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Published in:
Current Opinion in Biotechnology

DOI:
10.1016/j.copbio.2019.01.007

Publication date:
2019

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Metabolic engineering and synthetic biology employing *Lactococcus lactis* and *Bacillus subtilis* cell factories

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Metabolic engineering and synthetic biology approaches have prospered the field of biotechnology, in which the main focus has been on *Escherichia coli* and *Saccharomyces cerevisiae* as microbial workhorses. In more recent years, improving the Gram-positive bacteria *Lactococcus lactis* and *Bacillus subtilis* as production hosts has gained increasing attention. This review will demonstrate the different levels at which these bacteria can be engineered and their various application possibilities. For instance, engineered *L. lactis* strains show great promise for biomedical applications. Moreover, we provide an overview of recent synthetic biology tools that facilitate the use of these two microorganisms even more.

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Current Opinion in Biotechnology 2019, **59**:1–7

This review comes from a themed issue on **Tissue, cell and pathway engineering**

Edited by **Marjan De Mey** and **Eveline Peeters**

https://doi.org/10.1016/j.copbio.2019.01.007

Introduction

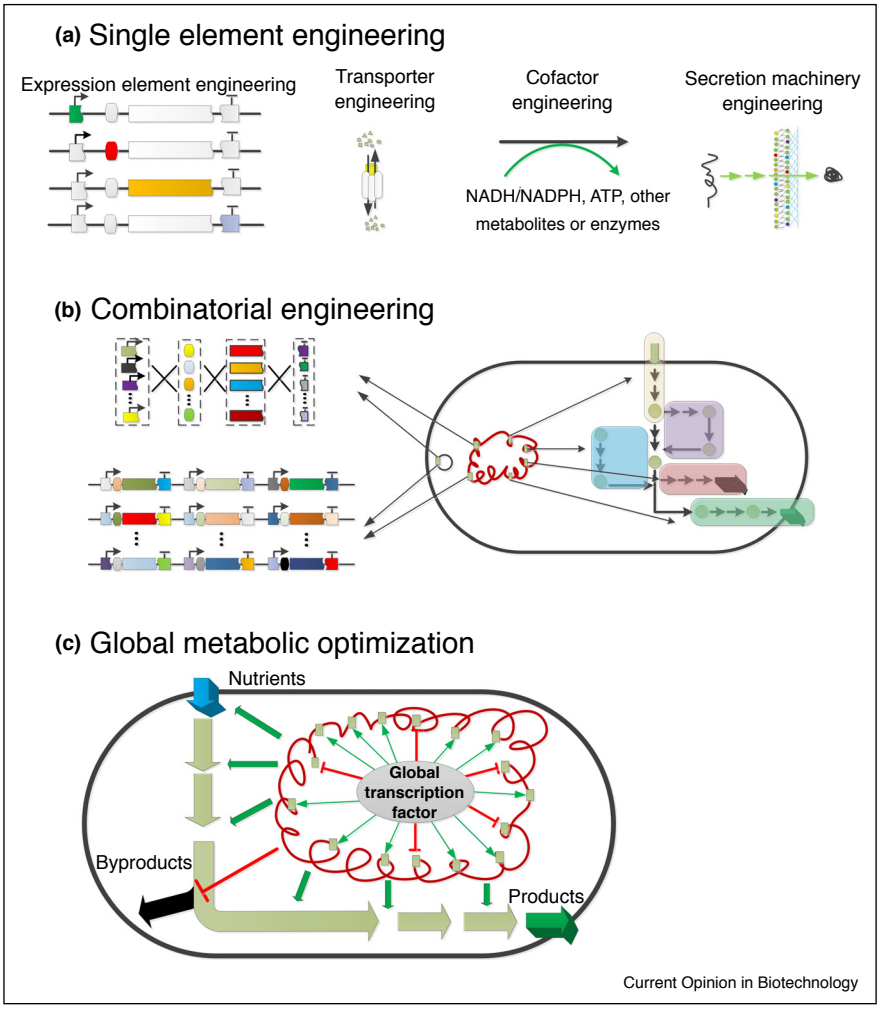
In the last few decades, metabolic engineering and synthetic biology approaches to improve industrial applications of microbes have delivered many breakthrough results [1]. However, most of this work has been performed with the main model organisms *Escherichia coli* and *Saccharomyces cerevisiae*. Here, we will focus on two other workhorses in biotechnology that is the food-grade bacterium *Lactococcus lactis*, and industrial chassis *Bacillus subtilis*. *L. lactis* is indispensable in dairy and health applications, being a production organism for antimicrobials, polyphenols, oral vaccines, and flavor-compound and texturizing compound. *B. subtilis* is an efficient metabolite and enzyme producer for various industrial applications. Moreover, the sporulation properties of the latter also offer opportunities for vaccine production by expressing antigens at the surface of spores. These microorganisms have some unique properties, which make them particularly suited for specific applications. Both *L. lactis* and *B. subtilis* lack immunogenic lipopolysaccharides that render them better hosts for expression of health-related products, like antimicrobial delivery or oral vaccines, than *E. coli* and other Gram-negative bacteria. Moreover, *L. lactis* model strain MG1363 produces only a low number of exoproteins and no exoproteases, while genome-reduced *B. subtilis* strains [2•] also lack extracellular protease activity, which make these hosts excellent enzyme producers, since the products will stably remain in the culture supernatants.

Metabolic engineering of *L. lactis*

For more than 20 years, the relatively simple metabolism of *L. lactis* has served as a target for metabolic engineering. The ability to metabolize a broad range of carbon sources, high glycolytic flux and tolerance to high concentrations of organic acids and alcohols makes it an excellent candidate for bioproduction of fine chemicals and food ingredients. Rewiring the pyruvate node by blocking competing enzymes and modifying the native glycolytic flux via ATP (adenosine triphosphate) and cofactor recycling, lead to strains efficiently producing diacetyl, acetaldehyde, acetoin, and so on. The ability of *L. lactis* to switch between fermentation and respiration when hemin is present was elegantly exploited as a way to regenerate NAD (nicotinamide adenine dinucleotide) during acetoin and 2,3-butanediol production [3•]. The most recent studies not only obtained the highest 2,3-butanediol level reported for *L. lactis* to date [4], but also demonstrated its potential for converting dairy waste streams into the value-added products (3*R*)-acetoin, 2,3-butanediol and biofuel ethanol with high yields encouraging further bioprocess optimization [4,5].

Many engineering efforts were put in food industry-related strain improvement, such as stress and phage resistance [6–8]. Rerouting and overexpression of various native and heterologous pathways in *L. lactis* (Figure 1a,b) have yielded efficient vitamin, polyol, EPS (extracellular polymeric substances), hyaluronic acid producers (reviewed in Ref. [6]). Development of protocols for cell propagation in emulsion droplets coupled to microfluidics

Figure 1



Metabolic engineering strategies for the production of industrial relevant products by *L. lactis* and *B. subtilis*. **(a)** Rational engineering of expression cassettes (from left to right, different colored boxes represent various promoters, RBSs, CDSs and terminators, respectively), transporters, cofactors, or secretion machinery components. **(b)** Modular or combinational optimization of different rational approaches in a multi-gene product synthetic pathway. **(c)** Global transcription machinery engineering (gTME) allows system-wide pathway modification for improving the metabolic capacity or chemical tolerance. All the three levels of metabolic optimization approaches have been done in *B. subtilis*, while only strategy (a) and (b) have been applied for the overexpression of products in *L. lactis*.

and other screening methods allowed selection of strains with higher biomass or better vitamin producers without any targeted genetic manipulation [9,10•].

Synthetic biology tools for *L. lactis*

Genetic engineering for strain optimization in *L. lactis* has been focused for many years on traits useful to improve industrial milk fermentations. Because of its beneficial properties and the expanding genetic toolbox (Table 1), *L. lactis* has also gained interest as an expression host for the production of heterologous proteins and therapeutic/antimicrobial peptides [11]. Various tools have been instrumental to enable the use of *L. lactis* as a production host. Depending on the purpose, either (artificial) constitutive or inducible promoters are employed to (over)express the gene or gene cluster of interest. The development of inducible gene expression systems has been extremely powerful for functional characterization or production purposes. Various systems have been developed, of which the NIsin-Controlled Expression system (NICE) is mostly used [12]. Also other systems such as Zirex (Zinc-regulated expression system) [13] and ACE (agmatine-controlled expression) [14] were developed that can be used alone or in combination to enable sequential expression. Riboswitches can be used as an OFF-switch to enable gene silencing and are currently under development for *L. lactis* [EP Hernandez *et al.*, unpublished data].

Table 1

Existing and novel synthetic biology tools for *L. lactis*

Purpose	Synthetic biology tool	Reference
Controlled gene expression	Nisin-controlled gene expression system (NICE) Zinc-regulated expression system (Zirex) Agmatine-controlled expression system (ACE) Stress-inducible controlled expression system (SICE) pH-responsive expression (P170)	[19••]
Constitutive gene expression	Constitutive native promoter library	[55]
Genome engineering	pSEUDO. Site-specific integration based on homologous recombination	[18]
	Recombineering. Marker-free method for chromosomal mutations/deletions using ssDNA oligo's	[17]
	Cre-*loxP* recombination system. Site-specific recombination system that allows multiple gene deletions in *L. lactis*	[56]
	CRISPR-Cas9/CRISPRi-based genome editing	[57]
Improved expression host	*L. lactis* NZ9000-4 (9k-4) with minimized genome and enhanced heterologous protein production	[58]
Improved protein secretion	Signal peptides (SP). A library of *usp45*-derived SP for efficient protein secretion	[59]

To introduce foreign DNA into *L. lactis*, electroporation is currently the golden standard. However, DNA transfer between two *L. lactis* species by conjugation was developed [15] and a non-conjugative vertical gene transfer has been observed as well (L Morawska, OP Kuipers, personal communication). Recently, the induction of competence genes created functional competence in *L. lactis* [16]. Different gene modification systems have enabled efficient chromosomal mutations by using recombineering [17]. Gene insertions or deletions were facilitated by double cross-over using the pCS1966 plasmid that is unable to replicate and that contains a selectable antibiotic marker and a counter-selectable orotate transporter. This can lead to markerless deletions or insertions [18]. An extensive overview of cloning vectors and tools for gene expression/modification is presented elsewhere [19••]. Currently, alternative natural methods are being developed that open up application options in, for example, the dairy industry. Phage contamination can be the cause of significant loss of bacterial activity in food fermentations and during the production of biochemicals. Antisense RNAs against crucial bacteriophage genes were previously used in *L. lactis* [20] to inhibit the efficiency of plaquing and burst size. In these studies, there was a limited design of these antisense RNAs, other than cloning different parts of the genes such that the opposite strand is transcribed as an antisense RNA. Recently, hundreds of novel RNAs with putative regulatory functions have been discovered [21] and advances in the field of regulatory RNAs have gained tremendous insight in the molecular mechanisms of how mRNAs can be silenced by degradation or by blocking of the Shine Dalgarno sequence to prevent translation, creating possibilities for a more designer approach using regulatory RNAs as a tool to affect gene expression. Cell penetrating peptides (CPPs) could provide a way to deliver phosphorodiamidate morpholino oligonucleotides (PMOs) to enable gene silencing. These CPP–PMO's are now allowed therapeutically [22], but can be applied to steer fermentation during the process itself in order to eliminate cell functions such as transporters or pathways. An illustrative example of synthetic biology making use of peptide modularity instead of DNA modularity was provided by lanthionine ring shuffling of lantibiotics, making use of over 25 different ring, hinge, and tail modules and a micro-alginate bead-based high throughput screening method to obtain new-to-nature lantibiotics. Some of the analyzed peptides possessed antimicrobial activity and were shown to have an unprecedented host range [S Schmitt *et al.*, unpublished data].

Use of *L. lactis* for biomedical applications

An established history of safe use in the food industry makes *L. lactis* an appealing organism for a number of biomedical applications. Absence of lipopolysaccharides and only a low number of exoproteins make it a better delivery vehicle or expression host than *E. coli* and other Gram-negative bacteria. Genetically modified (GM) *L. lactis* is used to (1) prevent and treat inflammatory bowel disease (IBD), diabetes, cancer by modulating inflammation via cytokine, anti-protease, antioxidant enzyme, antibacterial, and anti-antigenic peptide secretion; (2) fight infectious diseases and allergic reactions via modulation of immune responses and as a safer vaccine. GM *L. lactis* may even be used to deliver DNA molecules to mammalian cells as vaccines or as a form of gene therapy [23].

Steidler *et al.* have established proof of principle using *L. lactis* engineered to produce the anti-inflammatory cytokine interleukin-10 (IL-10) to treat IBD [24]. Using animal models, they showed that dietary administration of engineered bacteria is therapeutically effective. An elegant system for the containment of live GM bacteria was designed replacing the *thyA* gene with an expression cassette resulting in a strain that produces hIL-10 when is strictly dependent on thymidine or thymine for growth and survival. This strain was the first genetically modified organism (GMO) to reach clinical trials [25]. To date,

several engineered lactococci have reached clinical studies using similar safe containment strategy. Recently, a phase Ib/IIa study was announced to test the ability of *L. lactis* secreting IL-10 and proinsulin (AG019) [26•] to treat early onset type 1 diabetes. A phase 2 clinical trial of an oral rinse composed of a recombinant *L. lactis* strain engineered to secrete the mucosal protectant human trefoil factor hTFF1 was initiated [27]. An oral administration of *L. lactis* engineered to secrete anti-TNF-alpha nanobodies proved to be effective and safe against IBD in a phase I trial [28].

Although the NICE system proved to be useful in many cases, synthetic biology approaches required development of promoters that do not involve an external inducer and are constitutive or respond to factors which bacteria encounter in the mammalian body (Table 1).

A resource-conserving and environmentally sustainable production of plant natural products with health-beneficial properties using microbial cell factories is an attractive alternative to plant extraction or chemical synthesis. *L. lactis* was shown to be an excellent host for the expression of plant and fungal membrane proteins and soluble enzymes involved in polyphenol, terpenoid, and ester synthesis [6,29]. Functional pathways for the production of nutraceuticals resveratrol and anthocyanins were assembled. Employment of metabolic biosensors for malonyl-CoA allowed monitoring of intracellular precursor pool and suggested strategies to improve the product yield [29].

Metabolic engineering of *B. subtilis*

In addition to *L. lactis*, *B. subtilis* has been extensively exploited as a microbial cell factory for the overproduction of various industrially relevant products in the fields of food, pharma, and biotechnology. In the past few decades, numerous studies have been performed in attempts to develop this production host into a highly adaptable chassis with both high yields and a wide range of products [30].

Conventional approaches for improving the production capacity of *B. subtilis* include modifying the elements of synthetic pathways, that is gene copy numbers, promoters, RBSs (ribosome binding sites), CDS (coding sequences), and terminators [31•] or varying the availability of rate-limiting components, that is the composition of secretion machinery [32] (Figure 1a). However, these efforts, that are based on the rational modification of specific pathway or factors, always require a comprehensive understanding of the target metabolic networks and have limited success on strain improvement [33,34].

Metabolic engineering strategies that integrate newly developed systems and synthetic biology approaches have greatly facilitated the unlocking of phenotypes with desired cellular properties in *B. subtilis* [34]. Combinatorial pathway optimization, which enables to vary multiple critical pathway elements, can streamline metabolic engineering by reducing experimental efforts and the amount of *a priori* knowledge [35] (Figure 1b). The combinatorial engineering of promoters and RBSs [31•], and non-conserved sequences [36] demonstrated great potential in increasing reporter gene expression levels in *B. subtilis*. The divided parts of complex multi-gene product synthetic pathways have been modularly optimized for generating recombinant strains with improved production of *N*-acetylglucosamine [37]. Moreover, simultaneous engineering of the cell surface and the expressed targets lead to a further enhanced secretion efficiency of α-amylases in *B. subtilis* [38].

Global transcription machinery engineering (gTME), which allows multiple and simultaneous perturbations of the whole transcriptome, enables the increase of end-products by rerouting metabolic fluxes at a top layer of regulatory networks [1] (Figure 1c). This global optimization strategy can substantially simplify the strain enhancement design even without a complete picture of the underlying metabolic regulatory mechanisms [39]. A variety of global transcription factors in multiple microorganisms have been successfully engineered to elicit variants with improved metabolic capacity or chemical tolerance [40]. In a recent study, the gTME-based approach was applied for effectively and quickly unlocking *B. subtilis* variants by randomly mutagenizing the global N-regulator and C-regulator CodY and CcpA, respectively. The selected best phenotype, carrying crucial mutations among helix-turn-helix domains, reached a twofold increased overproduction of β-galactosidase. Furthermore, this improvement was demonstrated by the significantly enhanced overexpression of green fluorescent protein, a xylanase and a peptidase [41•].

Synthetic biology tools for *B. subtilis*

B. subtilis lends itself well to genetic manipulation due to its ability to become naturally competent and to take up both circular and linear forms of DNA. In addition, the genome can easily be changed for variable purposes (e.g. deletions, point mutations, insertions) by employing the mechanism of homologous recombination. Nevertheless, advances in technologies and increasing knowledge about the characteristics of a successful bacterial production host have driven the continuous development of useful *B. subtilis* strains and genome editing tools. This makes *B. subtilis* a very attractive bacterial host for academic and biotechnological purposes.

In addition to protease deficient *B. subtilis* strains (reviewed in Ref. [42]), which showed improved production yields of heterologous proteins (Table 2), the genome of *B. subtilis* has been reduced further in order to obtain a more simplified microbial chassis that possibly

Table 2

Existing and novel synthetic biology tools for *B. subtilis*

Purpose	Synthetic biology tool	Reference
Improved expression host	Protease-deficient *B. subtilis* strains	[42]
	Genome-minimized *B. subtilis* strains	[2•]
	Essential gene knockdown and non-essential gene knock out libraries	[45••,46]
Improved genetic competence	Inducible competence system to improve transformation efficiency	[44]
Expression elements	*Bacillus* BioBrick Box containing standardized vectors, reporters, promoters, epitope tags, and optimized fluorescent proteins	[47]
	A characterized phase-dependent endogenous promoter library	[48]
Controlled gene expression	Subtilin-regulated gene expression system (SURE)	[49]
	Inducer-free expression systems	[50]
Genome engineering	Markerless gene deletion system	[51]
	CRISPR-Cas9 system (pJOE8999)	[52•]
Databases	*Subti*Wiki. Database of genes, proteins, metabolic and regulatory pathways.	[53]
	BsubCyc. Database of metabolic pathways.	[54]

displays even higher production yields [43]. To date, a genome reduction of 36% has been achieved in *B. subtilis* [2•]. This led to two independent genome-minimized *B. subtilis* strains which have been subjected to multi-omics analyses and still display robust growth in complex medium. To cope with the gradual decrease in genetic competence as a consequence of genome reduction, an inducible competence system [44] was introduced in the minimal genome leading to a 20-fold increase in transformation efficiency compared to the reference strain *B. subtilis* 168. This, in combination with the preservation of commonly used loci for genomic integration, makes these Mini*Bacillus* strains attractive microbial hosts for heterologous expression. Currently, the Mini*Bacillus* strain PG10 is exploited for the heterologous production of lantibiotic peptides [AY van Tilburg *et al.*, unpublished data]. A major advantage of this lantibiotic production system in PG10 is the lack of extracellular serine proteases which results in the production of antimicrobial inactive precursor peptides which can be activated *in vitro* at a later stage.

Other useful *B. subtilis* strains can be found in the essential gene knockdown library [45••] and in two ordered and barcoded non-essential gene knock out libraries [46]. By providing information about gene functions, networks, and pathways in *B. subtilis*, these single-gene deletion strains can facilitate the design of tailor-made biological systems.

In the last decade, the genetic toolbox for genome engineering in *B. subtilis* has expanded significantly with the development of new tools as well as improvements of existing tools. Multiple toolboxes containing a variety of (standardized) elements for fine-tuning gene expression are nowadays available [47,48]. In addition, effort has been devoted to the generation of more tightly controlled expression systems (e.g. the subtilin-regulated gene expression (SURE) system [49]), as well as expression systems that are inducer-free [50] or which do not leave selectable markers or other scars behind [51]. Also, the CRISPR-Cas9 system has enriched the possibilities for genome editing in *B. subtilis* and other *Bacillus* species [52•]. By using the CRISPR-Cas9 system, disadvantages of markerless systems can be overcome, while deletions, mutations or insertions can easily be achieved at any place in the genome. Furthermore, various databases have been generated that provide a vast extent of information on the level of DNA, proteins, regulators, and metabolites in *B. subtilis* [53,54].

Conclusions and outlook

The low level of proteolytic activity, the availability of an extensive engineering toolkit, including strictly controlled promoters, and the great fermentative capacity of *L. lactis* make it an attractive host for flavor, antimicrobial peptide and metabolite production, since heterologous enzymes needed in pathway engineering will commonly be stably produced. *B. subtilis* can grow to high cell densities and is a great host for enzyme production, particularly in view of recent advances to minimize its genome reducing adverse proteolytic degradation and undesirable phenotypes such as sporulation and biofilm formation. A major advantage of these bacteria is also their food-grade status, although restrictive regulations on use of GMOs in food and environment still preclude some exciting applications, such as gut microbiota-modulating cultures, engineered probiotic strains or nutraceutical-producing cultures, and oral vaccines. Recent advances in CRISPR-Cas9 use and some foreseen medical applications, of engineered microbes might change this situation in the near future.

Conflict of interest statement

Nothing declared.

Acknowledgements

The authors thank Auke van Heel and Jakob Viel for productive initial discussions. SBvdM is supported by a grant from TKI Chemistry and NWO (project number 731.014.206), AvT is supported by EU H2020 grant on Rafts4Biotech, H.C. is supported by a grant from China Scholarship Council (CSC), while AS was supported by H2020 grant BachBerry.

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