Exploiting Prophage-mediated Lysis for Biotherapeutic Release by *Lactobacillus reuteri*

Laura M. Alexander¹, Jee-Hwan Oh¹, Donald S. Stapleton², Kathryn L. Schueler², Mark P. Keller², Alan D. Attie², and Jan-Peter van Pijkeren¹+

¹Department of Food Science, 1605 Linden Dr, University of Wisconsin-Madison, Madison, WI, 53706, US
²Department of Biochemistry, 433 Babcock Dr, University of Wisconsin-Madison, Madison, WI 53706, US

¹⁺To whom correspondence should be addressed:

Phone: +1 608 890 2640
Fax: +1 608 262 6872
Email: vanpijkeren@wisc.edu

**KEYWORDS**: Lactobacillus, Lactobacillus reuteri, lactic acid bacteria, probiotics, biotherapeutic, delivery vehicle, prophage, bacteriophage, leptin
ABSTRACT

*Lactobacillus reuteri* has the potential to be developed as a microbial therapeutic delivery platform because of an established safety profile, health-promoting properties, and available genome editing tools. Here, we showed that *L. reuteri* VPL1014 exhibits a low mutation rate compared to other gram-positive bacteria, which we expect will contribute to the stability of genetically modified strains. VPL1014 encodes two biologically active prophages, which are induced during GI transit. We hypothesized that intracellular accumulated recombinant protein can be delivered *in situ* following bacteriophage-mediated lysis. To test this, we engineered VPL1014 to accumulate leptin, our model protein, inside the cell. *In vitro* prophage induction of recombinant VPL1014 released leptin into the extracellular milieu, which corresponded with bacteriophage production. We also employed a plasmid-system that does not require antibiotic in the growth media for plasmid maintenance. Collectively, these data provide new avenues to exploit native prophages to deliver therapeutic molecules.
IMPORTANCE

Lactic acid bacteria (LAB) have been explored as potential biotherapeutic vehicles for the past twenty years. To secrete a therapeutic in the extracellular milieu one typically relies on the bacterial secretion pathway, i.e. the Sec pathway. Overexpression of a secreted protein can overload the secretory pathway and impact the organism’s fitness, and optimization of the signal peptide is also required to maximize the efficiency of the release of mature protein. Here, we describe a previously unexplored approach to release therapeutics from the probiotic Lactobacillus reuteri. We demonstrate that an intracellular accumulated recombinant protein is released following prophage activation. Since we recently demonstrated that prophages are activated during gastrointestinal transit, we propose this method will provide a straightforward and efficient approach to deliver therapeutics in vivo.
INTRODUCTION

Lactic acid bacteria (LAB) are a diverse group of gram-positive, non-spore forming bacteria. Representative genera include Lactococcus, Streptococcus, Lactobacillus, Leuconostoc, and Pediococcus. Lactic acid is the main end-product of glucose fermentation, which in homofermentative LAB yields two molecules of lactic acid per molecule of glucose, whereas heterofermentative LAB convert glucose to a mixture of carbon dioxide, ethanol and lactic acid (1). LAB can be found in various food-related ecosystems, including plant materials and traditional fermented foods (e.g. Kimchi), and are of interest to the food industry as several LAB strains produce antimicrobial molecules, i.e. bacteriocins (2). Some of these bacteriocins are effective in killing foodborne pathogenic bacteria, including Listeria monocytogenes (3). Due to the long history of safe consumption, the U.S. Food and Drug Administration deemed many LAB strains as Generally Recognized as Safe (GRAS) (4). Safety of the probiotic itself, combined with the fact that several strains have health-promoting properties, have put LAB in the spotlight to be genetically modified as production factories of recombinant, therapeutic proteins.

Members of the genera Lactococcus and Lactobacillus are excellent hosts to produce enzymes, biofuels, prophylactics, and therapeutics (5–8). Due to the ability of LAB to survive gastrointestinal transit and interact with mucosal environments, oral or intranasal LAB-mediated delivery of vaccines and therapeutics is an attractive alternative to intravenous or intramuscular administration of antigenic molecules (9). Recombinant LAB have demonstrated efficacy in animal models for the delivery of vaccines to target Clostridium difficile (10), Helicobacter pylori (11–13) human
papillomavirus (14–16), and influenza viruses (17–21). *Lactococcus lactis*, a species commonly found in milk products, has been extensively explored as a delivery vehicle and has been engineered to produce a variety of therapeutics, including interleukin-10 (IL-10) (22, 23), leptin (24), and the HIV-1 virucide cyanovirin protein (25). Phase I clinical trials with recombinant *L. lactis* secreting IL-10 demonstrated that the treatment was safe, but no significant decrease in disease activity in patients suffering from Crohn’s disease was observed (23). While further investigation is needed to better translate success in animal models to human applications, it is evident that *L. lactis* paved the way to develop LAB as therapeutic delivery vehicles, including for *Lactobacillus reuteri*.

*Lactobacillus reuteri* is a gut symbiont species found in the intestine of various vertebrates, including humans, pigs, cattle, rodents, sheep, and birds (26–31). The organism has evolved to thrive in the intestine, and select strains exhibit probiotic features including modulation of inflammation (32–35), prevention of bone loss in menopausal females (36), and production of reuterin, an antimicrobial molecule that has activity against *Escherichia coli* O157:H7 and *Listeria monocytogenes*, for example (37, 38). Genetic tools such as single-stranded DNA recombineering (39), CRISPR-Cas genome editing (40), and a counterselection marker (41) have been developed for *L. reuteri*, and provide the species the potential to be developed as a therapeutic delivery vehicle.

To secrete therapeutic molecules from bacteria, research groups have exclusively exploited the secretory pathway. The secretory pathway is an export machinery responsible for transporting a variety of proteins into and across the plasma...
membrane of bacteria (42). For biotherapeutic delivery, a signal peptide targets the therapeutic protein for secretion and is recognized by signal peptidase I (SPaseI), a transmembrane protein that facilitates translocation of the therapeutic fusion protein across the bacterial cell membrane and cleaves the signal peptide (43). The mature protein either remains associated with the cell, is anchored to the cell surface, or is released into the extracellular space (43). However, exploiting the secretory pathway to secrete high levels of recombinant protein can impose a burden on the cell. Additionally, the design of the fusion protein comes with several challenges. For example, the amino acid composition of the signal peptide combined with the N-terminal sequence of the mature protein are critical for optimal processing of the SPaseI (44). The SPaseI efficiency in *E. coli*, *B. subtilis* or select lactobacilli does not always extend to other lactobacilli, and the extraordinary genetic diversity of members of the genus *Lactobacillus* likely contributes to this (45–49). Clearly, passage of recombinant proteins through the secretory pathway can be a bottleneck to efficiently deliver content, and thus requires optimization to maximize efficiency (43, 44).

Another important consideration to engineer bacteria as biotherapeutic delivery vehicles is finding alternatives to antibiotic selection for recombinant plasmid maintenance. Antibiotic alternatives should eliminate both the need for antibiotics in the growth medium and concerns about spreading antibiotic resistance genes to the host microbiota. Engineered auxotrophy provides an elegant solution to this problem. By modifying the bacterium for auxotrophy to an essential amino acid, for example, the relevant gene can then be supplied *in trans* on the desired plasmid expression system.
Examples of this include the use of triosephosphate isomerase in *E. coli* (50), threonine auxotrophy in *L. lactis* (51), and thymidine synthase in *Lactobacillus acidophilus* (52).

In this study, we explored the potential of *L. reuteri* VPL1014 as a therapeutic delivery platform. Rather than using the secretion pathway to secrete proteins into the environment, we exploited native prophages of *L. reuteri* to lyse the bacterium and to release our model protein, leptin. Finally, we employed a thymidine synthase-based plasmid system that can be stably maintained in the cell without the need for antibiotic selection.

**RESULTS**

*L. reuteri* VPL1014 has a low mutation rate and survives gastrointestinal transit in a mouse.

One of the long-term goals of our research group is to develop lactic acid bacteria (LAB) as a platform to deliver therapeutics in the gastrointestinal (GI) tract. Potential strains for this purpose ideally 1) have a low mutation rate to preserve the genetic integrity, 2) can survive GI transit, and 3) are genetically accessible. First, we performed a mutation rate analysis on 10 select LAB, including *Lactococcus lactis*, to determine the number of mutations acquired per cell per generation. We observed that *L. reuteri* VPL1014 exhibits the lowest mutation rate ($8.7 \times 10^{-10}$ mutations/cell/generation), which was 9.4-fold lower than that of *L. lactis* ($9.4 \times 10^{-10}$ mutations/cell/generation) (Fig. 1A). The mutation rate of *L. reuteri* VPL1014 is 3.4-fold lower than that of the second lowest strain, *Lactobacillus salivarius* ($2.9 \times 10^{-10}$ mutations/cell/generation), the latter being comparable to the mutation rates of the
seven remaining lactobacilli strains, ranging from $4.29 \times 10^{-10}$ (L. gasseri ATCC 33323) to $6.06 \times 10^{-10}$ (L. plantarum BAA-793) mutations/cell/generation. Thus, the mutation rate varies considerably within the genus Lactobacillus.

Next, we compared a sub-set of LAB—L. reuteri VPL1014, L. plantarum BAA-793, L. rhamnosus ATCC 53103 and L. lactis NZ9000—for their ability to survive passage through the mouse GI tract. To identify and quantify the strains, we isolated rifampicin-resistant derivatives either by selecting strains that have naturally acquired mutations to render rifampicin resistance (L. rhamnosus, L. plantarum) or by mutating the rpoB gene to yield a rifampicin-resistant phenotype, which we accomplished by single-stranded DNA recombineering [L. lactis (LC:rpoB(H486N)) and L. reuteri (LR:rpoB(H488R))] (39). We administered the bacteria to mice (n=6/group) for two consecutive days with $10^8$ CFU per day, and 16 hours after the final gavage we quantified the viable bacteria in the feces. L. rhamnosus and L. plantarum were most robust in their ability to survive GI transit ($10^6$ CFU/100 mg feces), while L. reuteri and L. lactis were recovered at $10^5$ and $10^4$ CFU/100 mg feces, respectively (Fig. 1B). L. lactis MG1363—the precursor strain of NZ9000—has successfully been developed as a therapeutic delivery vehicle in clinical trials (23). We concluded that the intermediate survival capacity of L. reuteri VPL1014 is not a limiting factor to develop this strain as a therapeutic delivery vehicle. The combination of the low mutation rate, the ability to survive passage through the GI tract at levels that exceed those of the established delivery vehicle L. lactis, and the extended genome editing toolbox that has been developed for use in L. reuteri (single-stranded DNA recombineering, CRISPR-Cas genome editing and a recently developed counterselection marker (39–41)) led us to...
select *L. reuteri* VPL1014 for the development of a therapeutic delivery vehicle. To evaluate *L. reuteri* VPL1014 as a delivery vehicle, we chose the hormone leptin as our model molecule.

**Secreted Leptin is inefficiently processed by *Lactobacillus reuteri* VPL1014.**

Leptin is produced by adipose tissue and modulates appetite in humans and mice by signaling satiety in the brain (53, 54). First, we engineered *L. reuteri* VPL1014 to produce 3x-FLAG tagged murine leptin, codon optimized for expression in *L. reuteri*, from the multi-copy plasmid pJP028 (LR/pSP-Leptin-3xFLAG). By Western blot analysis, we demonstrated that recombinant leptin was produced by LR/pSP-Leptin-3xFLAG. However, our results suggested that the signal peptide was not processed efficiently; the size of the majority of recombinant protein corresponded to the unprocessed precursor, while a small fraction of protein yielded the expected size for mature leptin (Fig. 2a). These findings were substantiated after we engineered *L. reuteri* to express leptin lacking a signal peptide (LR/pLeptin-3xFLAG): Western blot analysis demonstrated that 3x-FLAG tagged leptin was produced at the expected size (19 kDa; Fig. 2a). To circumvent the use of a signal peptide to release the recombinant protein, we decided to pursue the development of *L. reuteri* to accumulate leptin within the cell for subsequent delivery.

First, we determined to what extent recombinant protein can be accumulated in the cells. To quantify the amount of leptin that was accumulated intracellularly, we lysed ~16-hour culture of LR/pLeptin-3xFLAG by bead beating and subjected the cell-free supernatant to ELISA. Our results showed that approximately 132±27.8 ng of leptin per
1 mg of cells (by dry cell weight) was produced by recombinant *L. reuteri* (Fig. 2b). As expected, we did not detect leptin in the lysate derived from *L. reuteri* VPL1014 harboring the pJP028 vector control plasmid (pCtl). Now that we determined that leptin can be accumulated to ng/mg concentrations in culture media, there is opportunity to release recombinant protein following cell lysis. This approach would alleviate the concern of inefficient secretion and/or processing of the signal peptide and relieve the pressure on the bacterial secretion system to secrete recombinant protein. Therefore, we explored the use of bacteriophages to release recombinant protein from *L. reuteri*.

**Exploiting prophages to release therapeutic molecule**

We recently demonstrated that *L. reuteri* 6475, a precursor of VPL1014, encodes two biologically active prophages—bacterial viruses that are integrated in the bacterial genome (55). During GI transit, the prophages are activated, leading to production of bacteriophages and approximately a 10-fold reduction in *L. reuteri* survival (55). We reasoned we could exploit bacteriophage-mediated lysis to release a therapeutic molecule. To establish proof-of-concept we used an untagged version of the same construct as described above (LR/pLeptin) and performed a mitomycin C induction experiment. Mitomycin C is a DNA-damaging agent that induces the SOS response of bacteria, which in-turn activates lysogenic phage to lead to phage-mediated cell lysis (56, 57). LR/pLeptin and wild-type harboring an empty-vector control (LR/pCtl) were induced with mitomycin C (0.5 µg/ml) at OD$_{600}$ = 0.3. Before induction (T$_0$), and 5 hours after MitC induction (T$_5$), we harvested culture supernatants to quantify leptin. We chose T$_5$ as our end-point in the analysis as no further reduction in optical density was
observed compared to T6 and beyond. At T5, we recovered 18.8-fold more leptin in the supernatant of the induced culture compared to the uninduced control (51.6\text{induced} \text{ng/ml}, P < 0.001). This suggested that prophage activation promotes the release of intracellular accumulated protein from LR/pLeptin.

Next, we examined the dynamics of phage-induced lysis and leptin release. We induced LR/pLeptin with mitomycin C and tracked plaque-forming units (PFU/ml), leptin release, and growth (OD\text{600}) every hour post-induction. We report leptin release as the percentage of leptin detected in the supernatant compared to the total amount of leptin present in the supernatant and cells. As expected, induction of LR/pLeptin resulted in an increase in the amount of PFUs over time (Fig. 3a). Compared to two-hours post-induction (T2), we observed at three-hours post-induction (T3) an exponential increase in PFU/ml (3.8 log\text{T2} vs 6.9 log\text{T3}, P < 0.02; Fig. 3a), which corresponded to a significant increase in the percent of leptin released (1.21\%\text{T2} vs 16.1\%\text{T3}, P < 0.05; Fig. 3b). At the following time points, T4-T5, the cell density was reduced (OD\text{600} = 0.79\text{T3} vs 0.66\text{T4} and 0.63\text{T5}), signifying cell lysis (Fig. 3c). Following 5 hours post-induction, 38\% of leptin was released in the culture supernatant. In the uninduced LR/pLeptin control, PFUs increased slightly at three hours post-induction (3.9 log(PFU/ml)\text{T2} vs 4.7 log(PFU/ml)\text{T3}, P < 0.05), while the percent of released leptin remained steady (0.62\%\text{T2} vs 0.58\%\text{T3}, P > 0.05) (Fig. 3). Together, these data suggest that bacteriophage-mediated lysis contributes to leptin release. To further substantiate this, we expressed leptin in LR\Delta\Phi1\Delta\Phi2, a derivative which lacks prophages (55). Mitomycin-C induction of LR\Delta\Phi1\Delta\Phi2/pLeptin culture did not induce lysis, and leptin release was marginal as we recovered only 1.9\% and 3.82\% leptin at T3 and T5, respectively (Fig. 3). Collectively,
we demonstrated that an exponential increase in phage production releases recombinant protein in the extracellular milieu, which provides a novel approach to deliver therapeutics. However, LR/pLeptin requires further optimization prior to in vivo studies. For example, as of now, we require antibiotics to maintain the recombinant plasmid in the cell. To overcome this, we focused next on an approach to eliminate the need for antibiotics in the growth medium.

**pMut:thyA stably maintains LR/pLeptin in the absence of antibiotic selection**

The gene *thyA* encodes thymidylate synthase, which converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), also known as thymidine (52). Previously, it was demonstrated that plasmid expression of *thyA* in a genetic background that lacks *thyA* renders stable plasmid maintenance (52, 58, 59). To establish proof-of-concept in *L. reuteri*, we first inactivated *thyA* by single-stranded DNA recombineering, and confirmed the resultant strain was auxotrophic for thymidine (data not shown). Next, we modified the leptin expression vector, which contains gene cassettes that encode resistance to chloramphenicol and erythromycin. We replaced the gene cassette encoding chloramphenicol resistance with *thyA*, and transformed the resultant construct into LRΔ*thyA:rpoB*(H488R) to yield LRΔ*thyA:rpoB*(H488R)/pLeptin-ThyA. For this experiment, we purposely maintained the marker encoding erythromycin resistance, which allowed us to accurately determine plasmid stability within the population. As controls, we included an empty-vector control (pCtl), and the vector encoding leptin lacking *thyA* (pLeptin). To determine plasmid stability, we passaged the strains for ~100 generations in MRS without antibiotic selection, after which we
determined the total number of CFUs and the number of CFUs which are resistant to erythromycin. After ~100 generations, we confirmed that 12±7.5% and of cells retained the empty vector control (pCtl) while we did not recover any cells that retained the plasmid expressing leptin (pLeptin), whereas the plasmid expressing ThyA was present in 77±12% of cells in the population (pLeptin-ThyA) (Fig. 4). We also performed this experiment in modified MRS (mMRS) without antibiotic or thymidine, and observed that after 50 generations, pCtl-ThyA and pLeptin-ThyA were retained at 62±20% and 100±11%, respectively, while pLeptin was lost after 25 generations (Fig. 4, inset). Thus, the combination of LRΔthyA:rpoB(H488R) and in trans expression of ThyA increased plasmid stability compared to the controls. In conclusion, LRΔthyA:rpoB(H488R)/pLeptin-thyA now constitutes a strain that does not require antibiotic in the growth media for plasmid retention, which we will exploit in future _in vivo_ studies.

**DISCUSSION**

In this study we explored the novel approach of exploiting _L. reuteri_ VPL1014 prophage for the release of intracellularly accumulated biotherapeutic. Based on its genetic stability, _in vivo_ survivability, and genetic accessibility, we pursued _L. reuteri_ VPL1014 as a delivery platform. After engineering _L. reuteri_ VPL1014 to produce leptin within the cell, we demonstrated that we can exploit phage-mediated lysis to release leptin, while we also developed a plasmid-system that does not require antibiotic selection.

An important selection criterium to develop _L. reuteri_ VPL1014 as a biotherapeutic delivery platform was the low mutation rate of this strain. It remains
speculative what the underlying mechanisms are that explains our finding, but it is possible that differences in the activity of MutS, a conserved protein that repairs mismatches that occur during replication (60, 61), could contribute to the differences in the mutation rates of lactobacilli. We determined that L. reuteri 6475—a precursor of VPL1014—and DSM20016T have similar mutation rates (data not shown), and our finding could provide a potential explanation of the clonal nature of human-derived L. reuteri strains 6475, JCM1112 and DSM20016T, which were isolated from different continents yet their genomes are nearly identical (62). From a practical standpoint, our finding is interesting because the low mutation rate of L. reuteri VPL1014 will result in a recombinant strain that will acquire few single-nucleotide-polymorphisms (SNPs) when compared to other LAB strains, including L. lactis that has a mutation rate that is nearly 10-fold higher than L. reuteri. Thus, we expect that the low mutation rate of L. reuteri VPL1014 will contribute to the genetic stability of our engineered strains.

Although the mutation rate of L. reuteri VPL1014 was one of the main selection criteria to use this strain for be further developed as a therapeutic, we observed that the ability to survive gastrointestinal transit was intermediate compared to other strains tested. In fact, L. plantarum BAA-793 and L. rhamnosus GG survived GI transit better than L. reuteri VPL1014. Logically, differences in the ability to survive exposure to acids, i.e. stomach and bile, may explain our observation. In addition, differences in prophage activation during GI transit could contribute to different efficiencies to survive GI transit. Recently, we demonstrated that prophages in L. reuteri are activated during GI transit. A mutant lacking the prophage genomes was recovered at approximately 8-fold higher levels than L. reuteri harboring prophages (55). At this point, we cannot compare our
recent findings to the literature as studies pertaining to prophage activation of gut symbionts during GI transit are in their infancy. However, both *L. plantarum* and *L. rhamnosus* encode biologically active prophages, as demonstrated by mitomycin C induction experiments (data not shown), which means that prophages cannot be excluded as a contributing factor to differences in GI survival. Less surprising was our finding that *L. lactis* was least robust during GI transit. *L. lactis* is commonly found in milk products and has not evolved to thrive in the intestinal environment, unlike *L. reuteri* for example (63, 64). Although *L. lactis* NZ9000 does not carry biologically active prophages that can contribute to reduced GI survival (65), it has been shown that *L. lactis* is more sensitive to bile acids (66), which could partially explain the organism’s reduced ability to survive GI transit.

Induction of prophages in *L. reuteri* during GI transit, thereby causing cell lysis in the gut and releasing intracellular content, can be used to deliver therapeutics. Recently published findings showed that intracellular accumulation of interleukin-22 (IL-22) in *L. reuteri* leads to the release of IL-22 during gastrointestinal transit, as demonstrated by induced gene expression of *regIIIγ*, a gene regulated by IL-22 (67). With a daily dosage of $10^7$ CFU of recombinant *L. reuteri* for 7 days, Hendrikkx et al. observed a decrease in liver damage in a model of murine alcohol-induced liver disease (67). Despite this exciting result, we do not currently have evidence that this method would result in systemic delivery of a therapeutic, which would be necessary for leptin to acquire biological efficacy. Achieving systemic delivery may also depend on the properties of the biotherapeutic molecule itself, such as polarity and hydrophobicity. For any
potential therapeutics, individual *in vivo* studies must be conducted to demonstrate delivery and efficacy.

There may be multiple advantages to using phage-mediated lysis as an approach. First, we have shown that diet can alter bacteriophage production in *L. reuteri* (55), which opens up the exciting opportunity to explore diet to control the release of therapeutics. Second, prophage-mediated delivery of therapeutics could reduce the total number of viable recombinant bacteria, thereby contributing to biological containment. However, studies to maximize biological containment to fully eradicate recombinant *L. reuteri* remain needed: although *L. reuteri* has proven to be safe for consumption (68–74), strategies need to be in place to halt the release of recombinant protein to both better control dosage and a possible undesirable side-effect. Third, using bacteriophages to release therapeutic molecules alleviates the need to use the native secretion system to deliver a protein. This means there is no need to identify the optimal signal peptide for secretion, which is known to be dependent on the sequence of the mature protein and can be a time-consuming screening process (44). Also, we expect that we are imposing a lower burden on the cell metabolism.

In future studies, we plan to address biological containment of our genetically modified organism. We will insert the leptin gene into the *L. reuteri* chromosome under the control of the EF-Tu promoter, and maximize the level of phage-mediated lysis by hijacking the phage regulatory proteins. We expect this will result in a food-grade, antibiotic-free, biologically contained delivery platform. At minimum, the concept of engineering *L. reuteri* for the intracellular accumulation of a product combined with phage-mediated lysis can be applied for studies to explore the local effect of molecules.
that would be otherwise difficult to deliver to the GI tract. The exploitation and study of probiotic prophage can result in both an effective, biologically contained therapeutic delivery vehicle and provide further insight into the role of phage in probiotic efficacy. In conclusion, our work presents a novel method to accomplish therapeutic delivery by exploiting phage-mediated lysis for therapeutic release by *L. reuteri* VPL1014.

**MATERIALS AND METHODS**

**Bacterial Strains and Media.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* EC1000 was used as an intermediate cloning host and was cultured at 37°C in lysogeny broth (LB, Teknova). Competent cells of *E. coli* EC1000 were prepared as described previously (75). *Lactobacillus reuteri* lacking prophages, LRΔΦ1ΔΦ2 (VPL4121) and LRΔΦ1ΔΦ2ΔattB1ΔattB2 (VPL4090, "lytic host") were constructed previously (55). Construction of the rifampicin resistant strains used in the *in vivo* survival experiment was achieved through single-stranded DNA recombineering that targeted the *rpoB* gene (*L. lactis* VPL4005) and LR:*rpoB*(H488R) (VPL4126) as described previously (39) or by random mutant isolation (*L. rhamnosus* VPL4141 and *L. plantarum* VPL4142). Lactobacilli were grown in De Man, Rogosa and Sharpe (MRS) medium (Difco, BD BioSciences) under hypoxic conditions (5% CO₂, 2% O₂) on agar plates at 37°C or in broth (static) at 37°C in a conventional aerated incubator. *L. reuteri* competent cells were prepared as described previously (40). *L. lactis* was grown in M17 broth (Difco) supplemented with 0.5% (w/v) glucose at 30°C. As needed, erythromycin was supplemented at 5 μg/ml for *L. reuteri* strains and 300 μg/ml for *E. coli* EC1000. Chloramphenicol was added as needed at 5 μg/ml or 20 μg/ml for *L. reuteri* and *E. coli* EC1000, respectively. Rifampicin was added as
needed at 25 µg/ml. To select for LRΔthyA:rpoB(H488R) (VPL4143) we used modified MRS lacking beef extract (mMRS-BE), which has the following ingredients: peptone (10 g/L, BD BioSciences), yeast extract (5 g/L, IBI Scientific), Tween-80 (1 ml/L, Sigma-Aldrich), ammonium citrate dibasic (2 g/L, Sigma-Aldrich), dipotassium phosphate (2 g/L Fisher Scientific), sodium acetate (1 g/L, Sigma-Aldrich), magnesium sulfate (0.1 g/L, Fisher Scientific), manganese sulfate (0.05 g/L, Sigma-Aldrich), and glucose (100 mM, Sigma-Aldrich). mMRS-BE was supplemented with trimethoprim (40 µg/ml) and/or thymidine (50 µg/ml) as needed.

**Mutation Rate Analysis.** Bacterial cultures were incubated for 16 h, sub-cultured to 10^3 CFU/ml and subsequently split into 24-wells (1 ml/well) in a deep-well 96-well plate (Celltreat). Following 48 h incubation, the total viability and the total number of rifampicin-resistant cells were determined by spread plating. The mutation rate was calculated with FALCOR, (Fluctuation AnaLysis CalculatOR, http://www.keshavsingh.org/protocols/FALCOR.html) using the Ma-Sandri-Sarkar maximum likelihood estimator (MSS-MLE), as described previously (76).

**Bacterial Survival Following Gastrointestinal Transit.** Twenty-four 6-week old male B6 mice (C57BL/6J) were purchased from Jackson Laboratory (Bar Harbor, ME). Prior to the start of the experiment, animals were adjusted to the new environment for one week. Animals were individually housed at an environmentally controlled facility with a 12 h light and dark cycle. Food (standard chow, LabDiet, St Louis, MO) and water were provided ad libitum. Mice (n=6/group) were gavaged for two consecutive days with...
100 µL PBS suspension containing $10^9$ CFU/ml of rifampicin-resistant *L. reuteri* 6475, *L. lactis* NZ9000, *L. rhamnosus* GG, or *L. plantarum* BAA-793. Fecal samples were collected from the bedding 16 h after the last oral administration and weighed.

The fecal material was resuspended in PBS to 100 mg/ml and plated on MRS-agar plates (or GM17 for *L. lactis*) containing 25 µg/ml rifampicin. Cell viability counts were normalized per $10^8$ CFU.

**Heterologous expression of leptin: LR/pLeptin.** All oligonucleotides are listed in Table 2. To construct LR/pLeptin, we amplified the backbone of pJP028 (derived from pNZ8048) with oVPL1200-oVPL1286, followed by DpnI treatment (Thermo Scientific). The sequence encoding murine leptin was obtained from NCBI (ADM72802.1). We codon optimized the leptin sequence for expression in *L. reuteri* with OPTIMIZER (77, 78), followed by gBLOCK synthesis (IDT-DNA) (Table 2), and amplification with oligonucleotide pair oVPL1348-oVPL1349. The codon optimized sequence for leptin is available under accession number MK297322 (GenBank). All amplicons were purified (GeneJET PCR purification kit, ThermoFisher), quantified (Qubit Fluorometric quantification, Life Technologies) and phosphorylated (T4 polynucleotide kinase, ThermoFisher), followed by ligation-cycle reaction (LCR) as described previously (79).

To clone leptin, we used bridging oligonucleotides oVPL1350 and oVPL1351. The resulting LCR mixture was transformed into *E. coli* EC1000 (VPL3481) and cloning of the leptin gene into pJP028 was confirmed by PCR (Taq polymerase, Denville Scientific) using oligonucleotides oVPL329-oVPL363. The resultant construct was named pNZ-SP-Leptin.
Purified pNZ-SP-Leptin plasmid was amplified with oVPL1199-oVPL1408 and was used as a template to construct pEFTu-SP-Leptin. A native constitutive promoter, EF-Tu, was amplified with primers oVPL1447-oVPL1448 from pJG001 (gift from Robert Britton) (41). Amplicons were subjected to LCR as described above with bridging oligonucleotides oVPL1409 and oVPL1410. The resulting plasmid, pNZ-EFTu-SP-Leptin, hereafter called pSP-Leptin, was transformed into L. reuteri VPL1014, resulting in LR/pSP-Leptin (VPL3585).

To generate a derivative lacking signal peptide (pLeptin), we amplified the backbone of pSP-Leptin with oVPL1810-oVPL1448, oligonucleotides that are located directly upstream and downstream of the sequence coding for the signal peptide. The resulting amplicon was fused by blunt-end ligation (T4 DNA ligase, Fisher Scientific) and transformed into L. reuteri VPL1014 and LRΔΦ1ΔΦ2 to yield LR/pLeptin (VPL3791) and LRΔΦ1ΔΦ2/pLeptin (VPL31067), respectively. The control plasmid was prepared by amplifying the backbone of pSP-Leptin with oVPL1408 and oVPL1286, oligonucleotides that are located directly upstream of the signal peptide and downstream of the sequence coding for leptin, followed by self-ligation and transformation into L. reuteri to yield LR/pCtl (VPL3583).

For Western blot purposes, we inserted the sequence encoding a 3X-FLAG tag to the 3'-proximal end of the leptin gene in plasmids pSP-Leptin and pLeptin. To accomplish this, we performed PCR using oligonucleotides oVPL2112 and oVPL2113, which are located on the 3' end of the leptin gene and just downstream of leptin on the plasmid backbone. A tag of 66 bp was included on the 5'-end of each primer, which following self-ligation, resulted in the DNA sequence coding for a 3X-FLAG tag. The
resulting plasmids were named pSP-Leptin-3XFLAG and pLeptin-3XFLAG, and established in L. reuteri VPL1014 to yield LR/pSP-Leptin-3XFLAG (VPL3752) and LR/pLeptin-3XFLAG (VPL3795), respectively.

**Construction of LRΔthyA.** We inactivated thyA in a rifampicin-resistant derivative of L. reuteri VPL1014 (LR:rpoB(H488R)) by single-stranded DNA recombineering as described previously (39). We previously engineered L. reuteri VPL1014 to be rifampicin resistant to assess survival following GI transit (LR:rpoB(H488R)) (VPL4126) (55). LR:rpoB(H488R) expressing RecT was transformed with 100 μg of oVPL1670 to generate an in-frame stop codon in thyA, using methods described previously (39). To identify cells in which thyA was inactivated, we used positive selection with trimethoprim. Trimethoprim is toxic to cells producing ThyA because it prevents the reduction of the byproduct dihydrofolate to tetrahydrofolate, thus inhibiting bacterial DNA synthesis (80). The selection media is also supplemented with thymidine to allow ΔthyA mutants to grow. Therefore, LRΔthyA:rpoB(H488R) mutants were selected for by plating serial dilutions onto mMRS-BE supplemented with trimethoprim (40 μg/ml) and thymidine (50 μg/ml). The genotype of the colonies was confirmed by mismatch amplification mutation assay (MAMA) PCR (81, 82) with oVPL1671, oVPL1672, and oVPL1673, followed by sanger sequencing.

**Construction of pLeptin-thyA.** To develop an expression vector without the need for antibiotic in the growth media, the gene conferring chloramphenicol resistance in the pJP028 backbone was replaced with thyA (52). We amplified pLeptin with oVPL2351
and oVPL2352 to generate a plasmid backbone lacking the chloramphenicol resistance gene. To complement thyA in *L. reuteri* lacking thyA, we then put the thyA gene under the control of the *L. reuteri* pMutL promoter, a promoter located upstream of the gene encoding MutL, which is involved in DNA repair (83). We amplified pMutL:thyA with oVPL736 and oVPL1725 using pSIP411:pMut:thyA as template, which we subsequently fused by blunt-end ligation (T4 DNA ligase) to the pLeptin backbone to generate pLeptin-thyA. The resulting construct, pLeptin-thyA was transformed into *E. coli* EC1000 to yield VPL31131. Purified pLeptin-thyA plasmid was transformed into LRΔthyA:rpoB(H488R), resulting in LRΔthyA:rpoB(H488R)/pLeptin-thyA (VPL31133). Transformants were selected on mMRS-BE agar harboring 5 µg/ml erythromycin (Em 5). LRΔthyA:rpoB(H488R)/pLeptin:thyA was then used for a plasmid stability experiment. Backbone control vector was prepared by amplifying pLeptin-thyA omitting Leptin with oVPL1408-oVPL1286. This amplicon was then treated as above and self-ligated with T4 ligase before transformation into LRΔthyA:rpoB(H488R), resulting in LRΔthyA:rpoB(H488R)/pCtl-thyA (VPL31134). For comparison in the plasmid stability experiment, pLeptin lacking thyA was transformed into LRΔthyA:rpoB(H488R), resulting in LRΔthyA:rpoB(H488R)/pLeptin (VPL31135).

**Protein preparation, ELISA, and Western Blotting**

*Western Blot.* Intracellularly accumulated leptin from LR/pLeptin-3XFLAG and secreted leptin from LR/pSP-Leptin-3XFLAG was analyzed by Western Blot. Protein samples were prepared from ~16 h cultures. LR/pLeptin-3XFLAG cells were harvested by centrifuging 1.5 ml of culture (1 minute at 21130 x g), dry cell-weight was measured for
normalization purposes, and the cell pellet was washed once in 1.5 ml distilled water, and resuspended in 500 µL lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100). Approximately 100 µL of zirconia glass beads (BioSpec) was added to the suspension. Cells were vortexed six times for 30 seconds with 30 second intervals on ice. Lysates were harvested by transferring the supernatants into a fresh tube, adding 1 ml of lysis buffer, and centrifuging at 8210 x g for 10 minutes. Samples were analyzed immediately or stored at -20°C until use. LR/pSP-Leptin-3XFLAG samples were prepared by centrifuging 1.5 ml of culture (1 minute at 21130 x g) after which we collected the supernatant. Protein from LR/pSP-Leptin-3XFLAG supernatants were precipitated as previously described (84). Samples were loaded onto Bolt TM 4–12% Bis-Tris Plus gels (Life Technologies) and transferred onto iBlot nitrocellulose membrane (Thermo Scientific). The membrane was washed for 1 h in Tris-buffered saline plus Tween 20 (TBST) and 0.5% w/v milk (blocking buffer) and then hybridized at 4°C for ~16 h with rabbit anti-Flag antibody (PA1-984B, Thermo Scientific) diluted 1:500 in blocking buffer. Following three washes with TBST for 5 minutes each, HRP-conjugated secondary antibody (Anti-Rabbit) was diluted 1:1000 in blocking buffer and incubated with the membrane for 2 h. Following another three washes with TBST, Clarity™ Western ECL substrate (BioRad) was added and incubated with the membrane for 5 minutes before imaging for chemiluminescence (BioRad Chemi-Doc™ Touch Imaging System).

ELISA. Leptin ELISA (R&D Systems) was performed as suggested by the manufacturer. To measure intracellular leptin from overnight cell culture, we processed samples from
LR/pLeptin in an identical manner as described above for preparing Western blot samples, while bacterial supernatant samples from the mitomycin C induction experiment were harvested by centrifugation (5 minutes, 21130 \( \times g \)), followed by filter sterilization with 0.22 \( \mu m \) filters (Millipore). For the mitomycin C induction experiment, total leptin was measured by combining supernatant with cell lysate sample. Percent leptin released was calculated by comparing the amount of leptin in the supernatant to the total. The final optical density was measured with a microplate reader (Molecular Devices, SpectraMax Plus 384; 450nm/570 nm) within 30 minutes of the completion of the ELISA. A standard curve was generated using JMP software to calculate leptin concentrations.

**Quantification of bacteria and bacteriophages.** For Mitomycin C induction, overnight (~16 h) cultures were diluted to OD\(_{600}\) = 0.1, and at OD\(_{600}\)=0.3, Mitomycin C was added (0.5 \( \mu \)g/ml) (Sigma-Aldrich). Samples were harvested every hour post-induction for 5 h to determine CFU/ml and PFU/ml. For PFU/ml analysis, cells were centrifuged (21130 \( \times g \) for 1 minute) and supernatants were filter-sterilized (0.22 \( \mu m \), Millipore). As a lytic host we used *L. reuteri* \( \Delta \Phi1 \Delta \Phi2 \Delta \text{attB1} \Delta \text{attB2} \) (VPL4090) (55), which was prepared as follows: ~16 h culture of lytic host was centrifuged at 3200 \( \times g \) for 5 minutes and washed once in an equal volume with phage diluent (16 mM MgSO\(_4\), 20 mM Tris-CL, pH 7.5 in dH\(_2\)O), followed by resuspension in phage diluent to OD\(_{600}\) = 2.0. Subsequently, we added 10 mM CaCl\(_2\) to the bacterial suspension. We mixed an equal volume of the lytic host suspension and phage samples (200 \( \mu L \) each) in a 15 ml conical tube and incubated the mixture at 37°C for 1 h. We added 3 ml of 0.2% (w/v) agarose harboring
10 mM CaCl₂, which was gently inverted three times, which was poured onto MRS agar supplemented with 10 mM CaCl₂, followed by 15 h incubation at 37°C.

**Plasmid Stability Assay.** Overnight (~16 h) cultures of LRΔthyA:rpoB(H488R) harboring pLeptin-thyA, pCtl-thyA, or pLeptin were diluted to 0.1% in MRS without antibiotic. Following an 8 h incubation, serial dilutions from each culture were plated onto plain MRS and MRS plates harboring 5 µg/ml erythromycin (MRS-Em) for cell viability counts. Cells were passaged twice a day in MRS±Em for ~100 generations. Plasmid stability was assessed every ~7 generations by calculating the ratio of colonies recovered on MRS-Em plates and the total amount of viable cells recovered on MRS plates without antibiotic selection.

**Statistics.** Data representation was performed using DataGraph 4.3 (Visual Data Tools, Inc., Chapel Hill, North Carolina, United States, https://www.visualdatatools.com). Statistical comparisons were performed using paired t-test, one-way ANOVA, and Tukey's HSD (JMP Pro, version 11.0.0). Three biological replicates were performed for all in vitro studies. All samples were included in the analyses, and experiments were performed without blinding.

**FUNDING**

The Van Pijkeren Laboratory is grateful for support from the UW-Madison Food Research Institute, National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number 1R21AI121662, National Institute of Food and
Agriculture, U.S. Department of Agriculture, Hatch Project under award number MSN1856150, and UW-Madison Institute of Clinical and Translational Research funded by the National Center for Advancing Translational Science award UL1TR000427. LA was supported by NIH National Research Service Award T32 GM007215 and the Science and Medicine Graduate Research Scholars Program.

ACKNOWLEDGEMENTS

We thank BioGaia AB (Stockholm, Sweden) for providing *L. reuteri* ATCC PTA 6475.

REFERENCES

1. Keles G, Demirci U. 2011. The effect of homofermentative and heterofermentative lactic acid bacteria on conservation characteristics of baled triticale–Hungarian vetch silage and lamb performance. Anim Feed Sci Technol 164:21–28.

2. Ayad EHE, Verheul A, Wouters JTM, Smit G. 2002. Antimicrobial-producing wild lactococci isolated from artisanal and non-dairy origins. Int Dairy J 12:145–150.

3. Vijayakumar PP, Muriana PM. 2017. Inhibition of *Listeria monocytogenes* on ready-to-eat meats using bacteriocin mixtures based on mode-of-action. Foods 6(3):22.
4. Mattila-Sandholm T, Mättö J, Saarela M. 1999. Lactic acid bacteria with health claims—interactions and interference with gastrointestinal flora. Int Dairy J 9:25–35.

5. Mercenier A, Müller-Alouf H, Grangette C. 2000. Lactic acid bacteria as live vaccines. Curr Issues Mol Biol 2:17–25.

6. Brown L, Pingitore EV, Mozzi F, Saavedra L, Villegas JM, Hebert EM. 2017. Lactic acid bacteria as cell factories for the generation of bioactive peptides. Protein Pept Lett 24:146–155.

7. Bron PA, Kleerebezem M. 2018. Lactic acid bacteria for delivery of endogenous or engineered therapeutic molecules. Front Microbiol 9.

8. van pijkeren J-P, Barrangou R. 2017. Genome editing of food-grade Lactobacilli to develop therapeutic probiotics. Microbiol Spectr 5.

9. Wang M, Gao Z, Zhang Y, Pan L. 2016. Lactic acid bacteria as mucosal delivery vehicles: a realistic therapeutic option. Appl Microbiol Biotechnol 100:5691–5701.

10. Guo S, Yan W, McDonough SP, Lin N, Wu KJ, He H, Xiang H, Yang M, Moreira MAS, Chang Y-F. 2015. The recombinant Lactococcus lactis oral vaccine induces protection against C. difficile spore challenge in a mouse model. Vaccine 33:1586–1595.

11. Hongying F, Xianbo W, Fang Y, Yang B, Beiguo L. 2014. Oral immunization with recombinant Lactobacillus acidophilus expressing the adhesin Hp0410 of...
Helicobacter pylori induces mucosal and systemic immune responses. Clin Vaccine Immunol CVI 21:126–132.

12. Li X, Xing Y, Guo L, Lv X, Song H, Xi T. 2014. Oral immunization with recombinant Lactococcus lactis delivering a multi-epitope antigen CTB-UE attenuates Helicobacter pylori infection in mice. Pathog Dis 72:78–86.

13. Zhang H, Qiu Y, Zhao Y, Liu X, Liu M, Yu A. 2014. Immunogenicity of oral vaccination with Lactococcus lactis derived vaccine candidate antigen (UreB) of Helicobacter pylori fused with the human interleukin 2 as adjuvant. Mol Cell Probes 28:25–30.

14. Li Y, Li X, Liu H, Zhuang S, Yang J, Zhang F. 2014. Intranasal immunization with recombinant Lactococci carrying human papillomavirus E7 protein and mouse interleukin-12 DNA induces E7-specific antitumor effects in C57BL/6 mice. Oncol Lett 7:576–582.

15. Kawana K, Adachi K, Kojima S, Taguchi A, Tomio K, Yamashita A, Nishida H, Nagasaka K, Arimoto T, Yokoyama T, Wada-Hiraike O, Oda K, Sewaki T, Osuga Y, Fujii T. 2014. Oral vaccination against HPV E7 for treatment of cervical intraepithelial neoplasia grade 3 (CIN3) elicits E7-specific mucosal immunity in the cervix of CIN3 patients. Vaccine 32:6233–6239.

16. Ribelles P, Benbouziane B, Langella P, Suárez JE, Bermúdez-Humarán LG. 2013. Protection against human papillomavirus type 16-induced tumors in mice using
non-genetically modified lactic acid bacteria displaying E7 antigen at its surface.

17. Li R, Chowdhury MYE, Kim J-H, Kim T-H, Pathinayake P, Koo W-S, Park M-E, Yoon J-E, Roh J-B, Hong S-P, Sung M-H, Lee J-S, Kim C-J. 2015. Mucosally administered *Lactobacillus* surface-displayed influenza antigens (sM2 and HA2) with cholera toxin subunit A1 (CTA1) Induce broadly protective immune responses against divergent influenza subtypes. Vet Microbiol 179:250–263.

18. Shi S-H, Yang W-T, Yang G-L, Cong Y-L, Huang H-B, Wang Q, Cai R-P, Ye L-P, Hu J-T, Zhou J-Y, Wang C-F, Li Y. 2014. Immunoprotection against influenza virus H9N2 by the oral administration of recombinant *Lactobacillus plantarum* NC8 expressing hemagglutinin in BALB/c mice. Virology 464–465:166–176.

19. Waki N, Matsumoto M, Fukui Y, Suganuma H. 2014. Effects of probiotic *Lactobacillus brevis* KB290 on incidence of influenza infection among schoolchildren: an open-label pilot study. Lett Appl Microbiol 59:565–571.

20. Lei H, Peng X, Zhao D, Ouyang J, Jiao H, Shu H, Ge X. 2015. *Lactococcus lactis* displayed neuraminidase confers cross protective immunity against influenza A viruses in mice. Virology 476:189–195.

21. Chowdhury MYE, Li R, Kim J-H, Park M-E, Kim T-H, Pathinayake P, Weeratunga P, Song MK, Son H-Y, Hong S-P, Sung M-H, Lee J-S, Kim C-J. 2014. Mucosal vaccination with recombinant *Lactobacillus casei*-displayed CTA1-conjugated
consensus matrix protein-2 (sM2) induces broad protection against divergent influenza subtypes in BALB/c mice. PloS One 9:e94051.

22. Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E. 2000. Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. Science 289:1352–1355.

23. Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon J, van Deventer SJH, Neirynck S, Peppelenbosch MP, Steidler L. 2006. A phase I trial with transgenic bacteria expressing Interleukin-10 in Crohn's Disease. Clin Gastroenterol Hepatol 4:754–759.

24. Bermúdez-Humarán LG, Nouaille S, Zilberfarb V, Corthier G, Gruss A, Langella P, Issad T. 2007. Effects of intranasal administration of a leptin-secreting Lactococcus lactis recombinant on food intake, body weight, and immune response of mice. Appl Environ Microbiol 73:5300–5307.

25. Liu X, Lagenaur LA, Simpson DA, Essenmacher KP, Frazier-Parker CL, Liu Y, Tsai D, Rao SS, Hamer DH, Parks TP, Lee PP, Xu Q. 2006. Engineered vaginal Lactobacillus strain for mucosal delivery of the human immunodeficiency virus inhibitor cyanovirin-N. Antimicrob Agents Chemother 50:3250–3259.

26. Abbas Hilmi HT, Surakka A, Apajalahti J, Saris PEJ. 2007. Identification of the most abundant Lactobacillus Species in the crop of 1- and 5-week-old broiler chickens. Appl Environ Microbiol 73:7867–7873.
27. Dellaglio F, Arrizza S, Ledda A. 1981. Classification of citrate fermenting Lactobacilli isolated from lamb stomach, sheep milk and pecorino romano cheese. Zentralblatt Für Bakteriol Mikrobiol Hyg Abt Orig C Allg Angew Ökol Mikrobiol 2:349–356.

28. Leser TD, Amenuvor JZ, Jensen TK, Lindecrona RH, Boye M, Møller K. 2002. Culture-independent analysis of gut bacteria: the pig gastrointestinal tract microbiota revisited. Appl Environ Microbiol 68:673–690.

29. Brooks SPJ, McAllister M, Sandoz M, Kalmokoff ML. 2003. Culture-independent phylogenetic analysis of the faecal flora of the rat. Can J Microbiol 49:589–601.

30. Salzman NH, de Jong H, Paterson Y, Harmsen HJM, Welling GW, Bos NA. 2002. Analysis of 16S libraries of mouse gastrointestinal microflora reveals a large new group of mouse intestinal bacteria. Microbiol Read Engl 148:3651–3660.

31. Reuter G. 2001. The Lactobacillus and Bifidobacterium microflora of the human intestine: composition and succession. Curr Issues Intest Microbiol 2:43–53.

32. Liu Y, Fatheree NY, Mangalat N, Rhoads JM. 2010. Human-derived probiotic Lactobacillus reuteri strains differentially reduce intestinal inflammation. Am J Physiol Gastroint Liver Physiol 299:G1087-1096.

33. Lin YP, Thibodeaux CH, Peña JA, Ferry GD, Versalovic J. 2008. Probiotic Lactobacillus reuteri suppress proinflammatory cytokines via c-Jun. Inflamm Bowel Dis 14:1068–1083.
34. McCabe LR, Irwin R, Schaefer L, Britton RA. 2013. Probiotic use decreases intestinal inflammation and increases bone density in healthy male but not female mice. J Cell Physiol 228:1793–1798.

35. von Schilide M-A, Hörmannsperger G, Weiher M, Alpert C-A, Hahne H, Bäuerl C, van Huynegem K, Steidler L, Hrcir T, Pérez-Martínez G, Kuster B, Haller D. 2012. Lactocepin secreted by *Lactobacillus* exerts anti-inflammatory effects by selectively degrading proinflammatory chemokines. Cell Host Microbe 11:387–396.

36. Britton RA, Irwin R, Quach D, Schaefer L, Zhang J, Lee T, Parameswaran N, McCabe LR. 2014. Probiotic *L. reuteri* treatment prevents bone loss in a menopausal ovariectomized mouse model. J Cell Physiol 229:1822–1830.

37. El-Ziney MG, van den Tempel T, Debevere J, Jakobsen M. 1999. Application of reuterin produced by *Lactobacillus reuteri* 12002 for meat decontamination and preservation. J Food Prot 62:257–261.

38. Talarico TL, Casas IA, Chung TC, Dobrogosz WJ. 1988. Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. Antimicrob Agents Chemother 32:1854–1858.

39. van Pijkeren J-P, Britton RA. 2012. High efficiency recombineering in lactic acid bacteria. Nucleic Acids Res 40:e76.

40. Oh J-H, van Pijkeren J-P. 2014. CRISPR-Cas9-assisted recombineering in *Lactobacillus reuteri*. Nucleic Acids Res 42:e131.
41. Zhang S, Oh J-H, Alexander LM, Özçam M, van Pijkeren J-P. 2018. d-Alanyl-d-alanine ligase as a broad-host-range counterselection marker in vancomycin-resistant lactic acid bacteria. J Bacteriol 200.

42. Tsirigotaki A, Geyter JD, Šoštaric’ N, Economou A, Karamanou S. 2017. Protein export through the bacterial Sec pathway. Nat Rev Microbiol 15:21–36.

43. Ray P, Dev I, MacGregor C, Bassford P. 1986. Signal Peptidases, p 75–102. In Wu HC, Tai PC (ed), Protein Secretion and Export in Bacteria. Springer, Berlin, Heidelberg.

44. Nilsson I, von Heijne G. 1992. A signal peptide with a proline next to the cleavage site inhibits leader peptidase when present in a sec-independent protein. FEBS Lett 299:243–246.

45. Canchaya C, Claesson MJ, Fitzgerald GF, van Sinderen D, O’Toole PW. 2006. Diversity of the genus Lactobacillus revealed by comparative genomics of five species. Microbiology 152:3185–3196.

46. Claesson MJ, Van Sinderen D, O’Toole PW. 2007. The genus Lactobacillus — a genomic basis for understanding its diversity. FEMS Microbiol Lett 269:22–28.

47. Heilig HGHJ, Zoetendal EG, Vaughan EE, Marteau P, Akkermans ADL, Vos WM de. 2002. Molecular diversity of Lactobacillus spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Env Microbiol 68:114–123.
48. Siezen RJ, Tzeneva VA, Castioni A, Wels M, Phan HTK, Rademaker JLW, Starrenburg MJC, Kleerebezem M, Molenaar D, Vlieg JETVH. 2010. Phenotypic and genomic diversity of Lactobacillus plantarum strains isolated from various environmental niches. Environ Microbiol 12:758–773.

49. Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker D, Hughes J, Goh Y, Benson A, Baldwin K, Lee J-H, Díaz-Muñiz I, Dosti B, Smeianov V, Wechter W, Barabote R, Lorca G, Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent J, Hutkins R, O’Sullivan D, Steele J, Unlu G, Saier M, Klaenhammer T, Richardson P, Kozyavkin S, Weimer B, Mills D. 2006. Comparative genomics of the lactic acid bacteria. Proc Natl Acad Sci 103:15611–15616.

50. Velur Selvamani RS, Telaar M, Friehs K, Flaschel E. 2014. Antibiotic-free segregational plasmid stabilization in Escherichia coli owing to the knockout of triosephosphate isomerase (tpiA). Microb Cell Factories 13:58.

51. Glenting J, Madsen SM, Vrang A, Fomsgaard A, Israelsen H. 2002. A plasmid selection system in Lactococcus lactis and its use for gene expression in L. lactis and human kidney fibroblasts. Appl Env Microbiol 68:5051–5056.

52. Fu X, Xu J-G. 2000. Development of a chromosome-plasmid balanced lethal system for Lactobacillus acidophilus with thyA Gene as Selective Marker. Microbiol Immunol 44:551–556.
53. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM. 1995. Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269:543–546.

54. Pellemounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F. 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269:540–543.

55. Oh J-H, Alexander LM, Pan M, Schueler KL, Keller MP, Attie AD, Walter J, van Pijkeren J-P. 2019. Dietary fructose and microbiota-derived short-chain fatty acids promote bacteriophage production in the gut symbiont Lactobacillus reuteri. Cell Host Microbe, https://doi.org/10.1016/j.chom.2018.11.016.

56. Ackermann HW. 1998. Tailed bacteriophages: the order Caudovirales. Adv Virus Res 51:135–201.

57. Heidelberger C, Chaudhuri NK, Danneberg P, Mooren D, Griesbach L, Duschinsky R, Schnitzer RJ, Pleven E, Scheiner J. 1957. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. Nature 179:663–666.

58. Ross P, O’Gara F, Condon S. 1990. Thymidylate synthase gene from Lactococcus lactis as a genetic marker: an alternative to antibiotic resistance genes. Appl Environ Microbiol 56:2164–2169.

59. Kim EB, Son JS, Zhang QK, Lee NK, Kim SH, Choi JH, Kang SK, Choi YJ. 2010. Generation and characterization of thymidine/D-alanine auxotrophic recombinant Lactococcus lactis subsp. lactis IL1403 expressing BmpB. Curr Microbiol 61:29–36.
60. Nag N, Rao BJ, Krishnamoorthy G. 2007. Altered dynamics of DNA bases adjacent to a mismatch: a cue for mismatch recognition by MutS. J Mol Biol 374:39–53.

61. Schofield MJ, Hsieh P. 2003. DNA mismatch repair: molecular mechanisms and biological function. Annu Rev Microbiol 57:579–608.

62. Walter J, Britton RA, Roos S. 2011. Host-microbial symbiosis in the vertebrate gastrointestinal tract and the Lactobacillus reuteri paradigm. Proc Natl Acad Sci 108:4645–4652.

63. Kimoto H, Nomura M, Kobayashi M, Mizumachi K, Okamoto T. 2003. Survival of lactococci during passage through mouse digestive tract. Can J Microbiol 49:707–711.

64. Frese SA, Benson AK, Tannock GW, Loach DM, Kim J, Zhang M, Oh PL, Heng NCK, Patil PB, Juge N, MacKenzie DA, Pearson BM, Lapidus A, Dalin E, Tice H, Goltsman E, Land M, Hauser L, Ivanova N, Kyrpides NC, Walter J. 2011. The Evolution of host specialization in the vertebrate gut symbiont Lactobacillus reuteri. PLOS Genet 7:e1001314.

65. Wegmann U, O’Connell-Motherway M, Zomer A, Buist G, Shearman C, Canchaya C, Ventura M, Goesmann A, Gasson MJ, Kuipers OP, Sinderen D van, Kok J. 2007. Complete genome sequence of the prototype lactic acid bacterium Lactococcus lactis subsp. cremoris MG1363. J Bacteriol 189:3256–3270.
66. Kimoto-Nira H, Kobayashi M, Nomura M, Sasaki K, Suzuki C. 2009. Bile resistance in *Lactococcus lactis* strains varies with cellular fatty acid composition: analysis by using different growth media. Int J Food Microbiol 131:183–188.

67. Hendrikx T, Duan Y, Wang Y, Oh J-H, Alexander LM, Huang W, Stärkel P, Ho SB, Gao B, Fiehn O, Emond P, Sokol H, Pijkeren J-P van, Schnabl B. 2018. Bacteria engineered to produce IL-22 in intestine induce expression of REG3G to reduce ethanol-induced liver disease in mice. Gut, 0:1–12. doi:10.1136/gutjnl-2018-317232.

68. Wolf BW, Wheeler KB, Ataya DG, Garleb KA. 1998. Safety and tolerance of *Lactobacillus reuteri* supplementation to a population infected with the human immunodeficiency virus. Food Chem Toxicol 36:1085–1094.

69. Weizman Z, Alsheikh A. 2006. Safety and tolerance of a probiotic formula in early infancy comparing two probiotic agents: a pilot study. J Am Coll Nutr 25:415–419.

70. Jones ML, Martoni CJ, Di Pietro E, Simon RR, Prakash S. 2012. Evaluation of clinical safety and tolerance of a *Lactobacillus reuteri* NCIMB 30242 supplement capsule: A randomized control trial. Regul Toxicol Pharmacol 63:313–320.

71. Jones ML, Martoni CJ, Tamber S, Parent M, Prakash S. 2012. Evaluation of safety and tolerance of microencapsulated *Lactobacillus reuteri* NCIMB 30242 in a yogurt formulation: A randomized, placebo-controlled, double-blind study. Food Chem Toxicol 50:2216–2223.
72. Mangalat N, Liu Y, Fatheree NY, Ferris MJ, Van Arsdall MR, Chen Z, Rahbar MH, Gleason WA, Norori J, Tran DQ, Rhoads JM. 2012. Safety and tolerability of *Lactobacillus reuteri* DSM 17938 and effects on biomarkers in healthy adults: results from a randomized masked trial. PLoS One 7:e43910.

73. Valeur N, Engel P, Carbajal N, Connolly E, Ladefoged K. 2004. Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract. Appl Environ Microbiol 70:1176–1181.

74. Hoy-Schulz YE, Jannat K, Roberts T, Zaidi SH, Unicomb L, Luby S, Parsonnet J. 2016. Safety and acceptability of *Lactobacillus reuteri* DSM 17938 and *Bifidobacterium longum* subspecies *infantis* 35624 in Bangladeshi infants: a phase I randomized clinical trial. BMC Complement Altern Med 16:44.

75. Sambrook J, Russell DW. 2006. Transformation of *E. coli* by electroporation. CSH Protoc 2006.

76. Hall BM, Ma C-X, Liang P, Singh KK. 2009. Fluctuation AnaLysis CalculatOR: a web tool for the determination of mutation rate using Luria–Delbrück fluctuation analysis. Bioinformatics 25:1564–1565.

77. Puigbò P, Guzmán E, Romeu A, Garcia-Vallvé S. 2007. OPTIMIZER: a web server for optimizing the codon usage of DNA sequences. Nucleic Acids Res 35:W126–W131.
78. Puigbò P, Romeu A, Garcia-Vallvé S. 2008. HEG-DB: a database of predicted highly expressed genes in prokaryotic complete genomes under translational selection. Nucleic Acids Res 36:D524-527.

79. Kok S de, Stanton LH, Slaby T, Durot M, Holmes VF, Patel KG, Platt D, Shapland EB, Serber Z, Dean J, Newman JD, Chandran SS. 2014. Rapid and reliable DNA assembly via ligase cycling reaction. ACS Synth Biol 3:97–106.

80. Brogden RN, Carmine AA, Heel RC, Speight TM, Avery GS. 1982. Trimethoprim: a review of its antibacterial activity, pharmacokinetics and therapeutic use in urinary tract infections. Drugs 23:405–430.

81. Cha RS, Zarbl H, Keohavong P, Thilly WG. 1992. Mismatch amplification mutation assay (MAMA): application to the c-H-ras gene. PCR Methods Appl 2:14–20.

82. Qiang YZ, Qin T, Fu W, Cheng WP, Li YS, Yi G. 2002. Use of a rapid mismatch PCR method to detect gyrA and parC mutations in ciprofloxacin-resistant clinical isolates of Escherichia coli. J Antimicrob Chemother 49:549–552.

83. Junop MS, Yang W, Funchain P, Clendenin W, Miller JH. 2003. In vitro and in vivo studies of MutS, MutL and MutH mutants: correlation of mismatch repair and DNA recombination. DNA Repair 2:387–405.

84. Sánchez B, Chaignepain S, Schmitter J-M, Urdaci MC. 2009. A method for the identification of proteins secreted by lactic acid bacteria grown in complex media. FEMS Microbiol Lett 295:226–229.
85. Leenhouts K, Buist G, Bolhuis A, ten Berge A, Kiel J, Mierau I, Dabrowska M, Venema G, Kok J. 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol Gen Genet MGG 253:217–224.

86. Collins J, van Pijkeren J-P, Svensson L, Claesson MJ, Sturme M, Li Y, Cooney JC, van Sinderen D, Walker AW, Parkhill J, Shannon O, O'Toole PW. 2012. Fibrinogen-binding and platelet-aggregation activities of a Lactobacillus salivarius septicaemia isolate are mediated by a novel fibrinogen-binding protein. Mol Microbiol 85:862–877.

87. Kuipers OP, de Ruyter PGGA, Kleerebezem M, de Vos WM. 1998. Quorum sensing-controlled gene expression in lactic acid bacteria. J Biotechnol 64:15–21.

TABLES

Table 1: Bacterial strains and plasmids used in this study

| Strains (Name/VPL) | Characteristics† | Source/Reference |
|-------------------|------------------|-----------------|
| **E. coli EC1000** | Derivative of *E. coli* MC1000 in which repA is integrated in (85) chromosome | |
| **L. reuteri VPL1014** | Human breast milk isolate | Lab stock |
| VPL4090 | Mutant lacking both active phages and attB sites, “Lytic Host” (55) | |
| VPL4121 | Mutant lacking both active phages, restored attB sites, LRΔΦ1ΔΦ2 (55) | |
| VPL4224 | Rif(+) mutant generated with oVPL236 for mutation in rpoB (55) gene (H488R) | |
| VPL4243 | Derived from VPL4224, mutations introduced in thyA(Y38-Q39S,M40L) with oVPL1670, This work | |
| **L. rhamnosus ATCC 53103** | Human fecal isolate | ATCC |
VPL4141 | Rif(+) natural mutant isolated from MRS-Rif25 plate | This work
---|---|---
*L. casei* BFLM218 | Human fecal isolate | (35)
*L. fermentum* ATCC 14931 | Fermented beets isolate | ATCC
*L. plantarum* ATCC BAA-793 | Human saliva isolate | ATCC
VPL4142 | Rif(+) natural mutant isolated from MRS-Rif25 plate | This work
*L. salivarius* CCUG 47825 | Human blood isolate | CCUG (86)
*L. gasseri* ATCC 33323 | Human intestinal isolate | ATCC
*Lactococcus lactis* subsp. cremoris NZ9000 | Dairy starter, derivative of MG1363, pepN::nisRK | (87)
VPL4005 | Rif(+) mutant generated with pVPL234 for mutation in rpoB gene (H486N) | This work
*L. jensenii* ATCC 25258 | Human vaginal isolate | ATCC
*L. acidophilus* ATCC 4356 | Human isolate | ATCC

### Plasmids

| Plasmid | Description | Source |
|---|---|---|
pVPL2042 | Em<sup>R</sup>, pNZ8048 derivative. Cm marker was replaced by Em marker | Van Pijkeren Lab stock |
pVPL3583 | pJP028 vector control (pCtl) | This work |
pVPL3585 | pJP028 derivative, pNZ-EFTu-SP-Leptin | This work |
pVPL3752 | pJP028 derivative, pJP-EFTu-SP-Leptin-3XFLAG | This work |
pVPL3791 | pJP028 derivative lacking signal peptide, pNZ-EFTu-Leptin | This work |
pVPL3795 | pJP028 derivative, pJP-EFTu-Leptin-3XFLAG | This work |
pVPL31131 | pJP028 derivative, pJP-EFTu-Leptin-ThyA | This work |
pVPL31134 | pJP028 derivative, pCtl:ThyA | This work |

VPL: Van Pijkeren Lab strain identification number. pVPL: Van Pijkeren Lab plasmid identification number.

†: *repA*: replication initiation protein; *attB*: phage insertion site; Em<sup>R</sup>: erythromycin-resistant; Cm<sup>R</sup>: chloramphenicol-resistant; Rif(+): rifampicin-resistant; *rpoB*: β subunit of RNA polymerase, homolog of LAR_1402 in *L. reuteri* JCM1112; *thyA*: thymidylate synthase, homolog of LAR_0739 in *L. reuteri* JCM1112. The locus tags listed can be found on https://www.ncbi.nlm.nih.gov.

*: ATCC: American Type Culture Collection
| Oligonucleotide Name | Sequence (5'-3')† | Target/Comment |
|---------------------|------------------|----------------|
| oVPL234             | gagatacaccagggtctacaggcagaaaaagcaggtttgttggtccataaaattg | Recombineering oligo for *L. lactis* rpoB mutant |
| oVPL236             | tcaacaccaggccaagcctgaagagagcctttctgcttcaccataaatgtggtgtttgatccatgaactgg | Recombineering oligo for *L. reuteri* rpoB mutant |
| oVPL329             | attaccttgacctcattactggttaac | Rev, for pJP028 insertion screening |
| oVPL362             | tgtatatgcctctaaatttttaaatg | Rev, for pJP028 insertion screening |
| oVPL363             | taatatgagaataatgccgactgtac | Fwd, for pJP028 insertion screening |
| oVPL736             | tgaatgagttgactcaaccttg | Fwd, amplified pMutL of *L. reuteri* |
| oVPL1199            | atgttatgagaataatgcaaggg | Fwd, amplified signal peptide on pJP028 |
| oVPL1200            | tgtatcagaagcaacactcctc | Rev, amplified signal peptide on pJP028 |
| oVPL1286            | tgtatctggactcataaattag | Fwd, amplified pJP028 backbone |
| oVPL1348            | gttcaattcataaattaggtcagat | LCR bridging oligo for leptin insertion for pJP028:SP:leptin |
| oVPL1349            | acattctggactaactcataattg | Rev, amplified murine leptin from G-Block, codon optimized for *L. reuteri* (77, 78) |
| oVPL1350            | ttcatggggatgaatgcttcctgaatcattactcattccaaagtcaagatgactgtctgcactg | LCR bridging oligo for leptin insertion for pJP028:SP:leptin |
| oVPL1351            | ttacacaaattagatgttttagccccagtttttaaatgggaatccagaaaattagaaagcaagag | LCR bridging oligo for leptin insertion for pJP028:SP:leptin |
| oVPL1408            | agaaaaaccgactgtaaaaaagcag | Rev, amplified backbone of pJP028 for promoter swap |
| oVPL1409            | agaaaaaccgactgtttaaaaaagcagtttttgagtttaatgggaatccagaaaattagaaagcaagag | LCR bridging oligo for swapping in EF-Tu promoter in pJP028 |
| oVPL1410            | gcacgcagaaaattagagtttaaatgggaatccagaaaattagaaagcaagag | LCR bridging oligo for swapping in EF-Tu |

Table 2. Oligonucleotides used in this study
| Primer Identification Number | DNA Sequence | Description |
|-----------------------------|-------------|-------------|
| oVPL1447                    | cgaattaatagaaaaacattagtcaaatc | Fwd, amplified EF-Tu promoter |
| oVPL1448                    | taatgaaaaacctctgataatttacaag | Rev, amplified EF-Tu promoter |
| oVPL1670                    | cgtaaaataggaaaaacctttgttaggtcacaatcaagc | Recombineering oligo for ΔthyA, introducing Y38-Q39S, and M40L; bolded nucleotides introduce 5 adjacent mismatches to wild-type sequence |
| oVPL1671                    | gcatatcttagaataagggctgac | Fwd, for screening of ΔthyA (Y38-Q39S, and M40L) |
| oVPL1672                    | ttgcttaggtcaatcgcaagcctt | Rev, for screening of ΔthyA (Y38-Q39S, and M40L) |
| oVPL1673                    | aaaaatggaacatgtgtgacatgga | Rev, for screening of ΔthyA (Y38-Q39S, and M40L) |
| oVPL1725                    | tttaactgctacgggagccttg | Rev, amplified pMut-thyA |
| oVPL1810                    | atggcttcaattcggggat | Fwd, amplified leptin and added ATG start codon (bold) |
| oVPL2112                    | acatcttttataatcactcggttctttataatc | Rev, amplified leptin and added 3XFLAG tag (bold) to CTD of leptin |
| oVPL2113                    | cacagatagttcataaagatgatgtgataaatgtctttgaaccaaataagg | Fwd, amplified pJP028 backbone and added 3XFLAG (bold) to CTD of leptin |
| oVPL2351                    | taatctgctttgatttgtctatcg | Rev, amplified pJP028 backbone omitting CmR cassette |
| oVPL2352                    | aaggaagataaatcctcataaagggcg | Fwd, amplified pJP028 backbone omitting CmR cassette |

VPL: Van Pijkeren Lab strain identification number. pVPL: Van Pijkeren Lab plasmid identification number. oVPL: Van Pijkeren Lab primer identification number.

* rpoB: β subunit of RNA polymerase, homolog of LAR_1402 in *L. reuteri* JCM1112; thyA: thymidylate synthase, homolog of LAR_0739 in *L. reuteri* JCM1112; LCR – ligation cycle reaction; **bold** – nucleotides added to primer (for example, start codon, stop
codon, FLAG tag). The locus tags listed can be found on https://www.ncbi.nlm.nih.gov.
*: ATCC: American Type Culture Collection

FIG 1 Assessment of potential biotherapeutic delivery vehicles. (a) Mutation rates of selected lactic acid bacteria as determined by the FALCOR method (6). L. reuteri (Lre) exhibits a low mutation rate relative to other lactic acid bacteria. L. reuteri (Lre), L. salivarius (Ls), L. gasseri (Lg), L. fermentum (Lf), L. jensenii (Lj), L. casei (Lc), L. rhamnosus (Lrh), L. acidophilus (La), L. plantarum (Lp), and L. lactis (Ll). *p<0.05, **p<0.01. Results shown are averages of three independent experiments ± standard error. (b) LAB survival following GI transit in a mouse. L. reuteri (LR:rpoB(H488R)), L. rhamnosus, and L. plantarum survived GI transit at least 10-fold better than L. lactis, p<0.001, Tukey’s HSD. Results shown are averages of three independent experiments ± standard error.
**FIG 2** *L. reuteri*-mediated leptin production. (a) Western blot results for intracellularly accumulated leptin indicated that leptin:3XFLAG is produced at the expected size, while the majority of secreted leptin is incorrectly cleaved. (I) LR/pLeptin-3XFLAG (19 kDa) and secreted leptin (SP) LR/pSP-Leptin-3XFLAG (23 kDa). (b) ELISA confirmed leptin production by LR/pLeptin. Ctl = LR/pCtl. Nd = not detected. Results shown are the average of three independent experiments ± standard error, normalized per 1 mg of dry cell pellet weight.
Figure 3

Leptin release from recombinant VPL1014 following Mitomycin C treatment. (a) Plaque forming units derived from leptin-producing VPL1014 culture. No plaque forming units were produced by induced or uninduced LRΔΦ1ΔΦ2/pLeptin. Results shown are averages of three independent experiments ± standard error. (b) ELISA data showing the percentage of total leptin (supernatant + cell lysate) released into the extracellular milieu. Results shown are averages of three independent experiments ± standard error. (c) Growth curves of uninduced LR/pLeptin, uninduced LRΔΦ1ΔΦ2/pLeptin, induced LR/pLeptin, and induced LRΔΦ1ΔΦ2/pLeptin.*p<0.05, **p<0.01, ***p<0.001 (Tukey’s HSD). Results shown are averages of three independent experiments ± standard error.
FIG 4 Plasmid stability of pLeptin-thyA construct in LRΔthyA:rpoB(H488R).

Plasmid stability is represented by the percentage of cells from plain MRS broth that retained pLeptin-thyA, pCtl-thyA, or pLeptin over the course of ~100 generations without antibiotic in the media. Inset: plasmid stability of pLeptin-thyA, pCtl-thyA, or pLeptin from mMRS without thymidine (no beef extract). p<0.01, Tukey’s HSD. Results shown are averages of three independent experiments ± standard error.