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# High-throughput genome editing tools for lactic acid bacteria: opportunities for food, feed, pharma and biotech

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**Keywords**

Genetic tool development, Food fermentation, Biotherapeutics, Phytotherapeutics, Synthetic biology, GMO regulation

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**Abstract**

This mini-review provides an overview of traditional, emerging, and future applications of lactic acid bacteria (LAB) and discusses how genome editing tools can be used to overcome current challenges in all these applications. It also describes currently available tools and how these can be further developed, and takes current legislation into account. Genome editing tools are necessary for the construction of strains for new applications and products, but can also play a crucial role in traditional ones, such as food and probiotics, as a research tool for understanding mechanistic insights and discovering new properties. Traditionally, recombinant DNA techniques for LAB have strongly focused on being food-grade, but they lack throughput and the number of genetically tractable strains is still rather limited. Further tool development in this direction will enable rapid construction of multiple mutants or mutant libraries on a genomic level in a wide variety of LAB strains. We also propose an iterative Design-Build-Test-Learn workflow cycle for LAB cell factory development based on systems biology, with "cell factory" expanding beyond its traditional meaning of production strains and making use of high-throughput genome editing tools to advance LAB understanding, applications and strain development.

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## Introduction

Lactic acid bacteria (LAB) are a phylogenetically diverse but functionally related group of bacteria comprising the families *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae* and *Streptococcaceae*. They are low-GC, Gram positive, facultatively anaerobic, non-sporulating and have a highly fermentative lifestyle, converting a range of sugars into mainly lactic acid. Their natural habitats range from plants and milk to major human and animal microbiota niches (Douillard and de Vos 2014). Their benefits of safety for human and animal consumption, metabolic versatility, wide ecological niche adaptation (including industrial scale fermentations), combined with their long history of use in different forms of biotechnology are fuelling the attention towards novel uses of these microorganisms. As a result, LAB applications are rapidly expanding from food fermentations and probiotics to therapeutic agents for animals, plants and humans as well as platform production strains for chemicals and fuels.

As the use of Genetically Modified Organisms (GMOs) in food products is controversial or not allowed by regulatory officials in many countries, the development of high-throughput genome editing tools for LAB has been limited compared to industrial strains such as *Saccharomyces cerevisiae* and *Escherichia coli*. Although LAB were a pioneer group studied for development of genetic tools, with many cloning vectors derived from them still routinely used (De Vos 2011), their tools have mainly focused on being food-grade and less on efficiency or throughput (Fang and O'Toole 2009). Furthermore, laboratory evolution and random mutagenesis have been widely applied to obtain LAB strains with improved properties for the classical food applications as strains resulting from these methods are not considered GMO. However, such methods do not result in targeted modifications and selection of the right strains is often laborious, despite bioinformatics tools being highly instrumental to narrow down the initial experimental strain selection (Bergsveinson, Kajala and Ziola 2017; Stefanovic, Fitzgerald and McAuliffe 2017; Walsh 2017). The development of high-throughput genome editing tools (as opposed to plasmid-based expression systems) for a wide variety of strains is crucial for both fundamental studies and applications to enable fast, targeted and stable genomic modifications.

In this mini-review, we aim to provide a concise overview of traditional, emerging and future applications of LAB and argue how the development of high-throughput genome editing tools for a wide variety of strains can be beneficial, regardless of their GMO-status in the final application.

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Furthermore, we provide an overview of currently available tools and suggest how these can be further developed to enable all these applications, taking also the current legislation into account. Finally, we propose an iterative Design-Build-Test-Learn workflow cycle based on systems biology, similar to what is currently used for industrial production platform strains. This will support rational strain development of LAB for all their applications, whether the end-use strain is considered a GMO or not. Worth to note is that the focus is on engineering single/pure strains and not on microbial community engineering, which has recently been reviewed elsewhere (Sheth 2016; Bober, Beisel and Nair 2018; Zerfaß, Chen and Soyer 2018).

## Applications of LAB and the contribution of genome editing tools

### *Food fermentations*

Fermentation of food and beverages has been carried out since the early days of human technology development (10000 BC), most likely for food preservation purposes (Prajapati 2003; Nair 2003) as lactic acid formation by LAB and the resulting pH-decrease inhibits spoilage microorganisms. Other major roles for LAB in food are related to improving properties such as nutritional value (*e.g.* vitamin and anti-oxidants production, probiotic agents), organoleptic quality (*e.g.* flavour formation) or technofunctionalities (*e.g.* polysaccharide formation). They are also key players in a primary processing of food and beverage ingredients such as cocoa and coffee beans (De Vuyst and Weckx 2016; Pereira, Soccol and Soccol 2016) and will significantly influence the final quality of the product (see also *Agro-applications*). With the longest commercial use and an estimated market growth of 7,2% for the next five years (Intelligence 2018), fermented food is one of the most important economical applications of LAB.

The industrialization of fermented products led to the use of commercial starter and adjunct cultures instead of using natural/"random" cultures, allowing for increased control and optimization of the process and products (Leroy and De Vuyst 2004; Johansen 2018; Macori and Cotter 2018). The estimated economic growth for fermented products puts pressure on the development of starter cultures for bringing new products into the market, making fast innovation and market access key to the competitiveness in the food sector. In order to have more "natural and clean" products, a reduction of additives is also sought for by the food industry (Johansen 2018). This can be achieved by having more

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in-process production of metabolic compounds (*e.g.* vitamins, exopolysaccharides) by the microorganisms used in the fermentation be it starter cultures or adjunct strains (see also *Production platforms*).

For food applications, recombinant DNA technology is strongly limited by regulations (EU 2003; Derkx 2014) and the negative consumer perspective towards GMOs. Hence, this field mainly relies on classical untargeted and laborious methods based on natural selection and evolution, such as mutagenesis and adaptive laboratory evolution (Derkx 2014; Bachmann 2015; Johansen 2018), which are considered non-GMO. Other non-targeted methods resulting in non-GMO strains are transduction and conjugation (Zeidan 2017) (further discussed in *Genome editing*). Another classical strategy for achieving new traits in food products is the screening of microbial collections. However, global access to microbial and genetic diversity is now limited (Bourdichon 2012; Salvetti and O'Toole 2017) by the Convention of Biological Diversity (CBD) detailed in the Nagoya Protocol (Darajati 2013), which is further complicated by uncertainties about the interpretation of the document (Johansen 2017). Thus, for achieving genetic variation as well as targeted and stable strain improvement to advance new properties, genomic manipulation gains even more relevance. Rational and fast strain development is, however, currently inhibited by the factors described above.

Nevertheless, the development of genome editing tools can strongly benefit food applications as a research tool, without the final GMO-strain ending up in the product (Figure 1). For example, after bioinformatics data have predicted a role for a gene in *e.g.* flavour formation, this can be confirmed (or rejected) by its deletion or attenuation. This is especially important for compounds of which the production is not yet fully understood, such as expolysaccharides (Zeidan 2017). Moreover, targeted mutagenesis can be applied to relevant genes for a certain trait to evaluate their function and phenotype. This can aid in establishing a more targeted selection pressure and screening method to select for naturally evolved strains towards this modification(s). This has been done, for example, with phage-resistance factor *YjaE* in *L. lactis* (Derkx 2014). Altogether, by having a better understanding of compound formation and microbial metabolism, more rational and accelerated efforts can be applied to achieve superior properties on food products.

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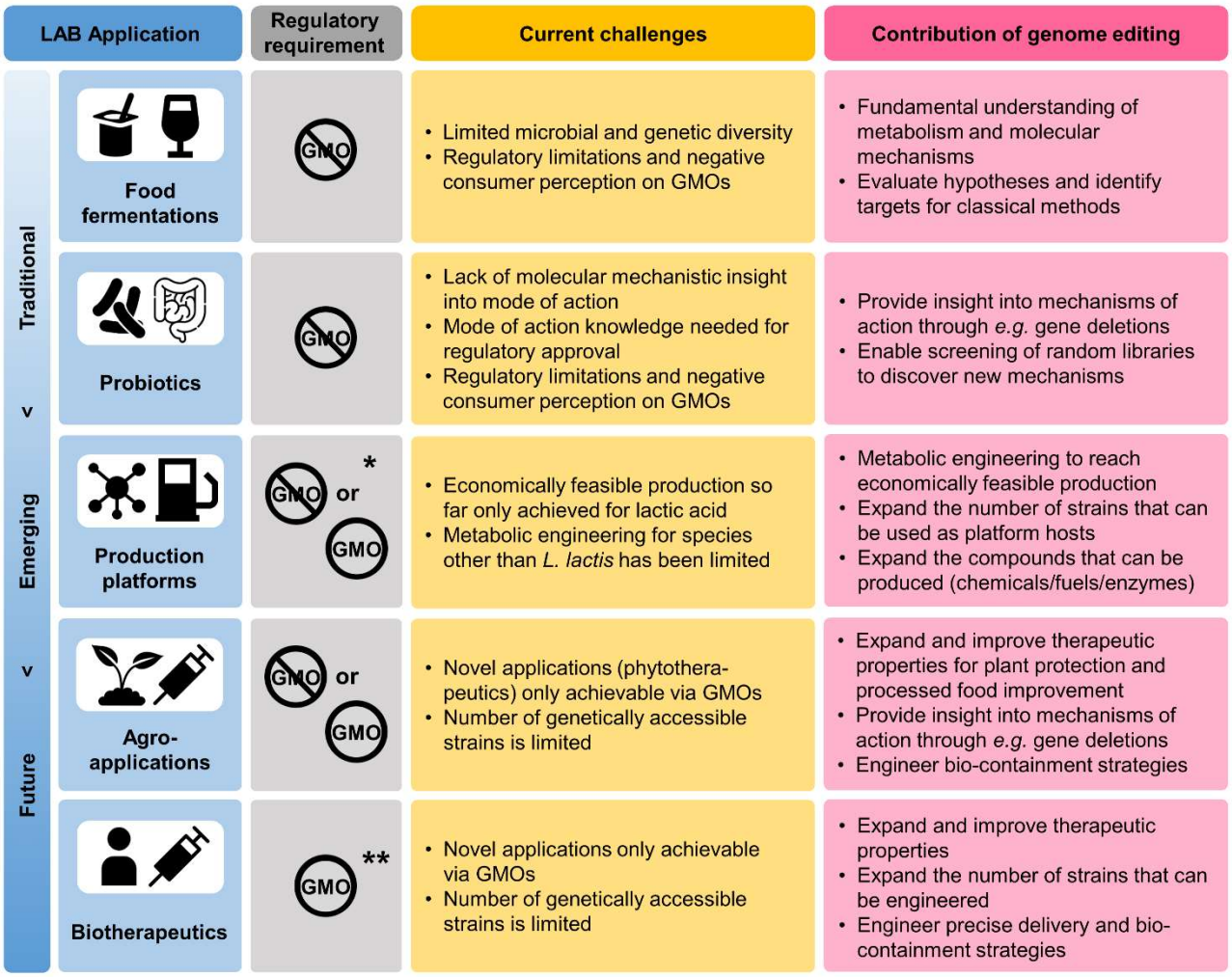


**Fig.1**

**Figure 1. Overview of traditional, emerging and future applications of LAB with the most important contributions of genome editing tools for each, including current regulatory requirements.** For all applications, genome editing provides the possibility to make tailored design strains with desired properties, but the direct use of GMO strains is currently limited; here we have depicted only possibilities within the current legislation. *For food ingredients and enzymes: mostly non-GMO via self-cloning. **Currently not approved, but GMOs are needed to reach the desired application.

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*Probiotics*

The World Health Organisation (WHO) has defined probiotics as live organisms that, when administered in adequate amounts, confer a health benefit on the host. Especially *Lactobacillus* species have attracted a lot of attention as probiotics, which are used as adjuvant or prophylaxis approach against conditions ranging as broad as neuropsychiatric disorders, cancer, irritable bowel syndrome and urinary tract infections (Reid 2017; Mays and Nair 2018). They are also used in a range of animal husbandries including chickens, cows, pigs and fish, to enhance productivity and reduce illnesses (Syngai 2016). The market for probiotics is ever-expanding, with a projected world-wide market size of $46.55 billion by 2020 (Salvetti and O'Toole 2017). Nevertheless, the complex molecular mechanistic modes of action of both probiotics and LAB-host-pathogen interactions are not yet fully understood (Lebeer 2018). After the implementation of EU legislation on health claims in 2009, no probiotics have been granted the right to claim health benefits in the EU. A vast amount of scientific literature indicates beneficial effects of probiotics, and the European Food and Safety Authority (EFSA) considers most health claims to be (possibly) beneficial to human health, but so far in all cases considered the scientific substantiation insufficient and rejected all health claims (Dronkers 2018). The most important aspects for this are the lack of molecular and mechanistic knowledge of their mode of action *in vivo* and irreproducibility of trials, as well as a strong individual responses of the hosts, and strain-specificity (Glanville 2015; Salvetti and O'Toole 2017; Lebeer 2018).

Improving molecular insight into the (dis)functionality of probiotics and observed strain-specificity will be instrumental in achieving the right to health claims. Although genomics- and transcriptomics-based studies are valuable tools (also termed 'probiogenomics' in this context) and have provided important knowledge (Guinane, Crispie and Cotter 2016), using genome editing can provide further detailed and experimental insight (Figure 1). After bioinformatics predictions, mutant strains can be constructed and aid in obtaining *in vitro* molecular and mechanistic knowledge on the mode of action of probiotics. Evaluating the mutant strains in *in vivo* systems can be a crucial tool in further understanding, and in developing probiotics (Lebeer 2018). For example, in *Lactobacillus gasseri* SBT2055, knockout studies of cell-surface associated *apf1* and *apf2* showed that both factors promoted co-aggregation of *L. gasseri* with the pathogen *Campylobacter jejuni*, but only APF1 inhibited *C. jejuni* infection in poultry. This elucidated mechanisms of competitive exclusion and provided insight into the health-benefiting effects of *L. gasseri* as a probiotic (Nishiyama 2015). Other

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examples of using genome editing as a research tool to unravel probiotic effector molecules have recently been reviewed in (Lebeer 2018). The limited use of genome editing in this field can be explained because genome editing is often associated with GMOs and these are not allowed for probiotics, while it has thus far been overlooked as a research tool. Further development of genome editing tools for a larger number of strains and screening methods to allow high-throughput construction and selection of mutant libraries would potentially enable identification of novel, unpredicted factors. Once regulations allow, the tools could furthermore be used to create GMO/improved probiotics that could for example be combined with biotherapeutics.

*Industrial production platforms for green chemicals, fuels and enzymes*

A wide range of products can be made through bio-based production via microbial fermentation of biomass-derived sugars to replace fossil resources, such as (building blocks for) plastics, nylons, solvents, fuels, pharmaceuticals and food and cosmetic ingredients. Traditional work horses for this type of cell factories are *E. coli* and *S. cerevisiae*, mainly because genetic tools for these organisms are well-developed. With the advent of second (non-edible) and third (seaweed- or gas-based) generation substrates to replace the first one (pure sugars derived from edible biomass such as corn or sugar beet), and supported by an ever-expanding range of chemicals gaining interest, it becomes clear that the traditional cell factory organisms are not always necessarily the best. Alternative hosts are gaining interest because they can be used in different process conditions (*e.g.* elevated temperature, no aeration); increased tolerance towards low pH or inhibitory compounds (from substrate or product) (Boguta 2014); the ability and speed to uptake different carbon sources (*e.g.* pentoses); industrial robustness (Beckner, Ivey and Phister 2011; Geissler 2016); the capability to naturally produce certain compounds which can be cost optimized (*e.g.* vitamins) or have a favourable metabolism towards the insertion of a new metabolic route; and finally, the genetic plasticity and possibility to be genetically engineered (Bosma, Forster and Nielsen 2017; Sauer 2017). All these factors are totally or partially present in LAB and explain the growing interest in developing this microbial group in this field; several recent and extensive reviews are available that provide overviews of different aspects of this (Gaspar 2013; Mazzoli 2014; Bosma, Forster and Nielsen 2017; Sauer 2017).

One of the main advantages of LAB is their food-grade safety and adaptation to food-related growth environments, and hence the possibility to use them as production platforms in food-related

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processes. A recent example is the use of metabolically engineered *L. lactis* for ethanol production from the lactose in whey to valorize this waste product of cheese making (Liu 2016). Attempts have been made to ferment the whey-lactose with yeasts, but these suffer from low robustness and slow fermentation; using *L. lactis* proved a promising feasible solution on which the startup company Alcowhey was founded (Liu 2016; Jensen 2017). Another highly interesting application would be the in-process production of proteins or enzymes for food products by starter or adjunct strains (Matthews 2004) as well as the production and release of peptide and protein-like therapeutics for human, animal or plant usage (García-Fruitós 2012).

Although to a lesser extent than for chemicals and fuels, LAB are also starting to be considered for enzyme production – mainly due to their status as food-grade and safe organisms. Currently, *Bacillus subtilis* is the most-used host for enzyme production. As Gram positives, LAB and *B. subtilis* lack a second membrane and periplasm, which favors protein secretion. Whereas *B. subtilis* natively secretes a range of proteins including proteases, *L. lactis* has only one natively secreted protein and one protease, making it an interesting host for protein production (Morello 2007).

To be economically feasible, any production organism should produce a single product in high titre, yield and/or productivity. This means that metabolic engineering through genome editing is required, for which high-throughput engineering tools are required. Nevertheless, except for *L. lactis,* no extensive metabolic engineering has been performed to obtain fully economically competitive LAB cell factories for chemical and fuel production (Gaspar 2013; Mazzoli 2014; Bosma, Forster and Nielsen 2017; Sauer 2017). This is largely due to still underdeveloped genome editing tools for industrially relevant strains. For example, many Lactobacilli and Pediococci have shown to be more tolerant to several stresses compared to *L. lactis*, but lack genetic tools (Boguta 2014; Bosma, Forster and Nielsen 2017). Developing tools for such organisms is highly needed to make use of the wide variety of available LAB strains and their metabolic capacities.

*Agro-applications*

To feed the ever-growing world population, crop health becomes increasingly important. The use of pesticides is progressively regarded as undesired, making organic solutions more important. The production of crops for food and feed is tightly interconnected and microorganisms, including LAB, have many important roles in several parts of the processes. One way of improving plant health is the

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use of bacteria in biocontrol as organic pesticides against, for example, fungi. Traditionally, research on plant growth promoting organisms has focused on Rhizobia, *Bacillus* and *Pseudomonas*. LAB also form a part of the phytomicrobiome of several plant species, but have yet been underexplored (Axel 2012; Lamont 2017). Examples of biocontrol activities are the production of reactive oxygen species, bacteriocins and other antimicrobial compounds, competitive colonization of the plants (which protects them from pathogens), and alteration of the plant immune response (Gajbhiye and Kapadnis 2016; Konappa 2016; Lamont 2017). In many cases, the identity of the antimicrobial compound and which genes encode for it is unknown. Moreover, little is known about the molecular interactions between LAB and plants. Characterizing this further would provide new possibilities for biocontrol and improvement of plant growth and health (Lamont 2017), expanding LAB to a type of plant probiotics.

Plant health is closely related to food and feed, not only as supply chain but also for organoleptic and technofunctional properties in the final product. Metagenomic studies are starting to unveil the phytomicrobiome and factors that affect the epiphytic and endophytic microbial composition in food and feed plants, which become relevant for product processing such as sourdough fermentation of wheat flour (Minervini 2015). A better understanding of the phytomicrobiome of the raw material and the effects its components and metabolites have on food processing could guide new applications or technofunctionalities in the food industry. For example, studies have shown that the presence of LAB in silage used as cow feed affects the final taste and smell of the milk (Kalač 2011), and improved the amount of beneficial fatty acids, such as α-linoleic acid, in chicken meat (Hossain 2012). The use of LAB in animal feed could be further expanded into an integrated organic biorefinery approach. This has been shown in a study aimed at supplying sufficient protein into the animal feed chain by leveraging *Lactobacillus salivarius* fermentation of pressed clover grass juice as an environmentally friendly method to obtain high quality purified plant proteins. The residual pulp can be used as cattle feed, making this a fully integrated LAB-based bio/agrorefinery approach (Santamaría-Fernández 2017).

Altogether, the agro-industry is a promising application field and whereas the use of GMOs in organic farming is currently still out of the question, genome editing tools can be beneficial as a research tool in the same way as the probiotic field discussed above. They can contribute to the fundamental understanding of the strains, their relations to the plants and the mechanisms involved in altering the plant properties and the final processed product for food and feed.

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*Biotherapeutics*

One of the most promising novel applications of LAB, which is still under rapid development, is their medicinal use in therapeutics, prevention and diagnosis (Mays and Nair 2018). Particularly, their use as delivery agents of drugs and vaccines is gaining attention. LAB are particularly suitable since they are already recognized as health-improving agents (especially *Lactobacillus*) in probiotics and are safe for human consumption. Efforts using LAB as biotherapeutics have mostly focused on GIT-related ailments using the strains as oral vectors, leveraging their capacity to survive the stomach acids and adhere to the intestinal epithelium. Examples are treatments against irritable bowel disease (IBD) and gluten intolerance, with successful delivery of anti-oxidant enzymes and anti-inflammatory cytokines (De Moreno De Leblanc 2015). Other targets are related to metabolic disorders (*e.g.* diabetes, obesity and phenylketenuria) (Ma 2014; Durrer, Allen and Hunt von Herbing 2017), microbial infections (Hwang 2016) and oncological disorders or the side-effects of treatments (*e.g.* mucositis) (Carvalho 2017). Beyond the oral forms, LAB are also being developed for mucosal (vagina and mouth) delivery of molecules and as vaccines (Wang 2016), as well as for skin or wound treatment (Vågesjö 2018). Many LAB naturally produce antimicrobial peptides, (*e.g.* bacteriocins), which have applications as bio-controlling agents and immunomodulators in the GIT and are currently commercialized in the purified form for veterinary use (Ahmad 2017). These compounds have demonstrated high specificity and potency *in vivo* and are a potential alternative to fight the raising of antimicrobial resistance (Behrens 2017). Targeted delivery via synthetic biology can potenciate their use as antimicrobial agents of the future. Another attractive field is their use for diagnosis by acting as biosensors inside or outside of the body. For example, *L. reuteri* was engineered to detect a quorum sensing molecule produced by *Staphylococcus sp*. during pathogenesis in the nano- to micro-molecular range, which can be used to detect early nosocomial *Staphylococcus* contamination (Lubkowicz. 2018).

Stable and tuneable modifications via genome editing and synthetic biology are crucial in this field for the addition of the therapeutic compounds to the microbial delivery host, as well as for the insertion of regulation mechanisms, delivery strategies and biocontainment systems (Mays and Nair 2018) (Figure 1). The absence of genetic markers, such as antibiotics, in the final strain is essential to avoid potential risk of transfer of antibiotic resistance to pathogens inhabiting the host. Furthermore, the current tools are limited to a few strains; this needs to be expanded to a wider range of strains with

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better biotherapeutic potential. Most tools are available for the model strain *L. lactis*, while several Lactobacilli have proven a more interesting target group due to their prolonged survival and colonization in the GIT. However, their limited genetic accessibility and toolbox restrain their use (Allain 2015; Bron and Kleerebezem 2018). Furthermore, as with probiotics, better understanding of the interactions with the host on a molecular and cellular level are needed to fully use these biotherapeutics (Figure 1).

The microbial therapeutics and diagnostics market is estimated to occupy close to 79% of the therapeutics segment by 2030 with annual growths of over 80% from 2019 onwards, attracting boosts in funding and investment on the development of new microbial agents (Microbiome Therapeutics and Diagnostics Market (2nd Edition), 2017-2030 2017). As a new field, there are no commercially available LAB-biotherapeutics yet, besides the non-GMO ones composing the community in human faecal transplantations approved by the FDA (FDA 2016). This is expected to change soon as the first clinical trials by pharmaceutical companies with live engineered biotherapeutics using LAB are on-going (Bron and Kleerebezem 2018). Contrary to the traditional LAB applications, it is expected that the benefit as next-generation therapeutics might facilitate a favourable public opinion towards the acceptance of GMOs in this field. Although much more research is required regarding efficiency, fundamental questions and safety, LAB as biotherapeutics can bring a revolution in patient care and become an important asset towards autonomous and precise medicine (Mays and Nair 2018).

**Overview of high-throughput genome editing tools: current and future**

This section will provide a brief overview of currently available genome editing tools (Figure 2) and discuss how these can be further expanded to enable the wide range of LAB applications described above. It also includes transformation methods (Figure 2A) as the introduction of DNA is the crucial first step towards any genome editing. Subsequently, genome editing can be divided into two elements: genomic integration of the introduced DNA (Figure 2B), and selection for mutant cells or counter-selection against wild-type cells (Figure 2C). Further improvements of the genetic toolbox are gene silencing and developments in synthetic biology.

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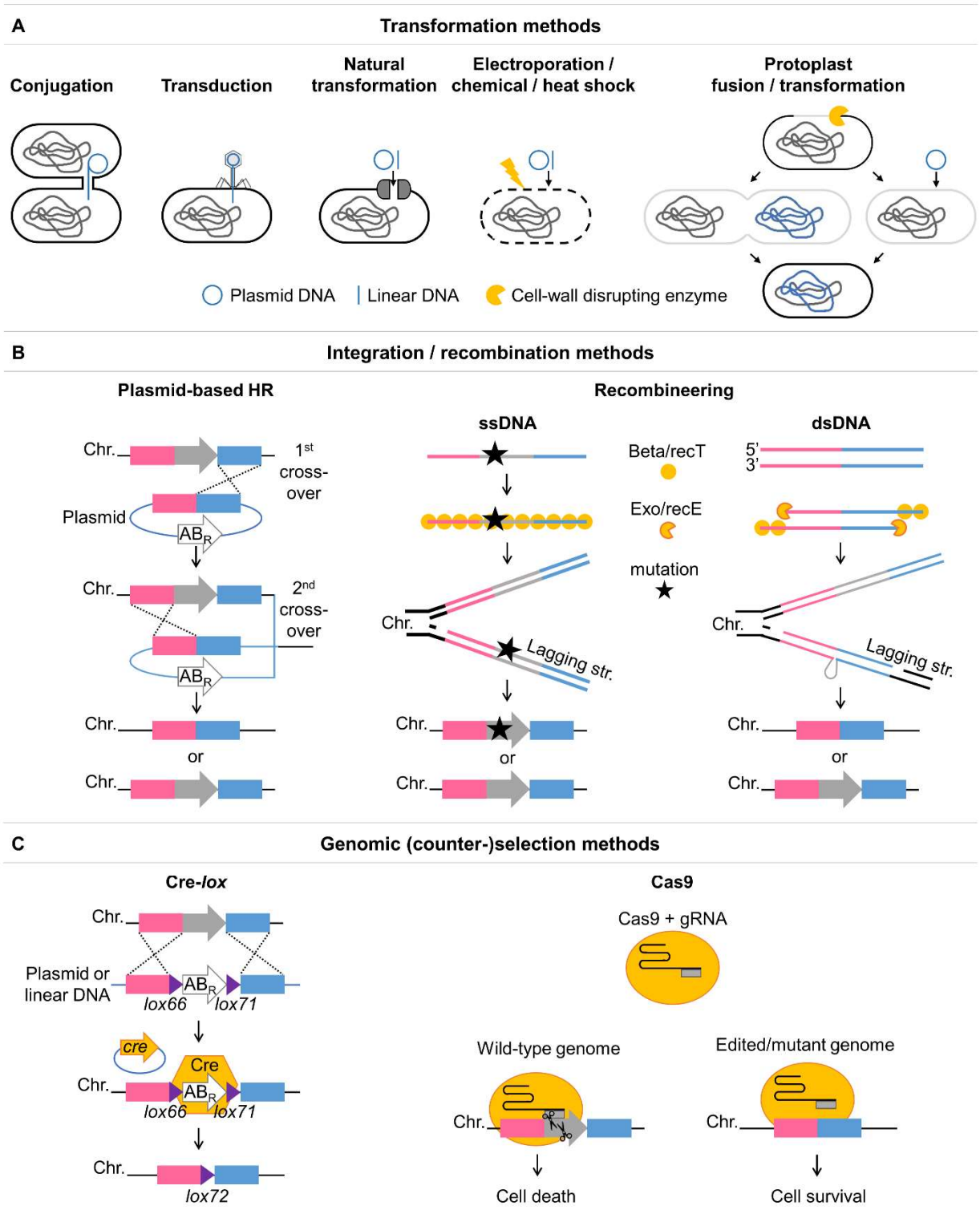


**Fig.2**

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**Figure 2. Schematic overview of transformation and genome editing methods.** Abbreviations: Chr.: chromosome; str.: strand; $AB_R$: antibiotic resistance; ssDNA: single stranded DNA; dsDNA: double stranded DNA; gRNA: guide RNA, which can be either a single guide (sgRNA) or a dual crRNA:tracrRNA. **A.** Transformation methods. For electroporation/chemical/heat shock transformation, the yellow flash indicates any of these external treatments (electrical pulse, chemical treatment or heat shock). For the protoplast-based method, the left arrow indicates protoplast fusion of two different cells and the right arrow indicates transformation of protoplasts. **B.** Integration/recombination methods, all currently reported for LAB. For plasmid-based HR, several variants are possible as further explained in the main text: non-replicating plasmids and thermosensitive plasmids can be used for the first crossover; counter-selectable markers such as *oroP* can be added to the plasmid for the second crossover and plasmid curing. A marker can be introduced within the homologous regions but this does not result in clean mutations. Without marker insertion (as depicted here), the result can be either wild-type or mutant, which need to be verified by PCR. **C.** Genomic (counter-) selection methods reported for LAB to select for mutants via marker insertion and removal (in the case of Cre-*lox*), or against wild-types (in the case of Cas9). Both methods could be used in combination with any of the integration methods shown in B. The *cre* gene is usually transformed into the cells in a second transformation round after *lox-cat-lox* insertion. Cas9 and the gRNA are usually also introduced on a plasmid, which can be on the same plasmid as the homologous flanks when Cas9-expression is induced, or on separate plasmids in separate transformation rounds. Alternatively, Cas9 could be integrated and expressed from the genome (or a native CRISPR-Cas-system can perform this function), and the gRNA is expressed from a plasmid.

*Transformation (DNA transfer) and genetic accessibility*

Transformation (the process to introduce DNA) can be achieved via naturally occurring or artificial methods (Figure 2A). Naturally occurring methods, particularly conjugation, have been widely exploited to achieve non-GMO LAB strains (see next section) (Pedersen 2005; Derkx 2014). Conjugation is based on DNA transfer mediated by mobile genetic elements via direct physical contact ('mating') between a donor and a recipient cell. Conjugative plasmids and transposons are very common in LAB, but the details of conjugative mechanisms are not fully understood and this field still

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needs improvement to widen its applicability (Kullen and Klaenhammer 2000; Dahmane 2017). The size of the DNA region transferred with conjugation is large in comparison to other methods like transduction, where it is limited by phage capsid size (Bolotin 2004). Phage transduction enables genetic transfer across different microbial groups of LAB mediated by phages (Ammann 2008) and is a potential tool for human microbiome engineering (Sheth 2016). In natural competence, exogenous DNA translocates through a native, genetically encoded DNA uptake machinery formed by a multiprotein complex (Blokesch 2016). It is well-known in *Streptococcus* (Gardan 2009; Muschiol 2015), but only recently identified and achieved in *Lactococcus* strains (David 2017; Mulder 2017). The distribution and abundance of natural competence has likely been underestimated (Blokesch 2016) and the new findings in *Lactococcus* might pave the way for such transformation strategies in other LAB strains that are so far considered non-genetically accessible.

In artificial methods, cells need to be made competent through for example washing with cell envelope-weakening solutions, after which external agents are used for cell permeabilization and transformation. Electroporation uses high-voltage pulses and is the most suitable method for high-throughput purposes due to its simplicity, high efficiency and wide applicability (Landete 2017). Generalized electroporation protocols have been successfully used to transform a wide range of LAB strains. Although these studies indicate that the majority of LAB is genetically accessible through electroporation, efficiencies varied strongly among strains and protocols need to be optimized (Landete 2014; Bosma, Forster and Nielsen 2017). Many factors are known to affect electroporation efficiency in strain-specific ways, including growth medium, growth stage of harvest, buffers used and electroporation parameters (Serror 2002). A method with low efficiencies and less suitable for targeted modification but suitable for the large-scale exchange of genomic DNA for *e.g.* evolutionary engineering via genome shuffling, is protoplast fusion (Mercenier and Chassy 1988). This has been used in combination with laboratory adapted evolution (ALE) to generate a *Lactobacillus* strain more tolerant to acidic conditions (Patnaik 2002).

Bacteria have evolved defense strategies against foreign DNA elements, such as restriction modification (RM) and CRISPR-Cas systems or combinations of these (Dupuis 2013). In RM systems, a set of enzymes discriminates self and non-self DNA by methylating it (methyltransferases) and cleave the “invading” DNA (restriction endonucleases) (Vasu and Nagaraja 2013). Recent reports have even shown the existence of “phase-variable” RM systems in LAB, which result in variable methylation

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patterns (De Ste Croix 2017). Limitations for maintaining foreign DNA in cells have been mainly related to RM systems and are a bottleneck for genetic engineering. To overcome these barriers and improve transformation efficiencies, several strategies have been employed: 1) Deletion of the RM systems from the genome (Joergensen 2013); 2) Using a cloning host/DNA source with compatible methylation pattern or heterologously expressing the target strain's methylation genes (Spath, Heinl and Grabherr 2012); and 3) *In vitro* DNA methylation with host cell extracts (Teresa Alegre, Carmen Rodríguez and Mesas 2004). Bypassing the RM systems is necessary to introduce and maintain DNA in the cell (plasmid or linear) and enable further development of genome editing.

*Genome editing (DNA integration)*

Classically, LAB genome editing systems for targeted genomic modifications are based on integrative plasmids that use homologous recombination (HR) to insert or remove a gene of interest via two crossover events between the homologous regions on the plasmid and the genome using the cells' native recombination machinery (Figure 2B). The more recently developed method of recombineering employs phage-derived recombination systems which enable the direct integration of linear DNA into the genome (Figure 2B). Both methods are specific for the desired gene, as recombination occurs over the homologous regions between the introduced DNA and the genome (Figure 2B blue and pink). When the antibiotic marker on the plasmid is placed outside the homologous regions, these methods result in clean and marker-free mutations (Figure 2B). Using replicating plasmids as integration tool requires methods to distinguish between cells harboring the replicating plasmid and those which have integrated the plasmid in the genome. Non-replicating plasmids like pORI can be used in model strains of *L. lactis* (Law 1995), avoiding such a selection step, but this is only possible when both transformation and integration efficiencies are high enough to select immediately for the often low-frequency event of recombination in the transformed cells (Maguin 1992; Fang and O'Toole 2009). An often-used solution for this are conditionally replicating vectors, such as plasmids with a thermosensitive replicon like pG$^{+}$host (Maguin 1992; Maguin, Vost and Dusko Ehrlich 1996) and pTRK (Russell and Klaenhammer 2001). After transformation at the replicative temperature, the temperature is increased to disable plasmid replication and allow selection of integrants (Maguin 1992). To facilitate the second crossover and plasmid curing, a counter-selectable gene can be added to the plasmid that confers toxicity when a certain compound is added to the growth medium. Examples of

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this used in LAB are orotate transporter *oroP* with 5-fluoroorotate (Solem 2008), uracil phosphoribosyltransferase *upp* with 5-fluorouracil (Goh 2009), and dipeptide ligase *ddl* with vancomycin (Zhang 2018). It is important to note that this counter-selection is only against plasmid presence and not against wild-type genomes. Hence, these methods can result in both wild-types or mutants, depending on whether or not both flanks are used for crossing over.

Using recombineering instead of plasmid-based HR eliminates the need for double crossovers and curing of integrative plasmids as it relies on the direct introduction and integration of linear ssDNA or dsDNA oligos with the help of a phage-derived recombination system such as λ Red or RecET (Figure 2B) (Pines 2015). Recombineering has been established in *L. lactis, L. reuteri*, *Lactobacillus gasseri* (Van Pijkeren and Britton 2012), *Lactobacillus casei* (Xin 2018) and *Lactobacillus plantarum* (Yang, Wang and Qi 2015). It is very suitable for high-throughput editing as it does not require cloning of fragments into a plasmid, but is more challenging to establish than plasmid-based HR for two reasons: 1) The phage-derived components are strain-specific and suitable versions of the proteins need to be identified for each new strain, and 2) When aiming for clean gene deletions (*i.e.* without inserting a selection marker or *lox* sites into the genome), selecting mutants can be challenging because efficiencies of recombineering are generally low: for ssDNA recombineering in *L. reuteri*, the efficiency was 0.4-19% (Pijkeren and Britton 2014).

To increase efficiencies of obtaining mutants with both plasmid-based and recombineering methods, it is necessary to establish selection tools for mutants, or counter-selection tools against wild-types (Figure 2C). A frequently used method is Cre-*lox* (Figure 2C), which has for example been used for easy selection of dsDNA recombinants in *L. plantarum* (Yang, Wang and Qi 2015) and *L. casei* (Xin 2018). However, this system always requires two transformation rounds (one to insert the *lox*-marker-*lox* cassette and one to introduce the Cre recombinase) and does not result in clean gene deletions as a 45 bp long *lox72* sequence is left in the genome (Figure 2C). For creating clean gene deletions, the recently developed Cas9-based technology forms a strong counter-selection tool in bacteria (Figure 2C) (Mougiakos 2016). Cas9 is the endonuclease of Type II CRISPR-Cas systems, which in nature function as prokaryotic adaptive immune system (Barrangou 2007; Brouns 2008) but have recently gained fame as versatile genome editing tool. When directed to its target DNA by a provided guide RNA and recognizing its target next to a short DNA motif called protospacer adjacent motif (PAM), Cas9 creates blunt dsDNA breaks (Figure 2B). Whereas eukaryotes can repair such

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breaks by non-homologous end joining (NHEJ), this system is absent or not active in most bacteria (Bowater and Doherty 2006). Hence, they are unable to repair such breaks and this creates a powerful counter-selection tool against wild-type cells as these will be killed due to Cas9 cleavage (Figure 2C) (Mougiakos 2016). In *L. reuteri*, Cas9-based selection of mutants after ssDNA recombineering increased the efficiency from 0.4-19% to 100% (Oh and Van Pijkeren 2014).

Cas9-based editing has now been established for targeted genome editing in *L. reuteri* together with ssDNA recombineering (Oh and Van Pijkeren 2014), and together with plasmid-based HR in *L. lactis* (van der Els 2018) and *L. plantarum* (Leenay 2018). It has also been used for the removal of large mobile genetic elements without HR in *Streptococcus thermophilus* (Selle, Klaenhammer and Barrangou 2015) and *L. lactis* (van der Els 2018)**.** A major challenge of using Cas9 in bacteria is that its activity must be tightly controlled to allow HR-based genome editing before killing the wild-type cells, requiring tightly controllable promoters or multiple transformation rounds. A variant of Cas9 is the nickase $Cas9^{D10A}$, which only makes single stranded nicks instead of double stranded breaks in the genome due to a mutation in one of the two active sites of Cas9. Nicks in the genome are less lethal to the cells, and are furthermore suggested to enhance homologous recombination (Song 2017). The nickase was used together with an integrative plasmid in *L. casei* with an efficiency up to 65%, requiring only a single transformation round (Song 2017). Several alternative Cas9s and other CRISPR-Cas-systems are being characterized for genome editing, showing advantages such as wider applicability, specificity, stability, or less toxicity (Jiang 2017; Mougiakos 2017) and evaluating these in LAB might provide benefits. Furthermore, the repurposing of endogenous CRISPR-Cas systems, which are abundantly present in LAB, into Cas9-like counter-selection systems is a promising approach for broadening the number of species that can be engineered (Crawley 2018). Lastly, all reported genome modifications in LAB so far only make one modification at a time, while multiplexing (targeting multiple genes at the same time) would be crucial for many of the applications. Such multiplexing is complicated when using plasmid-based HR and would strongly benefit from establishing recombineering methods for a wider range of strains.

Next to genome editing, also gene silencing would be a highly desirable addition to the LAB genetic toolbox. A powerful tool for high-throughput gene silencing is “dead” Cas9 (dCas9), where both Cas9-active sites have been mutated, creating a catalytically inactive Cas9 that binds DNA but does not cleave it (Bikard 2013; Qi 2013). This tool can be used for tunable gene silencing but has not

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been exploited for LAB other than as proof of principle in *L. lactis* (Berlec 2018). This would be useful to for example screen phenotypes of silenced genes to select knockout or modification targets, or aid in unraveling fundamental questions as discussed for the probiotics. When using dCas9 for silencing, no HR is needed and multiplex silencing could hence be more easily achieved than multiplex editing.

Regarding synthetic biology developments, improving regulatory control systems of the metabolic routes is highly desirable for LAB, especially for bio-therapeutic applications. Particularly those systems using promoters that can be induced in the gut by the human host metabolites to control gene expression *in vivo* at the targeted location (Bober, Beisel and Nair 2018) as well as for bio-containment strategies, which are crucial for safety and for which much more research is still needed (Wegmann 2017). Systems based on quorum sensing or reciprocal transcriptional repression systems have been used for inducing autolysis in *E. coli* (Chan 2016; Hwang 2017) and could be adapted to LAB. Another option would be the use of Cas9 as a programmable self-killing agent as shown in *E. coli* (Ronda 2016). Gene circuits construction is also important for the development of bacterial biosensors, where engineered strains can detect certain molecules related to a disease in the human host.

Altogether, more efficient and advanced genome editing tools need to be developed for a wider range of LAB. This includes making more strains genetically accessible for transformation - preferably via electroporation, allowing for high-throughput methods - and establishing recombineering and CRISPR-Cas-based counter-selection methods for these strains to enable multiplex genome editing.

*GMO vs. non-GMO*

Regulations surrounding GMOs are complex and consumer acceptance plays an important role in the reluctance to use GMOs, especially in food. In the EU, GMOs are not allowed in the final product (*i.e.* as food, probiotics or bio- and phytotherapeutics), but are allowed as contained production hosts (*i.e.* as production host for chemicals, fuels and enzymes in which the organism remains within a factory/reactor) (Johansen 2018). Even if the microorganism does not end up in the final product but is used to produce food ingredients (*e.g.* enzymes), consumer acceptance plays a significant role and it is often important for food-grade enzyme production companies to be able to sell their enzymes as completely GMO-free (Derkx 2014). Hence, even contained microorganisms in such cases should be non-GMO.

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For these reasons, many tools for LAB have focused on using systems that are non-GMO according to current legislation. Next to strains created via random mutagenesis or laboratory evolution, the current EU legislation considers strains generated by natural gene transfer methods such as conjugation and transduction as non-GMO (provided none of the involved strains is a GMO) (Sybesma 2006; Johansen 2017). "Non-GMO" for contained use also includes so-called "self-cloning", which means the modification of a strain with DNA that is taken from the strain itself or from a very closely related strain and may involve the use of recombinant vectors as long as these consist of DNA from this same or closely related strain (Meacher 2000; Verstrepen, Chambers and Pretorius 2006; Landete 2017). Because of this, there is a focus on 'food-grade' vectors for LAB to meet these criteria. By definition, this also means that clean deletion mutants created with such LAB-vectors are considered non-GMO (De Vos 1999). Self-cloning is only allowed for contained use and the organisms created by such methods are not allowed in the final product (Sybesma 2006; Johansen 2018), and is therefore used mainly for food ingredients.

Regarding the advanced genome editing tools (*e.g.* recombineering and CRISPR-Cas) being developed for LAB, if the tool vectors come related species related to the target strain, they could be considered as a "self-cloning" category, having also the added advantage of being clean/marker-free if using appropriate counter-selection methods (*e.g.* Cas9, Figure 2C). Targeted genomic modifications would then end up with a similar genotype as the wild-type strain, plus or minus a specific gene which could have also being edited by a classical method such as random mutagenesis (Johansen 2017). It is then questionable whether a strain obtained via random mutagenesis (currently allowed for human consumption) is safer than a strain that was obtained via targeted and clean self-cloning methods (not yet-regulated for human consumption) containing the same mutations. For that reason, there is a strong call for a more case-by-case assessment and information dissemination for public awareness, next to a need for further investigation of potential long-term effects of GMOs (Sybesma 2006; Fears and Ter Meulen 2017; Johansen 2017; Csutak and Sarbu 2018).

## Conclusions and outlook

In prospect, genome editing could be used to create tailored strains for properties on demand and reach the "ultimately" desired LAB strain for any given application. For some cases, such as for production platforms, that is the currently followed direction. However, for more traditional

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applications related to human consumption, this possibility is inhibited by regulations and consumer opinion. Nevertheless, genome editing tools can be applied for advancing the strains in a more indirect way as a research tool, by improving our knowledge on the strain itself and its relations with its hosts, as well as guiding to better targets for modifications using more "natural" editing methods. Those "natural" methods do not need a GMO label and will be better accepted by the general public. As the field progresses, more examples of genome editing success stories demonstrating advantages on precision, efficiency, innovation and time-saving will emerge. If those are paired with demonstrable safety tests for human consumption and good communication with the general public, the possibility to make LAB strains by design might become a reality for any established or new application.

For all applications described here, whether the final strain is a GMO or not, the LAB can be considered as microbial cell factories providing function and benefit to health, food and economy, and a workflow could be applied similar to that used in the development of traditional industrial biotechnology strains such as *E. coli* and *S. cerevisiae* for green chemical production (Palsson 2015) (Figure 3). Such a systems biology-based workflow has been shown to speed up the process of cell factory development significantly by combining genome editing and synthetic biology, *in silico* prediction and models, and high-throughput methods/automation and is still being further advanced (Campbell, Xia and Nielsen 2017). To be applied to the wide variety of LAB applications described here, this workflow could be used as in a classical metabolic engineering approach, generating engineered GMO or non-GMO strains depending on the engineering method used, but also as a research tool for fundamental understanding of the strains by designing mechanistically targeted experiments with non-GMO strains as final result (Figure 1 and 3). For LAB to follow this accelerated strain development strategy, it is crucial to develop high-throughput genome editing and selection methods to build and test the strains. Also, the development of more and improved metabolic models and high-throughput screening and read-out tools for the desired phenotypes will be crucial. The latest advances in genome editing and biotechnological developments will undoubtedly provide breakthrough solutions for innovation in the wide and ever-expanding applications of LAB.

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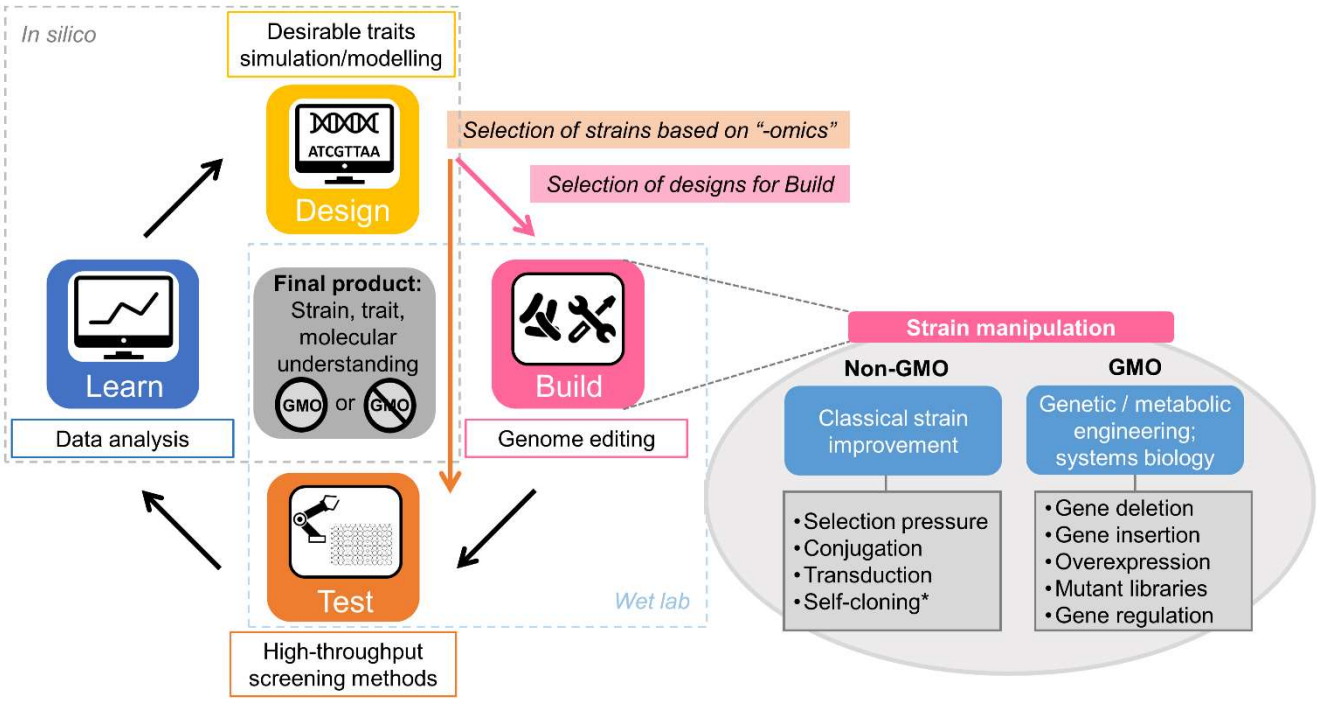


**Fig.3**

**Figure 3. Iterative Design-Build-Test-Learn workflow for cell factory development.** Proposed work flow generally applicable to all forms of cell factories discussed in this review based on systems biology for rational and advanced strain development. Adapted for LAB from the "classical" workflow described elsewhere (Palsson 2015; Campbell, Xia and Nielsen 2017). Strain manipulation can result in non-GMO strains obtained via classical gene manipulation methods, or in GMO strains via (high-throughput) genome editing tools. The GMO strains can also function as intermediate strains in the cycle as fundamental research tool, with the final strain being non-GMO selected/constructed via classical methods based on the knowledge gained through the intermediate GMO strains. *In the EU, self-cloning is allowed for contained use, but not for non-contained applications such as food and probiotics.

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**Conflicts of interest**

The authors declare to have no conflicts of interest.

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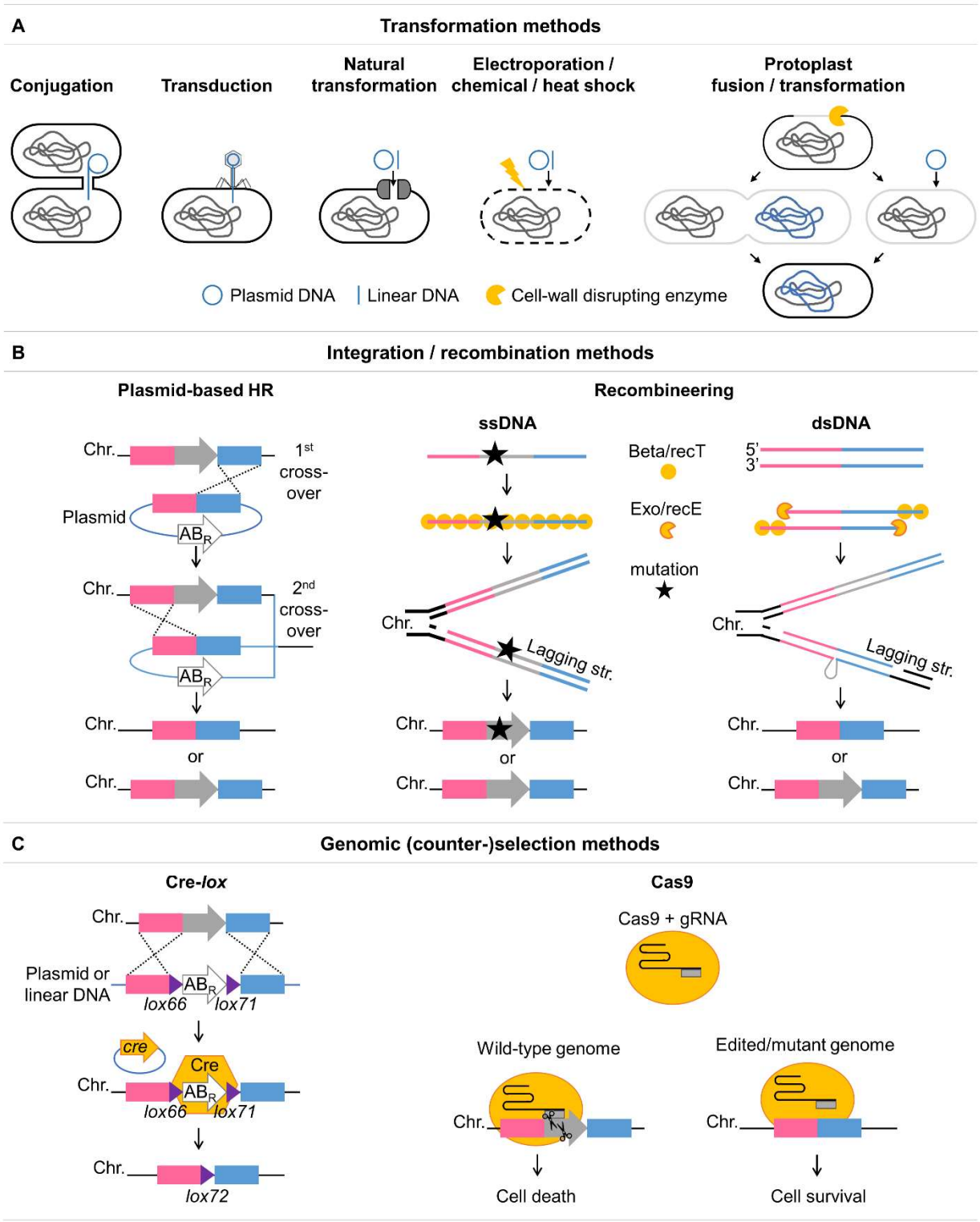


**Fig.2**

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**Figure 2. Schematic overview of transformation and genome editing methods.** Abbreviations: Chr.: chromosome; str.: strand; $AB_R$: antibiotic resistance; ssDNA: single stranded DNA; dsDNA: double stranded DNA; gRNA: guide RNA, which can be either a single guide (sgRNA) or a dual crRNA:tracrRNA. **A.** Transformation methods. For electroporation/chemical/heat shock transformation, the yellow flash indicates any of these external treatments (electrical pulse, chemical treatment or heat shock). For the protoplast-based method, the left arrow indicates protoplast fusion of two different cells and the right arrow indicates transformation of protoplasts. **B.** Integration/recombination methods, all currently reported for LAB. For plasmid-based HR, several variants are possible as further explained in the main text: non-replicating plasmids and thermosensitive plasmids can be used for the first crossover; counter-selectable markers such as *oroP* can be added to the plasmid for the second crossover and plasmid curing. A marker can be introduced within the homologous regions but this does not result in clean mutations. Without marker insertion (as depicted here), the result can be either wild-type or mutant, which need to be verified by PCR. **C.** Genomic (counter-) selection methods reported for LAB to select for mutants via marker insertion and removal (in the case of Cre-*lox*), or against wild-types (in the case of Cas9). Both methods could be used in combination with any of the integration methods shown in B. The *cre* gene is usually transformed into the cells in a second transformation round after *lox-cat-lox* insertion. Cas9 and the gRNA are usually also introduced on a plasmid, which can be on the same plasmid as the homologous flanks when Cas9-expression is induced, or on separate plasmids in separate transformation rounds. Alternatively, Cas9 could be integrated and expressed from the genome (or a native CRISPR-Cas-system can perform this function), and the gRNA is expressed from a plasmid.

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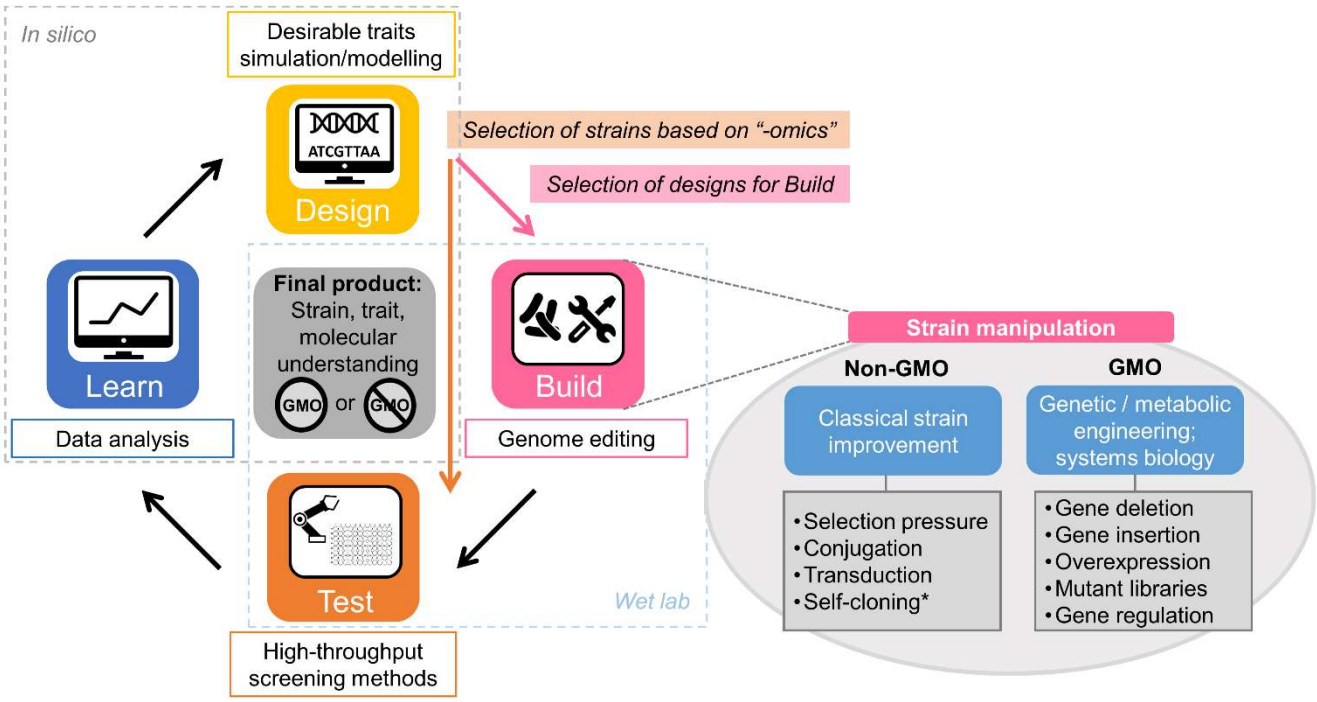


**Fig.3**

**Figure 3. Iterative Design-Build-Test-Learn workflow for cell factory development.** Proposed work flow generally applicable to all forms of cell factories discussed in this review based on systems biology for rational and advanced strain development. Adapted for LAB from the "classical" workflow described elsewhere (Palsson 2015; Campbell, Xia and Nielsen 2017). Strain manipulation can result in non-GMO strains obtained via classical gene manipulation methods, or in GMO strains via (high-throughput) genome editing tools. The GMO strains can also function as intermediate strains in the cycle as fundamental research tool, with the final strain being non-GMO selected/constructed via classical methods based on the knowledge gained through the intermediate GMO strains. *In the EU, self-cloning is allowed for contained use, but not for non-contained applications such as food and probiotics.