *B. subtilis* than using *E. coli* or *S. cerevisiae*.

However, *B. subtilis* still has some disadvantages in contrast to laboratory chassis and some other industrial strains. For example, its product yield is not high enough and its growth cycle is relatively long. As to synthetic biological enabling tools, their development is chiefly based on *E. coli* and *S. cerevisiae*. Thus, it is crucial to develop novel enabling techniques for the *B. subtilis* chassis [6, 38, 39]. By understanding the molecular mechanisms of *B. subtilis* related to productivity and robustness, as well as improving the existing enabling tools, we will be able to overcome its downsides and promote its application in biomanufacturing. Selecting appropriate chassis forms may be one way to mitigate the weakness of *B. subtilis*. We will discuss MGC first and then move on to CFS and SMC.

Genome-reduced chassis

The genome of *B. subtilis* has been sequenced and annotated, showing it is approximately 4.4 Mbp in length and contains around 4 100 genes [32, 40]. During evolution, *B. subtilis* continually absorbs foreign genetic resources by horizontal gene transfer [41]. Even though horizontal gene transfer may enhance bacterial adaptability to various niches, extra genes cause waste of energy and aggravate the complexity of product separation as well as purification.

Minimizing microbial genomes by deleting non-essential genes is expected to provide a predictable and

phenotypically-controllable chassis. Utilizing global transposon mutation, comparative genomics, single gene knockout, clustered regularly interspaced short palindromic repeat interference (CRISPR interference or CRISPRi) and other techniques, it is predicted that *B. subtilis* has about 300–500 essential genes [42–44]. Other methods, such as machine learning, may also be applicable for gene essentiality identification for *B. subtilis*. *SubtiWiki* listed 251 essential genes encoding proteins and two essential RNA-coding genes for *B. subtilis* as of November 2020 [45]. These essential genes are mainly involved in protein synthesis and quality control, metabolism, cell envelope synthesis and cell division, DNA replication and modification, chromosome maintenance, RNA synthesis, RNA degradation as well as protective functions [30, 46].

A chassis with a reduced genome may have some advantages for biomanufacturing. For instance, genome reduction can improve the genetic stability of the strain by eliminating some horizontally transferred sequences, prophages and mobile genetic elements. After the genome is streamlined, the complexity of substances in culture media is reduced, which is expected to diminish the viscosity of the fermentation broth and suppress foaming by decreasing the production of biosurfactants (e.g., surfactin, iturin, and fengycin), thereby lessening the energy consumption of the fermentation process and facilitating oxygen delivery. Additionally, *B. subtilis* contains at least 20 proteases and peptidases. The stability of the expressed proteins can be improved after some protease genes are deleted. For instance, chassis such as MGB874 and PG10 can increase protein yield and produce proteins that are difficult to express [47, 48]. The non-essential genes currently deleted mainly include these categories: (1) genes related to genomic instability, such as *SPβ* and *PBSX* prophage and *pro1-7*-type prophage; (2) regulatory genes; (3) stress-related genes; (4) large secondary metabolic operons, i.e., *pks* and *pps*; and (5) sporulation genes, such as *kinA*, *kinB*, and *kinC* [43, 49]. Genome reduction simplifies cell regulatory networks, similarly to “dimensionality reduction”, an approach computer uses to deal with complex variables [50]. Moreover, genome-reduced cells may have higher electroporation efficiency and lower mutation rates [30]. In addition, genome streamlining will make the culture medium easier to design, improve bioprocess atomic economy, and facilitate product separation.

Even after the genome is simplified, the physiological characteristics of the chassis may not be improved. Therefore, further debugging and optimization is needed. Adaptive laboratory evolution (ALE) and genome editing technology might meet these expectations [51]. However, chassis usually remove DNA fragments that cause genomic instability. Accordingly, it may reduce evolvability. Hence, we speculate that the efficiency of ALE to improve chassis product performance, robustness and environmental pressure adaptability might decrease.

The efficiency of genome editing affects the efficiency of genome reduction. Thus, developing effective genome editing tools is significant for chassis construction. Some novel technologies exploiting bacterial defense systems offer appealing tools for genetic manipulation. For example, Wu *et al.* developed a novel counter-selectable cassette based on the *B. subtilis* toxin-antitoxin system to improve the efficiency of genome editing [52]. Compared to zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and some recombinase-based technologies, CRISPR-Cas9 is more efficient and will likely play an important role in *B. subtilis* chassis engineering [53–56]. CRISPR-related genome editing techniques may replace some traditional methods such as reverse selection, double-crossover marker-free genome editing, recombinase-based genome editing techniques and genome editing for strains that are difficult to transform [57–60]. Importantly, multiplex genome editing can greatly improve the efficiency of genome editing [61, 62]. Recently, CRISPR-Cas12a has emerged as a candidate for multiplex genome editing due to better specificity and more efficient crRNA array processing in comparison to CRISPR-Cas9 [63, 64]. Additionally, a retron-based genome editing system was successfully applied in the evolution of targeted sequences in *E. coli*, which suggests that retroelement-based editing tools may also work in *B. subtilis* [65].

Cell-free chassis

CFSs mainly refer to *in vitro* protein expression systems based on purified enzymes or cell extracts. Broadly speaking, cell-free artificial metabolism, cell-free transcription-translation system, systems biocatalysis, and ribosome display system can be generally classified as CFSs [66–68].

In addition to genome-reduced cells, CFSs can also be developed as chassis. For example, Kelwick *et al.* advanced a CFS based on *B. subtilis* WB800N [67]. This system did not use exogenous mRNAs, ribosomes, or protease inhibitors. Instead, it utilized a more efficient glycerol 3-phosphoglyceride exogenous energy buffer system to characterize a *B. subtilis* promoter library and expressed 0.8 μ M reporter protein GFPmut3b, which is a $\sim 72\times$ fold-improvement when compared with its *B. subtilis* 168 counterparts. The lengthened reaction time was 150 min in contrast to previous reports (15–60 min). Tian *et al.* constructed a highly efficient *B. subtilis* cell-free system for N-acetylneuraminic acid (NeuAc) *de novo* synthesis, which had a 305-fold greater NeuAc synthesis rate than that *in vivo* and enabled fast pathway dynamics analysis [69]. They found that insufficient phosphoenolpyruvate supply was one of the NeuAc pathway’s bottlenecks. Their study demonstrates that CFS provides a rapid method for metabolic pathway diagnosis (Fig. 3).

Developing *B. subtilis* CFS can be very rewarding if scientists take full advantage of the established CFSs. Presently, there are several well-established cell-free system preparation protocols and some commercialized systems. For example, PURE (Protein Synthesis Using Recombinant Elements), a reconstituted system, can be considered as a set of “standardized parts [70-72].”

CFSs have many benefits over cellular systems. Traditionally, chassis are intuitively assumed as living cells that provide metabolic and energy resources. However, cells have natural limitations. For example, cell growth requires specific temperatures and culture conditions, and certain toxic proteins or membrane proteins cannot be expressed. In fact, biological parts could be exploited to produce “minimized cells” using a bottom-up process [73]. Thus, CFSs provide a new option for producing proteins and accelerating the DBTL cycle in some biofoundries [66, 74]. Additionally, CFSs can function over a wide range of operating parameters (temperature, pH, co-solvents, etc.), which can be homogeneous solution systems that are not limited by the kinetics of substrate diffusion or transmembrane active transport. Therefore, high yields are expected under the high load of substrates. In addition, CFSs are open systems that are easy to monitor, power, and modify by installing genetic circuits or metabolic pathways. Hence, CFSs offer an alternative to producing proteins that are difficult to express or toxic to the host, expanding the range of substrates.

CFSs are easy to prepare, preserve and measure. Hence, their application range is expanding to protein micro-fabrication, non-natural amino acid protein synthesis, protein glycosylation, non-ribosomal peptide synthesis, cyclic peptide synthesis, enzyme screening, prototyping of DNA regulation networks, biosensing, etc [68, 75-77]. CFSs are ideal for optimizing metabolic pathways and enabling artificial metabolic engineering to help produce substances that are incompatible with life systems [78]. If a CFS is constructed on the basis of minimized cells, its energy utilization may be more efficient. In addition, it is possible to further reduce genome size by optimizing the energy supply system and deleting scores of essential genes that are required for cell envelope, shape, and division [79].

Admittedly, CFSs have some drawbacks, for instance, high cost, short expression lifespan, and low efficiency in contrast to cellular systems due to requiring exogenous mRNAs, energy, and protein inhibitors. Additionally, the cell envelope of a CFS is removed, which may compromise compatibility between enzymes and modules. Consequently, biological reaction constraints should be re-examined in CFSs, such as the thermodynamic parameters and the optimum pH of enzymes. Fully exploiting thermostable enzymes developed through protein engineering may usher CFS into the new biomanufacturing age [80, 81].

Synthetic microbial consortia

SMC are artificial multi-cellular interaction systems that simulate the natural microbial community. Microbial communities often exhibit a population-level phenotype of complexity, relative stability, evolution and fluctuation. Traditionally, some fermentation products are produced by natural microbial communities, mainly mixed-species solid-state fermentation. Novel natural products can be discovered by studying the interactions between microorganisms [82]. In addition, designing artificial microbial communities can be useful for understanding and engineering microbiomes, that is, the whole microbiota in a human or animal body [83, 84]. Overall, SMC are possibly emerging as chassis for biomanufacturing (Fig. 4) [85-88].

B. subtilis -based SMC may be developed as chassis. For example, Hays *et al.* reported a stable (for weeks

to months) and robust platform for producing amylase and polyhydroxybutyrate (PHB) by engineering autotrophic sucrose-secreting cyanobacteria (*Synechococcus elongatus* PCC 7942) and heterotrophic microorganisms (*B. subtilis*, *E. coli*, or *S. cerevisiae*) [89]. These light-driven co-culture dyads revealed general design principles for the construction of robust autotroph/heterotroph consortia as photoproduction platforms, which can persist when challenged with specific perturbations. Additionally, it was reported that bioplastic producing *Ralstonia eutropha* 5119 and sucrose hydrolyzing *B. subtilis* were co-cultured as SMC that can manufacture poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer from sugarcane without precursor feeding [90]. Liu *et al.* built a consortium as a microbial fuel cell consisting of *B. subtilis* RH33 and *Shewanella oneidensis* MR-1, which generates bioenergy stably for 500 h [91]. Electron transfer efficiency of this consortium was enhanced because MR-1 could efficiently utilize the high concentration of riboflavin produced by RH33. These instances show that SMC may have advantages over MGC in terms of energy/carbon source utilization.

Apart from the above-mentioned multispecies system, there are some seminal reports discussing monospecies SMC, which are promising for microbial production. Hong *et al.* designed a genetic circuit based on the quorum sensing (QS) system, which can be used to achieve competition between strains, and replace the biofilm of another strain [92]. One advantage of a monospecies SMC is that there is no need to optimize the medium.

Research shows that SMC have many unique advantages over monoculture (pure culture) systems because they can utilize complex substrates, reduce the metabolic burden of hosts and lessen genetic burden by avoiding assembling too many genetic circuits [93, 94]. Currently, different types of chassis are applied toward biomanufacturing and enabled by integrating functional modules or rewiring synthetic gene regulatory networks. As functional modules and synthetic gene regulatory network complexity grows, it is difficult to coordinate these modules and networks within a pure culture system. Using SMC is advantageous for assembling different biological circuits, functional modules or metabolic pathways to different cells and avoiding incompatibility among synthetic biological devices/modules/pathways [95]. Hence, SMC compartmentalizes various functional modules so that different strains can perform distinct functions simultaneously with reduced interference in order to implement complex functions [96]. Meanwhile, new products can be synthesized by exploiting enzyme resources from diverse strains. SMC assist scientists to understand multi-cellular temporal and spatial dynamic behavior and population stability [88]. Additionally, SMC may be more resistant to environmental factors and able to respond to changing environments [92]. Moreover, SMC can increase the yield of biomass, which is desirable for biomanufacturing [97].

Introducing artificial cell-to-cell communication and metabolite exchange are the main principles for designing synthetic microbial communities [98, 99]. Cell-signaling mechanisms (such as QS) can be applied to create synthetic circuits across cells from the bottom up that enable cells to exhibit population behavior [100, 101]. Artificial microbial communities can also be reconstructed from the top down on the basis of natural microbial communities. Furthermore, systems biology offers insights into the rational design of synthetic microbial ecosystems [83].

By engineering *B. subtilis*' signal transduction and metabolite exchange, scientific workers can rationally engineer *B. subtilis* for applications in medicine, food and feed sectors [10, 11]. Although there are various interactions among cells, positive interactions (e.g., commensalism, mutualism) are desirable for biomanufacturing. Thus, it is valuable to investigate mechanisms for positive interactions as well as those factors impacting microbial ecosystems' genetic stability and long-term robustness. Additionally, biofilm-related molecular mechanisms and the multicellular nature of *B. subtilis* might be useful for designing monospecies SMC [102]. Furthermore, most reported SMC are composed of wild-type strains or engineered strains. If SMC were built from MGC, its physiological behavior could be more predictable than current counterparts.

Undoubtedly, current *B. subtilis*-based SMC also have some imperfections, for instance, few applications and difficulties in wiring logic computing [103-105]. Additionally, few *B. subtilis*-based SMC have been developed due to insufficient understanding of intercellular communications and interspecies metabolite utilization. However, we believe that SMC will offer new options as chassis for biomanufacturing and environmental

remediations through applying new approaches such as QS engineering and microfluidic chips [92, 106].

Concluding remarks

This review article suggests that *B. subtilis* chassis may have three categories: MGC, CFS and SMC. They have great challenges and potential in biomanufacturing 4.0. For example, genome-reduced *B. subtilis* chassis has demonstrated superior production characteristics to some extent. Moreover, with the development of highly efficient DNA synthesis, it is possible to synthesize the *B. subtilis* genome from the bottom up in the future [18]. In addition to MGC, other types of chassis can be expected to expand their application scenarios. For example, synthesizing some high-value proteins in CFS seems commercially feasible, although it is still in its infancy. In addition, well-developed *B. subtilis* -based SMC can be achieved by engineering QS systems, reconstructing artificial-metabolic circuits and exploiting metabolic cross-feeding in the forthcoming days [107].

To fully exploit *B. subtilis* chassis' potential in biomanufacturing in the future, we should focus on essentialome (*this term is coined herein to stand for a total set of essential genes*) engineering.

Currently, chassis engineering projects are chiefly aiming at exploring the way to streamline non-essential genes to increase "atomic economy" for biomanufacturing and to decrease the biological regulating network complexity to strengthen predictability and genetic stability. Existing essential genes usually belong to the paleome, which determines cell doubling [20]. The paleome codes translation and transcription machines and provides information services for biosynthesis. Ribosomes, the core device of translation machines, determine the efficiency of protein production [108, 109]. Notably, engineering ribosomes can increase translation efficiency and temperature range [110, 111].

Accordingly, these examples suggest that the next generation of chassis engineering should focus on the essentialome, which codes transcription and translation machines and other necessary components for life persistence. We speculate that essentialome engineering may be an effective approach to breaking through the limitations of existing chassis because constraints within chassis can be fundamentally changed. Through essentialome engineering, next-generation chassis will not contain the original set of essential genes; instead, they will be composed of novel transcription and translation machines developed by evolution engineering, genetic code expansion, genome recoding, synthetic chimeric genome or other techniques [110-112]. Next-generation chassis derived from essentialome engineering may have not only novel ribosomes to decode metabolic pathway information with high efficiency but also sharper senses to interpret environmental signals.

Taken together, there is a growing demand to develop *B. subtilis* -based chassis for biomanufacturing 4.0 [113]. These challenges in chassis engineering can be solved by the synthetic biologist community and enabling technology democratization.

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Conflicts of interest

All authors report no conflict of interest.

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