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Genome Editing of Food-Grade Lactobacilli To Develop Therapeutic Probiotics

JAN-PETER VAN PIJKEREN¹ and RODOLPHE BARRANGOU²

¹Department of Food Science, University of Wisconsin-Madison, Madison, WI 53706

²Department of Food, Bioprocessing, and Nutrition Sciences, North Carolina State University, Raleigh, NC 27695

INTRODUCTION

For thousands of years, lactic acid bacteria (LAB) have been interwoven with our food supply. The earliest evidence of milk use dates back to 7,000 years BCE (1), while cheeses were produced as early as 6,000 years BCE (2). Advances in our understanding of the microbial world in the past couple of centuries have enabled microbiology-based food manufacturing on an industrial scale. Not only are LAB used to ferment dairy products, but they are also applied to pickle vegetables, to cure meats, and to produce alcoholic beverages such as wine and sake (3, 4). This long history of safe consumption led to the consideration that many LAB strains are Generally Recognized As Safe (GRAS). As early as 1906, LAB were linked to the promotion of human health. The Russian Nobel laureate Élie Metchnikoff hypothesized that ingestion of yogurt prolonged life in Eastern European populations by reducing “putrefying” [sic] bacteria. His linkage of the perceived longevity of the Eastern European populations with consumption of fermented dairy products (5) made him the grandfather of modern probiotics. Probiotics are defined as “live microorganisms, which when administered in adequate amounts, confer a health benefit to the host” (6). Metchnikoff’s probiotic theory of life prolongation was never directly tested, and researchers reported in 1924 that LAB present in yogurt, specifically *Lactobacillus bulgaricus*, most likely do not reduce “putrifying” bacteria in the intestine because *L. bulgaricus* did not survive gastrointestinal (GI) transit (7). Other groups challenged this finding. Elli et al. demonstrated that both *Streptococcus thermophilus* and *L. bulgaricus* were present in human feces after yogurt ingestion (8).

Regardless, Metchnikoff’s theory was born that bacteria could be health-promoting through modifying the composition of the bacterial population that inhabits our intestine. In 2017, this translates into the use of (tailored) probiotics to modify the gut microbiota to promote human health. Today we can engineer LAB in general, and recent advances have made it possible to engineer select probiotic strains in a high-throughput manner. Also, we have an increased appreciation and understanding of the role of the gut microbiota in health and disease. Thus, the contemporary application of tailored probiotics to promote human health

brings Metchnikoff's theory a step closer to reality. In this article, we discuss emerging applications of LAB to promote human health. Specifically, we focus on lactobacilli as tailored probiotics. We will highlight the potential of clustered regularly interspaced short palindromic repeats (CRISPRs) and the CRISPR-associated enzyme (Cas) as a broadly applicable molecular tool to engineer probiotics. Also, we will discuss the potential of engineered probiotics to deliver engineered bacteriophages harnessing user-defined CRISPRs to selectively kill target pathogens. Such approaches open new avenues to modulate the microbiome composition and function and to alter microbial populations by both the removal of select pathogens and the addition of probiotic bacteria.

LACTOBACILLUS STRAIN SELECTION

The application of LAB as bacterial therapeutics was first demonstrated by Steidler et al. (9). The cheese bacterium *Lactococcus lactis* MG1363 was engineered to secrete murine interleukin-10 (IL-10). Oral administration of the recombinant lactic acid bacterium significantly reduced intestinal inflammation in two mouse models of disease. The authors demonstrated (i) the presence of murine IL-10 in the colon of IL10^{-/-} mice and (ii) that *de novo* synthesis of murine IL-10 by recombinant *L. lactis* during GI transit resulted in amelioration of intestinal inflammation. Subsequently, the *L. lactis* workhorse has been exploited to deliver a variety of recombinant proteins (10, 11) and DNA (12–14) and has paved the way to harness other microbes as delivery vehicles. As these studies clearly demonstrate that *L. lactis* can be a successful delivery vehicle, what rationale supports the development of a therapeutic delivery platform using a microbe other than *L. lactis*? One argument arises from the fact that *L. lactis* is a microbe that originates from a plant environment and has adapted to thrive in milk, not in humans (15). Because the optimal growth temperature of *L. lactis* is 30°C, some researchers may choose to select a strain that thrives at 37°C. A higher *in vivo* metabolic rate is predicted to yield increased therapeutic protein delivery, which may be desirable. Also, the efficiency of survival following GI transit can be a selection criterion. Several strains have been identified, including some lactobacilli, that better survive the conditions encountered during GI transit compared to *L. lactis* (16, 17).

Selection of strains that thrive at 37°C and can withstand the harsh *in vivo* conditions can thus be a key criterion. In addition, some research groups have exploited natural health-promoting strains, such as *Escherichia coli* Nissle 1917. Although not within the focus of this chapter, *E. coli* Nissle 1917 is notable as the only probiotic *E. coli* in use for over a century and is now being developed as a therapeutic delivery platform (18–20). A clear advantage is that researchers can tap into the most comprehensive genetic toolbox available for bacteria. *E. coli* Nissle 1917 is one of the few Gram-negative probiotics, in contrast to the plethora of Gram-positive probiotics that are widely represented by lactobacilli. While lactobacilli are being exploited as delivery vehicles, strain selection will be key for successful design of tailored probiotics.

Leveraging *Lactobacillus*'s Genetic Diversity for Strain Selection

Lactobacillus is an extraordinary, diverse genus that contains over 200 distinct species and is the largest genus within the LAB (21). Lactobacilli are found in a wide variety of environmental niches, including plants and animals, and several species are commonly found in the GI tract of mammals (22). Many strains can withstand the harsh conditions encountered during GI transit, i.e., stomach acids and bile acids, which makes select undomesticated lactobacilli strains attractive delivery vehicles (23–26). It is important to emphasize the use of undomesticated strains because prolonged *in vitro* incubation will lead to adaptation to the new environment, and some traits may be lost, e.g., traits required to thrive *in vivo*. For example, *E. coli* K-12 has lost its ability to thrive *in vivo* because it has been cultured for nearly a century under laboratory conditions (27, 28). Analogously, a *Lactobacillus* strain that was isolated from the intestine 50 years ago and cultured *in vitro* since, may therefore not be the best choice for use as an *in vivo* delivery vehicle.

This broad genetic diversity, even between strains of the same species, also contributes to variation in the efficiency of genome editing. The van Pijkeren laboratory demonstrated that different *Lactobacillus reuteri* strains yield transformation efficiencies that differ as much as 100-fold (unpublished data). Also, single-stranded DNA recombineering (SSDR) procedures, optimized for *L. reuteri* ATCC PTA 6475, have not yielded recombinants in *L. reuteri* DSM 17938 (unpublished data). The lack of recombinants in *L. reuteri* DSM 17938 is not linked to differences in transformation efficiencies. Therefore, the intraspecies variation in *L. reuteri* is enough to cause major differences in the genome editing ability.

That strain-to-strain differences impact the ability to engineer bacterial genomes is not unique to lactobacilli. For example, “wild” isolates from *E. coli* were found to be more resistant to genome editing than *E. coli* K-12 (29). Thus, differences between strains are obvious and need to be taken into consideration when developing a strain as a therapeutic delivery vehicle.

Probiotic Features: a Closer Look at Strain-Level Differences

One common misconception is that all members of the genus *Lactobacillus*, or a particular *Lactobacillus* species, are probiotic. Probiotic characteristics are typically strain-dependent. A major contributing factor is the aforementioned extraordinary genetic diversity, which is also prevalent at the intra-species level. Comparative and functional genomics have revealed several examples that demonstrate probiotic strain specificity. The two examples that we highlight here are *Lactobacillus salivarius* and *L. reuteri*.

L. salivarius UCC118 is a candidate pro-biotic microbe for which several probiotic features have been validated by *in vivo* animal trials, including anti-inflammatory properties and production of an antimicrobial peptide (30–35). A screen among 88 lactobacilli, which included 35 *L. salivarius* strains, revealed that the isolate *L. salivarius* CCUG 47825 bound fibrinogen at levels comparable to the Gram-positive pathogen *Staphylococcus aureus* (36). This is an undesirable characteristic because the interaction between a fibrinogen-binding protein and blood platelets can lead to platelet aggregation (37). Although strain UCC118 lacks the gene encoding the fibrinogen-binding protein, this gene is present but not

expressed in several *L. salivarius* strains. Thus, selection of a candidate delivery strain requires genome-mining strategies to eliminate strains encoding potential undesirable gene products. In addition, not all strains of the same species share probiotic characteristics. For example, the antimicrobial product reuterin and the anti-inflammatory product histamine, the latter coded by the *hdc* gene cluster, are mostly specific to a single clade of *L. reuteri* strains (38–41). In addition to proper strain selection, availability or development of a genetic toolbox will be key to developing tailored probiotics.

LACTOBACILLUS GENOME EDITING

One approach to enhance health-promoting properties is the ability to transform cells with exogenous DNA to genetically modify and engineer their genome. Depending on the type of application and the organism of choice, such engineering can be the first hurdle in the development of a LAB therapeutic. Several tools have been developed, but their application can be strain-dependent. Our discussion will not cover genetic engineering tools comprehensively, but we will briefly highlight some tools that have been developed and applied to lactobacilli. These include Cre-lox (42), bacteriophage integrases (43), introns (44), and two-plasmid integration systems (45, 46). To create DNA insertions or deletions following Campbell-like integration, the *upp*-encoded uracil phosphoribosyltransferase can be used in some strains to identify cells in which the second homologous recombination event occurred (47). Such tools have been very valuable to the LAB research community, but high-throughput engineering approaches to make subtle mutations in a LAB genome have been limited until more recently.

The Britton laboratory developed SSDR in *L. lactis*, *L. reuteri*, and *Lactobacillus plantarum* (48). The procedure was optimized for use in *L. reuteri* and *L. lactis*, while proof of concept was demonstrated in *L. plantarum*. SSDR allows the user to create single-nucleotide changes in the chromosome without the need for antibiotic selection. Only an oligonucleotide is needed to generate the desired mutation(s) in the chromosome, and no cloning is required. SSDR can also be applied to make small deletions (<250 bp) in the chromosome which also do not require selection. The design and application of SSDR were recently reviewed by the same authors (49). The van Pijkeren laboratory combined SSDR with CRISPR-Cas selection in *L. reuteri* (50). CRISPR-Cas further advanced the applicability of SSDR. Low-efficiency mutants are enriched in the population following CRISPR-Cas-mediated killing of the wild-type cells. Now, mutant genotypes can be identified that previously were not possible. For example, an oligonucleotide can be used to generate 1-kb deletions or to perform targeted codon mutagenesis.

The next section will discuss CRISPR and the CRISPR-Cas protein (51) because the CRISPR-Cas technology has driven the genome editing revolution. Indeed, we believe that LAB, and lactobacilli in particular, have an untapped potential to exploit CRISPR-Cas for genetic engineering purposes (21).

CRISPR-Cas Systems

CRISPR-Cas constitutes the adaptive immune system of bacteria to protect against invasive elements such as bacteriophages and plasmids (52). Entry of the foreign DNA into the

bacterial cell results in incorporation of small DNA fragments into the CRISPR locus as novel spacers integrated between CRISPR repeats (52). The CRISPR locus is transcribed and processed, yielding small CRISPR RNAs (crRNAs) that guide Cas effector nuclease toward complementary target sequences (53, 54). Subsequent exposure to foreign DNA for which the cell has a complementary crRNA results in the formation of a DNA-RNA duplex in a sequence-specific manner. The crRNA thus guides Cas nucleases to cleave the foreign double-stranded DNA, yielding sequence-specific targeting (55, 56). Next, the invasive DNA is degraded by host nucleases, which interrupts and prevents replication of the targeted DNA (57).

There are two classes and six main types of CRISPR-Cas systems, which both provide DNA-encoded and RNA-mediated targeting of nucleic acid in a sequence-specific manner (51) (Fig. 1). Of all CRISPR-Cas systems, the type II system is the most streamlined. Type II systems hinge upon only four *cas* genes, namely *cas1*, *cas2*, *cas9*, and *csn2/cas4*, and a dual crRNA:tracrRNA (58) complex that guides the Cas9 nuclease to generate a double-stranded break for sequence-specific cleavage of DNA (55, 56). In eukaryotes, upon genesis of a double-stranded break, the endogenous DNA repair machinery patches back the two cleaved DNA ends using non-homologous end-joining or replaces the native sequence by homology-directed repair using either a similar sequence within the genome (either another allele or a homologous sequence) or a provided template. Non-homologous end-joining-based repair typically yields random single nucleotide polymorphisms and short indels, whereas homology-directed repair enables precise modification of DNA sequences. Commonly, the sequence altered by the repair machinery is construed as an “edited” version of the wild-type sequence—hence the CRISPR-enabled genome editing craze leading to its widespread use in genetic engineering (59).

Since the first proof of concept in 2013 that the human genome can be edited using Cas9, a plethora of CRISPR-based technologies have been developed to alter the genome, the transcriptome, and the epigenome (60). Scientists around the globe implemented CRISPR-Cas in different scientific fields of study, with applications for bacteria, archaea, eukarya, and even viruses. In addition to the broad adoption of these technologies in plants and mammals, the microbiology community has started to harness these CRISPR-based tools for organisms that are widely used in medicine, biotechnology, and agriculture (61, 62). We believe there is potential for the implementation of that technology in GRAS LAB for the development of engineered biotherapeutic agents (63).

The CRISPR literature is too extensive to discuss in depth. Therefore, we suggest a few of the many reviews that discuss the biology and genetics of CRISPR-Cas systems (64, 65), as well as CRISPR-based technologies and their applications in genome editing and beyond (66, 67). Here, our intent is to focus on microbial applications in general, and specifically on genome editing for enhanced health-promoting abilities.

CRISPR-Cas Genome Editing in *Lactobacilli*

After CRISPR-Cas was discovered in 2007 (52), different groups started to use it in 2013 as a genome editing machine (61, 62). At the time of writing this review, CRISPR-Cas-based genetic engineering in *Lactobacilli* has only been applied in *L. reuteri* (50). A recent

extensive comparative genomics study revealed that CRISPR-Cas systems are widely distributed in the genus *Lactobacillus*. The authors of this work suggested that this may be due to phage predation, horizontal gene transfer, and the extensive genome remodeling, which collectively contribute to this genus's genetic diversity (21, 68, 69). Whereas CRISPR-Cas systems are present in approximately 46% of the total bacterial genomes present in CRISPR databases, the genus *Lactobacillus* encodes CRISPR-Cas systems in nearly 63% of the sequenced genomes (21). The wealth of CRISPR-Cas systems in lactobacilli provides researchers with the opportunity to use native Cas enzymes, combined with user-defined CRISPR arrays, to select for (low-efficiency) recombinant genotypes (70). This naturally encoded resource expands the genetic engineering potential of a group of organisms that has been historically challenging to engineer, including probiotic strains. We expect that the CRISPR toolbox, combined with technologies such as SSDR, will simplify and accelerate development of next-generation probiotics (50, 71).

LACTOBACILLI AS THE CHASSIS FOR TAILORED PROBIOTICS

Editing Native Genes To Alter the Probiotic Immune-Modulatory Profile

One approach to enhancing probiotic properties is to modify native genes that contribute to immunomodulation. Although an overall mechanistic understanding of probiotic features is still mostly lacking, the bacterial outer cell surface plays key roles in probiotic-host interactions (72). The outer cell surface of Gram-positive bacteria consists of a thick peptidoglycan layer that contains polymers of phosphate-alditol groups in addition to proteins and polysaccharides. The polymers of phosphate-alditol groups are also known as teichoic acids (TAs). TAs can be linked to the peptidoglycan (cell wall TAs) or to the membrane via glycolipids (lipoteichoic acids [LTAs]) (73, 74). The presence of TAs seems to be conserved in lactobacilli, though some species, including *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, and *L. reuteri*, do not produce cell wall TAs (75). The established role of TAs in immunomodulation offers a potential target for improving probiotic efficiency. For example, TAs act on mammalian Toll-like receptors that stimulate dendritic cells and subsequently lead to cytokine responses (76, 77). Also, TAs can impact the adhesion of bacteria to host cells (78). Thus, alteration of such cell wall components by genome engineering approaches could further enhance the probiotic profile of a strain. Whereas purified LTAs of *L. plantarum* WCFS1 elicited a proinflammatory response, integration of a suicide knockout vector in the gene responsible for D-alanylation of TAs shifted the immunomodulatory profile to anti-inflammatory. Moreover, the genetically modified strain provided enhanced protection in a murine colitis model (79). This elegant work and that of others (80–83) not only shed light on *Lactobacillus*-mediated immunomodulation, but also provides an exciting opportunity to explore this pathway to enhance probiotic features.

Chronic inflammation in the colon can lead to development of colon cancer. Engineered probiotics with an improved anti-inflammatory profile may be useful to prevent colon cancer. An example is a derivative of *Lactobacillus acidophilus* NCFM, which did not produce LTAs following deletion of the gene encoding a phosphoglycerol transferase (84). This mutant was generated by a two-plasmid system (45), which is an adaptation from the

pVE6007/pORI19 system (85). Mice prone to developing polyps in the colon due to a mutation in the adenomatous polyposis coli (*Apc*) gene developed significantly fewer polyps in the colon and ileum when administered the engineered probiotic lacking LTAs compared to the probiotic wild-type strain. The authors demonstrated that the wild-type and recombinant probiotic differentially modulated T-regulatory cells, which are known to play a fundamental role in the development of cancer.

In addition to modification of TAs, lacto-bacilli surface proteins—including cell wall-anchored proteins—also play a key role in immunomodulation; this topic has been extensively reviewed by others (86–88).

Increased understanding of the cell wall architecture of select probiotics will provide us with novel opportunities to tailor its structure for enhanced immune-modulatory properties. Basic human intervention studies with these tailored probiotics are much needed before we can fully embrace the potential of such engineered probiotics. Elegant gnotobiotic mouse models are available to characterize the interplay among (tailored) probiotics, the gut microbiota (89, 90), and the murine immune system. However, the very simple question remains: can a strong anti-inflammatory immune response observed in mice, after exposure to a human-derived tailored probiotic, be replicated in human subjects exposed to the same tailored probiotic?

Lactobacilli as Antimicrobial Production Factories To Alter the Microbiota

For decades, LAB have been exploited as microbial production factories for a wide array of proteins and metabolites (91–95). For *in vivo* applications, lactobacilli can be attractive vehicles to transport and deliver select molecules in the GI tract; however, strains need to be selected carefully. First, a strain must be able to survive passage through the GI tract. Second, one needs to be able to engineer the genome. Lactobacilli with these characteristics may function as robust mother-ships to transport and deliver therapeutics, including select antimicrobial molecules, in the GI tract. In this section we will describe the potential of lactobacilli as delivery vehicles of antimicrobial compounds to selectively alter the composition of the GI microbial community. Such lactobacilli-mediated therapies may serve as complementary or even alternative strategies to antibiotic treatment to combat antibiotic resistance, a major health threat in 21st century medicine.

Increased understanding of both the pathogenic nature of select microbes and the fundamental role of the GI microbiota in maintaining or promoting human health (96) has created a conundrum in health care. As of today, antibiotics are the primary line of treatment to eradicate bacterial pathogens. Yet application of broad-spectrum antibiotics typically results in major perturbations of the GI microbiota (97). These perturbations, especially in early life, have been correlated with long-lasting metabolic changes leading to undesirable outcomes such as obesity and allergic asthma (98, 99). Immunocompromised and elderly individuals are at increased risk of developing antibiotic-associated colitis and diarrhea (100). Also, historical overuse of antibiotics in both agriculture and health care has selected for microbes with antibiotic resistance (101). Efforts to identify novel antimicrobials, in both industrial and academic settings, have been mostly futile. A major hurdle in the identification and development of novel antimicrobials is the requirement of the molecule to

effectively penetrate the bacterial cell wall for subsequent activity (102). As an alternative to the development of novel antimicrobials, expanding the natural probiotic potential with native, or bacteriophage-derived, antimicrobials may offer a supplemental or alternative strategy to antibiotics. Next, we will discuss the application of probiotic-derived natural antimicrobials and bacteriophage-derived endolysins, i.e., “enzybiotics” to antimicrobial treatments (103).

Enhancing Native Antimicrobial Activity

LAB are well known for their extensive heterogenic repertoire of antimicrobial compounds, including bacteriocins (104). Bacteriocins are small ribosomally synthesized peptides that can inhibit or kill other bacteria. The functional diversity of this family of antimicrobials is large, as illustrated by the fact that bacteriocins can collectively target a wide array of Gram-negative and Gram-positive bacteria (105). Although several bacteriocin-containing products have been developed for commercial purposes, clinicians have not yet widely explored the application of bacteriocins to target pathogens.

Although narrow-spectrum bacteriocins would be preferred, broad-spectrum bacteriocins may be useful to alleviate a bacterial infection of unknown source. Bacteriocin-mediated impact on the gut microbiota composition can be substantial, as demonstrated by Abp118, a broad-spectrum bacteriocin produced by *L. salivarius* UCC118 (106). The microbiota of mice and pigs were compared between groups that were administered *L. salivarius* wild-type or *L. salivarius* *abp118*. The authors confirmed that the presence of the bacteriocin-producing lactobacilli alters the gut microbiota composition; however, there were no significant changes in the microbial diversity. Nevertheless, this study and that of others (107) demonstrated that a bacteriocin-producing probiotic can eradicate select members of the gut microbiota, providing a rationale to engineer bacteriocins for enhanced efficacy. One example is the most extensively characterized bacteriocin, nisin, which is produced by select *L. lactis* strains and streptococci. Here, we will focus on NisA, one of the six natural nisin variants. Members of the Hill laboratory subjected *nisA* to site-directed and saturation mutagenesis, and recovered mutants with enhanced activity against Gram-positive and Gram-negative pathogens (108, 109). These examples clearly demonstrate the value of “classical” genetic approaches for generating probiotics with improved function. The above-described methodology was based on modification of a plasmid-encoded nisin by PCR. Recent technological developments now enable codon saturation mutagenesis in the chromosome of select lactobacilli. For example, the van Pijkeren laboratory combined SSDR with CRISPR-Cas selection in *L. reuteri* to perform one-step codon saturation mutagenesis (50). A single transformation of an oligonucleotide containing the NNK motif (N= A/T/G/C and K= G/T) yielded a pool of recombinants in which a single codon was modified to encode all 20 amino acids. These approaches could be multiplexed to accelerate the discovery of probiotics with enhanced antimicrobial activity.

As an alternative to codon mutagenesis to identify novel antimicrobial variants, genetic engineering approaches can also be applied to improve the production of the antimicrobial. Improved production was previously achieved for reuterin, also known as 3-hydroxypropionaldehyde (3-HPA) (110). Select *L. reuteri* strains produce reuterin as an

intermediate during glycerol fermentation to yield 1,3-propanediol (111). Reuterin has broad-spectrum activity (38, 112, 113). The gene cluster responsible for 1,3-propanediol production (and thus reuterin) is the propanediol utilization (*pdu*) operon. By SSDR, six bases were modified in the promoter region driving expression of the *pdu* operon. The recombinant strain produced more reuterin (114), resulting in 3-fold-increased killing efficacy of *E. coli* compared to the wild-type strain (110). Also, deletion of the gene encoding 1,3-propanediol reductase, which is responsible for the conversion of reuterin to 1,3-propanediol, yielded approximately 4-fold more reuterin compared to the wild-type (115). Therefore, a double mutant derivative in which increased expression of the *pdu* operon is combined with deletion of the 1,3-propanediol reductase gene has the potential to yield a tailored probiotic with superior *in vivo* killing activity. As with any mutation that is either naturally acquired or engineered, it remains to be seen if the new genotype impacts *in vivo* fitness.

Enzybiotics

Bacteriophages are known to be the most ubiquitous form of life on planet Earth. In the GI tract alone, $\sim 10^{15}$ bacteriophages are predicted to be present. Except for filamentous bacteriophages, which the host excretes (116, 117), other bacteriophages require host lysis to release progeny phage. Two proteins play a key role in degrading the bacterial cell wall: holins and lysins (118). During bacteriophage replication, biologically active lysins are present in the cytosol but require expression of a membrane protein, holin, to release the virions from the cell. When holin levels are optimal, the lysin can access the peptidoglycan layer for cleavage that leads to bacterial cell lysis (119). So far, five main groups of lysins have been identified that can be distinguished from one another based on their cleavage specificity for the different bonds within the peptidoglycan (Fig. 2) (120). Structurally, lysins can consist of a single catalytic domain, which is typical for lysins derived from bacteriophages targeting Gram-negative bacteria (121). Bacteriophages targeting Gram-positive bacteria typically encode lysins that contain multiple domains: an N-terminal catalytic domain and a C-terminal cell wall-binding domain (122, 123). Few lysins have been identified with three domains (124).

Since their discovery over a century ago, bacteriophages have been exploited to kill select microbes via their production of holins and/or endolysins. Historically, bacteriophage therapy was mainly focused in Eastern Europe, whereas the wide application of antibiotics was preferred in the United States (125). The advantages of chemically synthesized molecules over biologically manufactured viruses include ease and consistency. However, the emerging and rapidly expanding threat of antibiotic resistance has led to a revival of the use of bacteriophages in therapy, though clinical success—specifically to target GI microbes—has so far been limited. Successful application of bacteriophage therapy to target microbes in the gut requires the bacteriophage to maintain biological activity following exposure to stomach acids and bile acids, which has proven to be problematic in general (126).

One approach that may hold promise is to use lactobacilli to deliver bacteriophage lysins *in situ* to kill the bacterial target. The basis for this approach is that (i) many lactobacilli are able to efficiently survive passage through the GI tract (23–26), (ii) the cell wall-binding

domain determines a narrow host range (127, 128), which makes them suitable for heterologous expression, and (iii) various *in vitro* and *in vivo* studies have demonstrated that heterologously expressed lysins can kill select Gram-positive bacteria (129–132). Some studies have reported that lysins can also be effective against Gram-negative bacteria despite the presence of an outer cell membrane (133, 134). Various databases provide a valuable resource to select lysins for heterologous expression. For example, EnzyBase2 contains over 2,000 enzybiotics encompassing 1,800 enzybiotics derived from natural resources (bacteriophages) and approximately 200 synthetic enzybiotics (135). Also, the PHAST (PHAge Search Tool) database provides a wealth of information on bacteriophage genomes, both complete and remnant, that allows users to search for bacteriophage-derived endolysins for heterologous expression (136). Though theoretically promising, these resources have not been fully explored.

The application of enzybiotics may be suitable for *Clostridium difficile* infections. *C. difficile* is the leading cause of nosocomial antibiotic-associated diarrhea, and a major contributing factor to *C. difficile* disease is antibiotics. Antibiotics cause a significant disruption of patients' GI microbiota, which allows *C. difficile* to quickly expand and results in toxin-induced diarrhea, among other clinical manifestations (137). In up to 30% of people previously treated for a *C. difficile* infection, every future antibiotic treatment will trigger another episode of *C. difficile* expansion, mostly instigated by hypervirulent strains. The emerging hypervirulent phenotype (138), identification of strains that are resistant to fluoroquinolones (139), and the realistic prospect that acquired antibiotic resistance will go beyond the documented fluoroquinolone resistance (140, 141) collectively create the potential for a *C. difficile* epidemic. A highly successful remedy for recurrent *C. difficile* infections is a fecal microbial transplant. Although FMT has a success rate of ~90%, it has become evident in the last decade that a variety of diseases are linked to differences in the gut microbiota composition (142), including obesity (143, 144), and widespread fecal microbial transplant application should be approached with caution. This is supported by a recent report describing the case of a woman who received a fecal microbial transplant and subsequently rapidly gained 37 pounds and became obese (145). Thus, more subtle and specific approaches to treating *C. difficile* are much needed. Therefore, approaches to eradicate *C. difficile* using approaches that do not disturb the microbiota, such as *in situ* delivery of enzybiotics, have the potential to become alternative treatment strategies. Successful expression of the *C. difficile* enzybiotic CD27L has been demonstrated in *L. lactis* (146), but proof of concept in mouse models of *C. difficile* infection is thus far lacking.

CRISPR-Based Antimicrobials

Though the biological purpose of CRISPR-Cas is to serve as an immune system against invasive nucleic acids in bacteria and archaea, they can be repurposed for other applications, including as programmable antimicrobials (147) (Fig. 3). Indeed, what renders CRISPR machines desirable for genome editing in eukaryotes makes them lethal antimicrobials in prokaryotes (148). The rationale for CRISPR-Cas lethality in bacteria is that many bacteria lack (efficient) nonhomologous end-joining systems (149). Therefore, self-targeting CRISPR spacers are highly lethal and are selected against during accidental acquisition of

spacers from the host chromosome (150). This is a tremendous opportunity for the repurposing of endogenous or exogenous CRISPR-Cas systems for self-targeting in bacteria, as programmable and specific antimicrobials (147). Proof of concept has been generated *in vivo* and *in vitro*, using both type I and type II CRISPR-Cas systems and harnessing both native and heterologous Cas nucleases (147, 151, 152).

However, the primary challenge is *in situ* delivery, especially in the GI tract. We envision that there is a significant potential for using engineered probiotic bacteria as “Trojan horses” for the local delivery of engineered bacteriophage that carry a CRISPR-cassette for self-targeting in pathogenic bacteria. One approach would be to engineer a hybrid between a plasmid and a bacteriophage to yield a phasmid for heterogenic bacteriophage production in the pro-biotic bacteria (Fig. 4). The generation of a phasmid was demonstrated 3 decades ago by fusion of the *E. coli* bacteriophage P2 with plasmid pBR322 (153). Replication of the phasmid could be established from either the plasmid replication proteins or from the bacteriophage replication proteins. Functional virions were produced by both replication methods. With the development of high-throughput assembling technologies, such as Gibson assembly (154), building synthetic DNA fragments such as phasmids, containing double-stranded DNA bacteriophage DNA, is a realistic approach. The CRISPR array, specific for the pathogen to be targeted, can be embedded in the bacteriophage genome for packaging, analogous to what has been described previously (151, 152). Once the phasmid is established in the probiotic, virions are produced in the cytosol. To release the virions *in situ*, a promoter that is activated upon exposure to an *in vivo* environmental cue (i.e., bile) can be fused to a probiotic-specific holin to lyse the engineered probiotic (155). Not only does efficient lysis result in therapeutic delivery, but it is also key to establishing biological containment. The released virions inject the DNA in the target pathogen to deliver the CRISPR array, which yields strain-specific killing when combined with the native Cas proteins. An advantage of the CRISPR approach is that CRISPR-Cas kills bacteria without lysis, which limits the release of toxins upon killing.

Without a doubt, the production of biologically active virions, aside from the potential hurdle to establish a large phasmid in the *Lactobacillus* cell, will be challenging, yet its potential to target pathogens in a strain-specific manner is unprecedented. Our vision is that the technological advances that have been made in the past decade, especially in the field of synthetic biology, combined with a growing research community studying lactobacilli, will inevitably lead to engineered probiotics that can modify the gut microbiota in a strain-specific manner.

FUTURE PERSPECTIVES

Overall, the examples we have provided illustrate how food-grade bacteria in general, and probiotic lactobacilli in particular, can be used to promote human health. As long as the scientific community continues to expand the genetic toolboxes for LAB, we envision that LAB will provide great potential to modulate the microbiota. In particular, the application of CRISPR-based technologies has the potential to eradicate target microbes in a strain-specific manner.

Advancing this field must include considerations of the ancillary forces and dimensions that drive and enable technological advances, such as intellectual property, regulatory processes, and consumer acceptance. The ongoing CRISPR intellectual property battles are somewhat hindering the adoption of this technology for food, agricultural, and clinical applications, given the lack of clarity around freedom to operate. Furthermore, regulatory approvals are still pending in some cases, and regulatory processes are unclear and/or yet to be defined in others. Lastly, consumer acceptance of genetically modified organisms remains a challenge that extends beyond the scientific dialogue, and much progress remains to be achieved. Nonetheless, the momentum of the ongoing microbiology renaissance remains strong, fueled by CRISPR technological advances and our increasing awareness of the microbiome.

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FIGURE 1.

CRISPR-Cas systems. Two primary classes of CRISPR-Cas systems have been established, based on the nature of the effector proteins that direct targeting: either multisubunit complexes (class 1) or single effector proteins (class 2). Each major type of effector protein drives select cleavage of target nucleic acid, generating single-strand exonucleolytic cleavage (type I), shredding (type III), unknown (type IV), blunt cleavage (types II and VI), or sticky-end dual nicking (type V).

FIGURE 2.

Endolysin target sites within the Gram-positive peptidoglycan matrix. A simplified overview of the peptidoglycan matrix in which the target sites of the five bacteriophage-derived endolysins are indicated with green arrows. The arrows refer to the following endolysin types: (1) muramidase, also referred to as lysozyme, (2) glucosaminidase, (3) amidase, (4) γ -endopeptidase, and (5) endopeptidase. The figure is adapted from reference 120.

FIGURE 3.

Repurposing CRISPR-Cas systems as antimicrobials. If endogenous CRISPR-Cas systems are natively present in the target organism (left), they can be repurposed and redirected toward self-targeting by delivering either CRISPR guide RNAs or synthetic CRISPR arrays that contain a self-targeting spacer that contains sequences homologous to those of the host chromosome. Alternatively, for organisms in which no CRISPR-Cas systems are universally present, or active (right), both the CRISPR arrays (or guide RNAs) and the Cas machinery (Cas effector nucleases such as Cascade or Cas9) can be delivered via plasmids or phages. Various types of CRISPR-Cas systems can be harnessed for lethal self-targeting (bottom), encompassing both class 1 and class 2 systems, exemplified by the type I-E system, hinging on the Cas3 exonuclease for extensive shredding of a DNA strand (bottom left), or by the type II-A system, hinging on the Cas9 endonuclease for genesis of double-stranded DNA breaks (bottom right).

FIGURE 4.

Probiotic dual-delivery system of CRISPR-coding bacteriophages. Conceptual overview of an engineered probiotic encoding phasmid-derived virions that harbor a CRISPR array to target pathogens upon release from the probiotic delivery host. Amplicons of a pathogen-derived double-stranded DNA bacteriophage are fused with a plasmid origin of replication (ORI), a probiotic auxotrophic marker, and a CRISPR cassette. The phasmid-encoded auxotrophic marker, when deleted from the bacterial chromosome, yields stable phasmid replication. The phasmid will reproduce virions, which encode engineered CRISPR arrays, in the cytosol of the cell. Release of the engineered virions can be achieved by placing a gene encoding a holin and/or endolysin protein, which is known to lyse the probiotic, under the control of a promoter that is activated upon sensing environmental cues, i.e., bile salts, in the small intestine. These already have been identified in bacteria (156), which can be adapted for use in probiotics. Successful lysis achieves both biological containment and delivery of the engineered virions *in situ*. When the virions attach to the target pathogen, DNA will be injected. Delivery of the user-defined CRISPR array will, combined with native Cas enzymes, result in strain-specific killing of the pathogen.