Stable Recombinant-Gene Expression from a *Ligilactobacillus* Live Bacterial Vector via Chromosomal Integration

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ABSTRACT Disease control in animal production systems requires constant vigilance. Historically, the application of in-feed antibiotics to control bacteria and improve performance has been a much-used approach to maintain animal health and welfare. However, the widespread use of in-feed antibiotics is thought to increase the risk of antibiotic resistance developing. Alternative methods to control disease and maintain productivity need to be developed. Live vaccination is useful in preventing colonization of mucosa-dwelling pathogens by inducing a mucosal immune response. Native poultry isolate *Ligilactobacillus agilis* La3 (previously *Lactobacillus agilis*) has been identified as a candidate for use as a live vector to deliver therapeutic proteins such as bacteriocins, phage endolysins, or vaccine antigens to the gastrointestinal tract of chickens. In this study, the complete genome sequence of *L. agilis* La3 was determined and transcriptome analysis was undertaken to identify highly expressed genes. Predicted promoter regions and ribosomal binding sites from constitutively expressed genes were used to construct recombinant protein expression cassettes. A series of double-crossover shuttle plasmids were constructed to facilitate rapid selectable integration of expression cassettes into the *L. agilis* La3 chromosome via homologous recombination. Inserts showed 100% stable integration over 100 generations without selection. A positive relationship was found between protein expression levels and the predicted strength of the promoters. Using this system, stable chromosomal expression of a *Clostridium perfringens* antigen, rNetB, was demonstrated without selection. Finally, two recombinant strains, *L. agilis* La3::P_{eff}-rnetB and *L. agilis* La3::P_{cwh}-rnetB, were constructed and characterized, and they showed potential for future application as live vaccines in chickens.

IMPORTANCE Therapeutic proteins such as antigens can be used to prevent infectious diseases in poultry. However, traditional vaccine delivery by intramuscular or subcutaneous injection generally has not proven effective for mucosa-dwelling microorganisms that live within the gastrointestinal tract. Utilizing live bacteria to deliver vaccine antigens directly to the gut immune system can overcome some of the limitations of conventional vaccination. In this work, *Ligilactobacillus agilis* La3, an especially effective gut colonizer, has been analyzed and engineered with modular and stable expression systems to produce recombinant proteins. To demonstrate the effectiveness of the system, expression of a vaccine antigen from poultry pathogen *Clostridium perfringens* was monitored over 100 generations without selection and found to be completely stable. This study demonstrates the development of genetic tools and novel constitutive expression systems and further development of *L. agilis* La3 as a live delivery vehicle for recombinant proteins.

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With a rapidly expanding human population, projected to reach 9.3 to 10.2 billion in 2050 (1, 2), economical and sustainable nutrient sources are vital. Poultry is a source of high-quality protein that can help meet these needs. Broiler chickens have many favorable characteristics, including efficient feed conversion ratio and lower ecological, water, feed, and space requirements than other production animals (3, 4). However, intensively farmed animals must be protected from the ever-present threat of infectious diseases. Broilers are colonized by a variety of chicken and human pathogens, such as *Salmonella enterica* (5), *Campylobacter jejuni* (6), and *Clostridium perfringens* (7, 8), posing challenges for the raising of productive, healthy, and food-safe birds. These bacteria have been controlled with antibiotics (8, 9); however, alternative strategies are required, as antibiotic use is driving the emergence of antibiotic resistance, which is posing a serious threat to human health. This has led to an industry-wide effort to find alternatives, driven by both regulatory requirements and consumer pressure to reduce antibiotic use (10).

For animal applications, conventional bacterin (whole killed cells) and toxoid (culture supernatant) vaccines, delivered by subcutaneous and intramuscular routes, have been widely used and are effective against many pathogens. However, they have been less successful against mucosa-dwelling pathogens. For example, only partial protection of poultry has been shown via the use of toxoid and bacterin vaccines against *C. perfringens* (11–13) and *C. jejuni* (14). Alternative vaccination methods are likely to be more effective, such as live bacterial delivery of vaccine antigens (15–17), which induces a mucosal IgA immune response (17–20) rather than systemic IgY and IgM responses (21). Another strategy to control mucosal pathogens involves the use of therapeutic molecules such as bacteriocins (22–25), host immune factors (26–28), and phage endolysins (29, 30).

Appropriately selected live bacterial vectors can colonize the mucosal layer and deliver therapeutic compounds directly to the ecological niche occupied by mucosal-dwelling pathogens. Live vectors have been administered orally, nasally, or via the lungs (16, 31, 32), making administration inexpensive and rapid. Delivery of vaccine antigens by live vectors, directly to the host immune system within the mucosal layer, can induce the production of secreted IgA antibodies against the vaccination target (33, 34).

Development of a successful live vector ideally requires stable expression of recombinant protein (20, 35). Given the proportional relationship between antigen dose and host immune responses (33, 36), expression stability is likely an important factor in determining vaccine efficacy. Dosage of antigen can be optimized at several levels, including choice of live bacterial vector, stable replication/maintenance of the expression cassette, and expression level of antigens (37–39), but maximizing expression while not reducing genetic fitness of the bacterial vector can be challenging. We hypothesize that the most effective live delivery bacteria are ones isolated from the commensal microbiota of the target host that can colonize and persist and are genetically tailored for constitutive antigen delivery.

The Gram-positive bacterial strain *Ligilactobacillus agilis* La3 (previously *Lactobacillus agilis*) (40) has properties that make it an excellent candidate for use as a live bacterial vector. This specific strain of *L. agilis* is a highly persistent colonizer of broiler chickens (41) and is readily culturable, transformable, and capable of heterologous protein expression (37). It is a non-spore-forming bacillus (42, 43) and is generally regarded as safe. *L. agilis* is commonly found in chickens (41, 44), with chicken isolates *L. agilis* JCM 1048 and *L. agilis* BCRC 10436 demonstrating potential as chicken probiotics (44–46).

The aim of the work reported here was to develop stable gene expression systems within *L. agilis* La3 to enhance its potential for use as a live vector to deliver therapeutic proteins to the gut mucosa of chickens. The *L. agilis* La3 genome and transcriptome

were characterized, and genetic tools were generated for heterologous protein expression, including a method to insert expression cassettes in the *L. agilis* La3 genome. Constitutively expressed native promoters from highly transcribed genes were identified, cloned, and used to direct expression of recombinant protein.

RESULTS

The *L. agilis* La3 genome. The 2,211,023-bp *L. agilis* La3 chromosomal genome was assembled using 88,005,605 bp (39.8× coverage). After polishing with PacBio reads and short-read error correction, the assembled *L. agilis* La3 genome produced a single, circularized 2,187,209-bp chromosomal genomic sequence with 41.3% GC content. *L. agilis* La3 also contains four plasmids, pLa3_1 (7,541 bp), pLa3_2 (4,743 bp), pLa3_3 (3,690 bp), and pLa3_4 (2,676 bp) (see Table S3 in the supplemental material).

The NCBI Prokaryotic Annotation Pipeline produced 2,055 coding sequences (CDS), whereas RAST produced 2,090 (Data Set S1). Within the SEED subsystem, 48% of CDS (990) could be assigned a broad gene function (Table S6). Closer analysis of the “virulence, disease and defense” group showed no known virulence or superantigen genes but mainly genes in the “resistance to antibiotics and toxic compounds” (27), “invasion and intracellular resistance” (11), and “adhesion” (2) groups. The genome also has a number of motility genes (43). The genome was found to contain eight rRNA gene operons. No known bacteriocins were present within the genome. Eight prophage regions within the *L. agilis* La3 genome were identified, only one of which appeared to encode an intact prophage.

The sequenced *L. agilis* La3 is similar to the other two published *L. agilis* strain genome sequences in terms of genome size, number of genes, and GC content (Table S4), despite differing isolation sources. The large core genome (73.48% of total gene content) is partially explained by the low number of genome sequences available for the analysis. The unique genes of *L. agilis* La3 (303) represent 14.74% of the coding sequences and consist of genes of unknown function, phage genes, integrases, and transposases.

RNA sequencing identified constitutively expressed genes. RNA sequence analysis was undertaken on both mid-logarithmic and early-stationary growth phases (Fig. S1) to identify constitutively, highly expressed genes (Fig. 1). The promoters of the most abundantly transcribed genes were targeted for use in recombinant expression systems tailored for use in *L. agilis* La3. These genes likely represent housekeeping genes for which expression occurs constantly throughout the growth cycle. The most abundant transcripts across both mid-logarithmic and early stationary phases were from the genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triosephosphate isomerase, elongation factor Tu, phosphoglycerate kinase, enolase, and a putative secreted protein.

Promoters were identified upstream of the abundantly transcribed genes. Putative promoter sequences were identified from a range of genes that were most actively transcribed (Table 1). For some of the genes, promoter sequences could not be identified, as the genes were embedded within operons, or the promoter sequence was not homologous enough to the consensus sequence to be identifiable.

Chromosomal insertion of expression cassettes via homologous recombination. In recombinant protein production systems, expression cassettes are commonly located on plasmids due to the ease of construction, ability to move between strains, and the possibility of high copy number. We first tested the suitability of a previously developed *Escherichia coli*/LAB shuttle vector, pTRKH2, as a platform for expression but found it to be very unstable in *L. agilis* La3. Therefore, a new *E. coli*/*L. agilis* shuttle vector was constructed by fusing the native *L. agilis* La3 plasmid, pLa3_4, with the *E. coli* plasmid, pGEM-T Easy, to produce the plasmid pVEcLa4. Passaging *L. agilis* La3-carrying plasmids, without antibiotic selection, was used to compare the plasmid stability of pVEcLa4 to the previously used shuttle plasmid pTRKH2 through ≥100 bacterial generations. The new shuttle plasmid, pVEcLa4, was significantly more stable (52.48% ± 19.19%) than pTRKH2 (7.45% ± 1.5%) ($P < 0.01$). Although pVEcLa4 was significantly

FIG 1 Graph showing the transcripts per million reads (TPM) of the most abundant gene transcripts present in *L. agilis* La3 when grown anaerobically at 42°C in MRS. Red bars represent logarithmic transcript abundance, and blue bars represent stationary transcript abundance.

more stable than pTRKH2, it still exhibited some instability; hence, it, too, would be less than ideal as a basis for stable recombinant gene expression in *L. agilis*.

To test the identified promoters and ensure stable replication of expression cassettes independent of selection markers, insertion into the chromosome of *L. agilis* was investigated. Candidate recombination sites in the chromosome were identified based on minimizing the impact on normal cell function and, thus, reducing the likelihood of gene integration events altering the key colonization and persistence characteristics of *L. agilis* La3. An integration site was identified, consisting of a 21-bp noncoding region between two converging transcribed genes, and a hypothetical protein (locus tag BEN83_08880) and a GMP synthase gene (locus tag BEN83_08885) were selected. This was between bp 1766620 and 1766640 within the *L. agilis* La3 chromosome (GenBank accession number [CP016766.1](#)) and did not disrupt any CDS. Insertion at this site would be unlikely to disrupt the upstream regulation of these CDS or impact expression of the surrounding genes. The RNA sequence analysis also revealed low transcriptional abundance across the site (data not shown), indicating that it was unlikely to be involved in production of any regulatory RNAs. The genomic and transcriptomic analyses supported the conclusion that this location was a suitable site for chromosomal integration.

The plasmid pTRKH2-LCER (Fig. 2) and subsequent pVEcXLa-based plasmids were constructed (Table S1) to facilitate chromosomal insertion of expression cassettes by homologous recombination. The pTRKH2 backbone demonstrated low stability within *L. agilis*. This was utilized to drive selection for chromosomal recombination. By passaging *L. agilis* transformed with pTRKH2 derivatives carrying chromosomal integration cassettes in the presence of chloramphenicol, the antibiotic resistance encoded within the integration cassettes was selected, while selection for the plasmid backbone, marked with a different antibiotic resistance gene (erythromycin resistance), was not maintained and was rapidly lost. After chromosomal insertional mutants were generated, both resistance phenotyping (chloramphenicol and erythromycin sensitivity) and PCR (Fig. S2) were used to screen colonies for those carrying the expected chromosomal inserts. The population was usually dominated by bacteria in which chromosomal

TABLE 1 Putative promoter sequences identified based on the Lactobacillus consensus sequence and PPP online portal

Operon/ORF	−35 region	TG region	−10 region	RBS	Start codon	Note	Source
Consensus sequence	TTGACA	Variable (15–20 bp)	TATAAT	Variable (GA rich)	AUG/GUG	Lactobacillus consensus sequences	109
GAPDH	TTGAAA	TTCAAAGTTAGAGATTATTT	GTTAAT...286 bp...	AAAGAGG...7 bp...	AUG	4 potential upstream promoters, start of gap operon	This study
	TTGAAA	GGAAGCAAGTTTAAAG	TAAAAT...222 bp...				
	CTTGCA	AAATATTTCITTTTGTGCTAT	AATCTT...116 bp...				
	TTAACT	GATAGGTAGCGATTGT	TATTAT...62 bp...				
Elongation factor Tu	TTFACA	AAGTTAAATTAGCCTCTA	TATAAT...130 bp...	CAGGAGG...10 bp...	AUG	2 possible upstream promoters	This study
	TTGGTT	TTCTCTTAGGAAAGTAG	TATAAT...55 bp...				
Enolase	TTGAAA	GTCTAAAGGAAAACCTT	TAGAAT...37 bp...	AAGGAGA...9 bp...	AUG	Inside gap operon	This study
Putative secreted protein	TGGTAA	ATTGGGAGAAAAATTATAAT	TATAAT...307 bp...	AGAAGGA...12 bp...	GUG	2 possible upstream promoters and inside operon	This study
	CTGACA	AGCCGGATCCAACGGTTA	AAGACA...88 bp...				
Cell wall-associated hydrolase	TTGTAA	CCTTTTGTACCTAATGCAA	TATTAA...37 bp...	GGGAGTGAA...6 bp...	TTG		This study

FIG 2 Schematic representation demonstrating the double-crossover event in *L. agilis* La3 using pTRKH2-LCER to generate insertional mutant *L. agilis* La3::P_{ldhL}-EGFP via homologous recombination and the corresponding Southern blot. (A) Plasmid map showing the left and right flanks (dark blue) (Continued on next page)

recombination had occurred within one to three subcultures. Chromosomal integration was confirmed to occur through homologous recombination via double crossover, as opposed to single crossover, by using a PCR primer pair 5 (Fig. 2). Chromosomal integration resulted in production of the strain *L. agilis* La3::P_{ldhL}-EGFP. Southern blot hybridization was performed to confirm recombination into the intended recombination site and to confirm that there had been no off-target integration. This was performed using DNA isolated from independent *L. agilis* La3::P_{ldhL}-EGFP colonies before and after they were passaged for ≥ 100 generations to confirm stable insertion (Fig. 2). Southern blot analysis demonstrated a single 2,856-bp band of the expected size. The probe did not hybridize to the negative *L. agilis* La3 wild-type control. Hybridization of the double-crossover insertion mutants before and after ≥ 100 generations under no selection indicated completely stable insertion at the targeted location within the chromosome. Figure S3 shows additional Southern blots proving recombination occurred at a single location within the chromosome.

Positive relationship between promoter strength and protein expression levels. The stable expression system for chromosomal integration was used to evaluate the predicted promoters. The first group of expression constructs that were made to assess promoter strength used the enhanced green fluorescent protein (EGFP) gene as the reporter. However, it was found that random nonsense mutations spontaneously arose at high frequency, indicating that high-level expression of EGFP was toxic to *L. agilis* La3 (data not shown). Therefore, an alternative fluorescent protein, CreiLOV (47), was chosen as a reporter to demonstrate activity of the selected promoters. CreiLOV has the added advantage of fluorescing without the need for oxygen and, hence, is particularly useful as it also has the potential for *in vivo* use in the anaerobic environment of the lower gut, the region where *L. agilis* La3 colonizes and where functional antigen delivery will be directed. The *creiLOV* sequence was codon optimized for *L. agilis* La3 (https://figshare.com/articles/Genbank_files_of_Lactobacillus_agilis_La3_genetic_tools/12111015) and cloned into pTRKH2-LCER to construct the pVEcXLa base plasmid (Fig. 3A).

The relative expression levels of CreiLOV directed by the five selected strong, constitutive promoters (P_{psp}, P_{gapr}, P_{enor}, P_{eftr} and P_{cwh}) within the pVEcXLa plasmid system were measured by relative fluorescence units (RFUs) in both *E. coli* and *L. agilis* La3 as a proxy for protein production levels. Constructs pVEcXLa-P_{psp}, pVEcXLa-P_{gapr}, pVEcXLa-P_{enor}, pVEcXLa-P_{eftr} and pVEcXLa-P_{cwh} were used for this experiment (Table S1). RFUs were measured in both *E. coli* JM109 (expression cassettes on pVEcXLa plasmids) and in *L. agilis* La3 (chromosomally integrated expression cassettes). Protein production in an *E. coli* system was determined, as this provided a promoter screening step before transformation into *L. agilis* La3. The pVEcXLa plasmids were introduced into *L. agilis* La3, and transformants were passaged until chromosomal recombination occurred. Surprisingly, the pVEcXLa-P_{psp} construct never recombined into the *L. agilis* La3 chromosome, despite the only difference between it and the other recombining plasmids being the promoter sequence. Therefore, for the *L. agilis* La3 fluorescence experiment, the plasmid-based expression cassette for the pVEcXLa-P_{psp} construct was used for analysis. When the effect size and relationship between protein expression levels (indicated by number of RFUs) and promoter strength (indicated by relative transcript abundance, or TPM) was examined, there was a strong positive relationship in *E. coli*

FIG 2 Legend (Continued)

allowing recombination of the *catP* and *egfp* genes into the chromosome, replacing 21 bp of noncoding DNA (between bp 1766620 and 1766640). The BEN83_08880 locus tag corresponds to a hypothetical protein while BEN83_08885 corresponds to a GMP synthase, both from the *L. agilis* La3 chromosome. The new mutant was resistant to chloramphenicol at 10 μ g/ml but erythromycin sensitive, as the erythromycin resistance gene (*Erm*) was not integrated. The purple text shows the primer sites (spans integration site using primer pair 7 [Table S2], made up of Crossover F and R Flank R Plas Clone), which allowed identification of *L. agilis* La3 chromosomal recombinants via PCR. (B) Overlay image of Southern blot to agarose gel electrophoresis image of the *L. agilis* La3 chromosomal digested with HindIII performed in biological triplicate. Lane 1, Generuler 1 kb Plus; lanes 2 and 3, *L. agilis* La3 WT; lane 4, *L. agilis* La3::P_{ldhL}-EGFP #1; lane 5, *L. agilis* La3::P_{ldhL}-EGFP #1, passaging ≥ 100 generations; lane 6, *L. agilis* La3::P_{ldhL}-EGFP #2; lane 7, *L. agilis* La3::P_{ldhL}-EGFP #2, passaging ≥ 100 generations; lane 8, *L. agilis* La3::P_{ldhL}-EGFP #3; lane 9, *L. agilis* La3::P_{ldhL}-EGFP #3, passaging ≥ 100 generations. The probe annealed to a band consistent with the expected size of 2,856 bp.

FIG 3 Visualization of the pVEcXLa plasmid and relationship between relative fluorescent units (RFUs) and transcripts per million reads (TPM) of identified promoters. RFUs are a proxy for protein production, while TPMs correspond to the promoter strength of the identified putative promoters. Colored dots show promoters associated with a TPM value: promoter-less pVEcXLa (pink), pVEcXLa-P_{psp} (yellow), pVEcXLa-P_{gap} (dark blue), pVEcXLa-P_{eno} (green), pVEcXLa-P_{eff} (light blue), and pVEcXLa-P_{cwha} (orange). (A) pVEcXLa contains a left and right flank allowing 100% stable, site-directed recombination into the *L. agilis* La3 chromosome. The chromosomal insertion cassette includes a codon-optimized *creiLOV* gene that is surrounded by unique restriction sites for cloning and the *catP* gene to select for chromosomal recombinants on chloramphenicol. Promoters were cloned upstream of CreiLOV to test their impact on protein expression levels. (B) *E. coli* expressing CreiLOV under different *L. agilis* La3 promoters showed a strong, positive relationship ($r=0.9657$, $P=0.0017$, $r^2=0.9325$). Error bars, $\pm$ standard errors of the means (SEM). (C) *L. agilis* La3 expressing CreiLOV did not show a distinct linear relationship ($r=0.6809$, $P=0.1365$, $r^2=0.4637$). For all experiments, $n=3$, performed in biological triplicate. Error bars, $\pm$ SEM.

JM109 ($r = 0.9657$, $P = 0.0017$, $r^2 = 0.9325$) when expressed from the pVEcXLa plasmids (Fig. 3C). The relationship between TPM and RFU was not strictly linear in *L. agilis* La3 ($r = 0.6809$, $P = 0.1365$, $r^2 = 0.4637$) (Fig. 3B), while Hedge's g indicated weak effect sizes were demonstrated for all promoters compared to the promoter-less pVEcXLa negative control (Table S5). High background fluorescence from *L. agilis* La3, combined with low fluorescence intensity of CreiLOV (https://figshare.com/articles/online_resource/Genbank_files_of_Lactobacillus_agilis_La3_genetic_tools/12111015?file=25498142), partially obscured the relationship between promoter strength and protein expression levels that was seen in *E. coli*. However, these results indicated that the promoters and ribosome binding site (RBS) were correctly predicted and could be correlated with expression levels.

Expression of recombinant *C. perfringens* toxin rNetB. With the stable chromosomally integrated native promoters demonstrated to be active, the expression strategy was used to express a relevant vaccine antigen. Recombinant NetB(S254L) [rNetB (S254L)], an inactivated form of the major *Clostridium perfringens* virulence factor for necrotic enteritis in chickens (48), was expressed to further evaluate the suitability of the selected promoters. Here, rNetB(S254L) will be referred to as rNetB. An *L. agilis* La3 codon-optimized *rnetB* sequence was cloned into each of the five pVEcXLa-Promoter-creiLOV plasmids, replacing *creiLOV* (Table S1). The five plasmids pVEcXLa-P_{psp}-rnetB, pVEcXLa-P_{gap}-rnetB, pVEcXLa-P_{eno}-rnetB, pVEcXLa-P_{eft}-rnetB, and pVEcXLa-P_{cwh}-rnetB were transformed into *L. agilis* La3, and then colonies were passaged and screened for chromosomal integration (data not shown). The plasmid-based cassette carrying the P_{psp} promoter, pVEcXLa-P_{psp}-rnetB, again did not result in chromosomal integration, indicating that the lack of integration may, in some unknown way, be related to the P_{psp} promoter. Of the recombinant strains, only *L. agilis* La3::P_{eft}-rnetB, *L. agilis* La3::P_{cwh}-rnetB, and *L. agilis* La3 (pVEcXLa-P_{psp}-rnetB) demonstrated rNetB expression (Fig. 4).

To determine the stability of rNetB chromosomal expression, a stability assay was performed in biological triplicate that showed 100% DNA stability for *L. agilis* La3::P_{eft}-rnetB and *L. agilis* La3::P_{cwh}-rnetB, while pVEcXLa-P_{psp}-rnetB was completely lost from *L. agilis* La3 due to an initial lack of recombination. The stability of rNetB expression was also evaluated. All independent isolates from both *L. agilis* La3::P_{eft}-rnetB (Fig. 4) and *L. agilis* La3::P_{cwh}-rnetB (Fig. 4) demonstrated rNetB expression in all randomly selected colonies from each strain, indicating consistent expression within each isolate population.

The amount of rNetB produced by the *L. agilis* La3 chromosomally integrated constructs was determined by comparison to a dilution series of known quantities of purified rNetB (Fig. S4). Both *L. agilis* La3::P_{eft}-rnetB and *L. agilis* La3::P_{cwh}-rnetB, both before and after passaging, were estimated to produce approximately 6 pg/ μ l.

DISCUSSION

Food safety is a primary concern when evaluating a live vaccine vector candidate for use in food production animals. The analysis of the coding potential of the *L. agilis* La3 genome was consistent with previous literature, which showed *L. agilis* can colonize the gut (49, 50), and there is no evidence of *L. agilis* causing disease (49–51). No known virulence factors were identified in the *L. agilis* La3 genome.

Previously identified as an isolate that can reliably colonize the gut of chickens (52), the transcriptome analysis provided some indication as to why this is the case. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor Tu, and enolase were the first, third, and fifth most abundant transcripts, respectively (Fig. 1). All are implicated in host binding and adhesion (53–55). GAPDH and enolase expressed by *Lactobacillus* species are positively charged and bind to negatively charged components of the cell wall at pH <5.6, human epithelial cells at pH <5, and fibronectin, plasminogen, and mucin, while releasing at higher pHs. Enolase and phosphoglycerate kinase (PGK) have also been shown to be adhesive to mucus (55). Elongation factor Tu is

FIG 4 Western blots of *L. agilis* La3 chromosomal recombinants expressing rNetB (32 to 34 kDa), harvested at early stationary phase (OD_{600} 5 to 6). (A) The outcomes of the five different promoter constructs expressing rNetB, performed in biological triplicate, prior to passaging. Lane 1, Precision Plus Protein Dual Xtra (Bio-Rad); lane 2, *L. agilis* La3 wild type #2 (negative control); lane 3, *L. agilis* La3 WT (negative control); lanes 4 to 6, *L. agilis* La3 (pVecXLa- P_{psp} -rnetB); lane 7, *L. agilis* La3:: P_{gap} -rnetB #2; lanes 8 and 9, *L. agilis* La3:: P_{eno} -rnetB; lanes 10 to 12, *L. agilis* La3:: P_{eff} -rnetB; lanes 13 and 14, *L. agilis* La3:: P_{cwh} -rnetB; lane 15, 0.69 ng rNetB. (B) rNetB expression from four independent *L. agilis* La3:: P_{eff} -rnetB transformants after passaging for ≥ 100 generations without selection. Three colonies from each of the four independent transformants were screened. Lane 1, Precision Plus Protein Dual Xtra (Bio-Rad); lane 2, *L. agilis* La3 wild type; lanes 3 to 6, *L. agilis* La3:: P_{eff} -rnetB transformant #1; lanes 7 to 10, *L. agilis* La3:: P_{eff} -rnetB transformant #2; lanes 11 to 14, *L. agilis* La3:: P_{eff} -rnetB transformant #3; lane 15, 0.69 ng rNetB. (C) rNetB expression from 4 independent *L. agilis* La3:: P_{cwh} -rnetB transformants after passaging for ≥ 100 generations without selection. Three colonies from each of the four independent transformants were screened. Lane 1, Precision Plus Protein Dual Xtra (Bio-Rad); lane 2, *L. agilis* La3 wild type; lanes 3 to 6, *L. agilis* La3:: P_{cwh} -rnetB transformant #1; lanes 7 to 10, *L. agilis* La3:: P_{cwh} -rnetB transformant #2; lanes 11 to 14, *L. agilis* La3:: P_{cwh} -rnetB transformant #3; lane 15, 0.69 ng rNetB.

also a surface protein that has been shown to bind strongly to several human intestinal cell lines and mucin at pH 5 but suffers a significant binding reduction at pH 7 (56). Another potential adhesion CDS in the *L. agilis* La3 genome is *degV*, which binds to short-chain fatty acids (57). The pH within the chicken gastrointestinal tract is 3.47 ± 0.139 in the gizzard, 6.43 ± 0.031 in the small intestine, 6.4 ± 0.057 in the large intestine, and 6.62 ± 0.162 in the cecum (58). However, *Lactobacillus* and, specifically, *L. agilis* have been shown to lower the pH of their environment by producing lactic acid (43, 45, 59). The high transcript abundance of these adherence genes may play a role in the probiotic impact (44, 45) of *L. agilis* by competing for epithelial cell binding sites, thereby reducing the chance of pathogenic infection (60). This, combined with the high transcription levels for lactic acid-producing L-lactate dehydrogenase, the 16th most abundant transcript in *L. agilis* La3 (Fig. 1), which has been confirmed experimentally via a reporter gene (37), means *L. agilis* La3 can alter its environment to increase acidity, thereby improving its adhesive abilities. The acidic environment produced may also reduce the ability of potential pathogens to gain colonization footholds by eroding their niche, which is more suited to itself and other lactic acid bacteria. One study showed a probiotic mix of *L. agilis* JCM 1048 (ATCC 43564) and *Ligilactobacillus salivarius* JCM 1230 inhibited growth of *Salmonella* spp. and *Campylobacter jejuni*, both of which make up most poultry-related foodborne illness incidences (61–63). It is hypothesized that the inhibitory activity is due to acid production. When the probiotic mix was given to chickens, a short-term significant increase of *Lactobacillus* isolates was

noted (44–46). The potential probiotic effects are not due to bacteriocins in *L. agilis*. Therefore, most likely *L. agilis* La3 modulates its environment to allow adhesion and successful colonization.

The presence of motility factors such as flagellar proteins (an important characteristic of the *L. agilis* strain) (43) likely allows *L. agilis* La3 to move through the mucosal layer of the gastrointestinal tract of the chicken. Combined with the bind-and-release mechanism of highly abundant host cell and mucus adhesion proteins, such as GAPDH, enolase, and elongation factor Tu, *L. agilis* La3 seems to be a highly flexible organism. Along with its ability to modulate the surrounding environment, this could partially explain why *L. agilis* is a highly specialized gastrointestinal colonizer across many different animals, including chickens (41, 44, 46, 64, 65), humans (49, 50), horses (66), pigeons (67), pigs, lemurs, and tapirs (42, 55, 64, 68). These adherence and motility genes may explain why *L. agilis* La3 is suitably adapted to persisting within the chicken gastrointestinal tract throughout the life of a chicken, across a diverse range of microbial populations and diets (41).

The transcription profiling of *L. agilis* La3 was undertaken to identify strong promoters. The goal was to identify constitutively transcribed promoters that were likely to be active *in vivo*, when *L. agilis* was colonizing the chicken gut gastrointestinal tract. It was not possible to obtain *L. agilis* cells from that *in vivo* environment, so, to increase the likelihood of identifying constitutively expressed genes, two different *in vitro* growth phases were investigated. The five predicted promoters (Table 1) associated with highly transcribed gene activity across different growth phases were confirmed to be promoters (and RBS) by demonstrating a positive relationship between transcript abundance and protein expression levels of CreiLOV. The relationship between increasing antigen dose and immune response has been previously shown (33, 34, 69, 70), so the success of a live vaccine relies heavily on the expression level of the antigen. These *L. agilis* La3 promoters all have higher TPM values than the L-lactate dehydrogenase promoter (P_{ldh}) previously used in *L. agilis* La3 expression constructs (37), which was found to be the 16th most abundant transcript (Fig. 1). The GAPDH promoter (P_{gap}) produced the highest constitutive transcript level and has been used previously to express high levels of heterologous protein in the algae *Dunaliella salina* (71). This promoter also produced the highest levels of CreiLOV in both *E. coli* JM109 and *L. agilis* La3 (Fig. 3B and C); however, no rNetB expression was identified. It is unclear why this occurred, although expression above a particular amount may be deleterious to *L. agilis* La3. Curiously, the relationship between promoter strength and protein expression of the predicted *L. agilis* La3 native promoters showed a stronger, positive relationship in *E. coli* than in *L. agilis* La3. It is possible that a more accurate measurement of CreiLOV expression in *L. agilis* La3 can be determined using antibody-based methods (72). Regardless, a moderately positive relationship ($r^2 = 0.4637$) was found between promoter strength and number of RFUs (Fig. 3B and C), indicating that a stronger promoter resulted in higher CreiLOV expression.

The pTRKH2-LCER and pVEcXLa chromosomal insertion system proved to be highly successful. Chromosomal recombination has been demonstrated many times within *Lactobacillus* species through site-specific *attP-attB* recombination (73), and homologous recombination (74) and has been occasionally used in developing live vectors (20, 75). The constructed pVEcXLa allowed stable chromosomal integration and formation of new *L. agilis* La3 strains, confirmed via antibiotic resistance profile, PCR, and protein expression. Surprisingly, the pVEcXLa- P_{psp} plasmid did not allow chromosomal integration despite passaging for >200 generations. The mechanism at work here is unknown but may be a sequence-specific effect of the 151-bp promoter region. Formation of DNA secondary structures was previously shown to impact recombination as well as gene regulation (76–78).

We utilized a validated vaccine antigen to demonstrate the potential of *L. agilis* La3 and pVEcXLa as a delivery platform. The necrotic enteritis essential virulence factor, NetB, is a 33-kDa protein (79). rNetB(S254L) is a reduced-activity mutated derivative of

NetB (48) that has been previously used as an immunogenic vaccine antigen, resulting in partial protection against necrotic enteritis (11, 13, 80–82). Successful demonstration of chromosomal rNetB expression by *L. agilis* La3, a native chicken colonizer, regulated by a highly active, constitutive, and native promoter demonstrates the potential value of *L. agilis* La3 as a live delivery vector. Three of the five constructed plasmids resulted in expression of rNetB (pVEcXLa-P_{psp}-rnetB, pVEcXLa-P_{eff}-rnetB, and pVEcXLa-P_{cwah}-rnetB), with the latter two demonstrating 100% stability, both in terms of the chromosomal insert and rNetB expression levels.

The chromosomal insertion site was chosen partially based on its relatively low level of transcription. The working hypothesis was that by utilizing a lowly transcribed region with no identified protein coding sequences, the chances of an insertion in the region disrupting any normal cellular function would be low. Considering the results obtained, it is likely this low transcriptional area has structural barriers to high expression and may explain the low levels of rNetB expression in *L. agilis* La3::P_{eff}-rnetB and *L. agilis* La3::P_{cwah}-rnetB. Variation in protein expression of up to around 300-fold has been demonstrated by inserting an identical expression cassette into multiple different regions of the *E. coli* chromosome (83). This shows the importance of chromosomal location for therapeutic gene expression. However, given that the CreiLOV expression results indicated a positive relationship between transcript abundance and protein expression levels, it is likely that they are the strongest promoters. The data presented here showed that promoters and ribosomal binding sites taken from highly transcribed genes do not equate to high expression levels when expressed in a different place within the chromosome.

In summary, we have constructed novel expression systems for the highly effective poultry gut colonizer *L. agilis* La3, allowing stable expression of recombinant protein. Useful extensions of this work to further develop *L. agilis* La3 as a live vector for poultry should include efforts to express a variety of vaccine antigens from notable pathogens such as *C. perfringens* or *C. jejuni*, as well as assessment of alternative chromosomal insertion sites to maximize recombinant protein expression, before testing the developed live vaccine strains in animal vaccination experiments.

MATERIALS AND METHODS

Growth conditions. *L. agilis* La3 (41) was grown anaerobically at 37°C in de Man, Rogosa, and Sharpe (MRS) medium (Oxoid), with antibiotics as required (unless otherwise stated, chloramphenicol, 10 µg/ml; erythromycin, 10 µg/ml). All *Escherichia coli* strains were grown at 37°C in lysogeny broth (LB) medium (1%, wt/vol, tryptone, 1%, wt/vol, NaCl, and 0.5%, wt/vol, yeast extract [Oxoid]) and shaken at 250 rpm. Antibiotics were added as required (chloramphenicol, 30 µg/ml; erythromycin, 10 µg/ml). Growth curves were performed as described in Data Set S1 in the supplemental material.

Plasmid construction. Plasmids were constructed via restriction enzymes (NEB) and T4 ligase (NEB) and are summarized in Table S1. Where stated, fragments used for cloning were PCR amplified using Platinum *Taq* (Invitrogen) per the manufacturer's instructions using primers detailed in Table S2. Constructs were confirmed by Sanger sequencing. Plasmids were isolated from *E. coli* JM109 using an ISOLATE II plasmid minikit (BIOLINE) kit per the manufacturer's instructions. Gel electrophoresis and transformation of *E. coli* were performed as described in Data Set S1.

Transformation of *L. agilis* La3. *L. agilis* La3 transformation was performed using a modified version of a previously published method (37). Briefly, overnight 30-ml cultures of *L. agilis* La3 were grown and inoculated 1:6 into fresh MRS liquid medium with 2% (wt/vol) glycine and grown at 37°C for 90 min. Cells were then centrifuged at 4,000 × *g* for 2 min at 4°C and supernatants removed via pipetting. Cells were washed with 40 ml of ice-cold ultrapure water and then resuspended in 40 ml ice-cold 50 mM EDTA and incubated for 5 min on ice. Cells were then washed in 40 ml 0.3 M sucrose and resuspended in 100 µl 0.3 M sucrose containing 3 µg plasmid DNA. This mixture was added to an ice-cold Gene Pulser 0.2-cm electroporation cuvette (Bio-Rad). Cells were electroporated using a Gene Pulser and Pulse Controller (Bio-Rad) at 1.5 kV, 200 Ω, and 25 µF. Cells were immediately diluted 1:20 into 1.3 ml of pre-warmed MRS liquid medium in 1.5-ml microtubes and incubated for 3 h at 37°C. Culture (200 µl) was gently spread onto MRS plates containing appropriate antibiotics and incubated anaerobically at 37°C for 24 to 72 h. All solutions were sterilized through a 0.22-µm Millex-GP syringe filter unit (Merck) before use. Plasmid pTRKH2 (Table S1) was used as a positive DNA control for transformations. DNA stability assays and Southern blotting were performed as described in Data Set S1.

Genome sequencing and assembly. For long-read data, *L. agilis* La3 was grown overnight in 10 ml MRS and 10 µl subcultured into five 1.5-ml MRS broth samples until mid-log phase (optical density at 600 nm [OD₆₀₀] of 1.8). DNA was extracted using the MagAttract HMW kit (Qiagen) per the manufacturer's instructions. DNA was concentrated and cleaned using Agencourt AMPure XP beads (Beckman Coulter) per the manufacturer's instructions, with one exception: 0.5 µl instead of 1.8 µl beads per 1 µl

of DNA was used and eluted into 84 μ l ultrapure water. Size selection at 20 kb was performed using BluePippin size selection (Sage Science), and DNA was prepared for sequencing using SMRTbell Template Prep kit 1.0 (Pacific Biosciences) and sequenced on the PacBio RS II at Macrogen (South Korea). For short-read data, chromosomal DNA was isolated from *L. agilis* La3 using the ISOLATE fecal DNA kit (Bioline) per the manufacturer's instructions. The Nextera XT DNA Library Prep kit (Illumina) was used per the manufacturer's instructions to prepare DNA for sequencing, with size selection at around 600 bp. The MiSeq was run using the 2 $\times$ 300-bp reagent kit, and 588,547,918 raw bases and 2,736,962 raw reads were generated. The *L. agilis* La3 genome is approximately 2.2 Mb, indicating 267-fold coverage. Sequence data and accession numbers from this study can be found in Table S3. DNA was also sequenced on the Genome Sequencer FLX system (454 Life Sciences/Roche, Branford, CT, USA) using 3-kb paired-end reads.

Sequencing data were analyzed on Biolinux 8 (84). Output PacBio bax.h5 files were concatenated into a .bam file using bax2bam version 0.0.8 (pitchfork release, 6 October 2016) (85) and then converted to fastq format using the bamToFastq command from bedtools version 2.17.0 (pitchfork release, 6 October 2016). All PacBio reads were incorporated for assembly using Canu Assembler version 1.3 with the following options: "stopOnReadQuality=false", "genomeSize=2.2m", and "-pacbio-raw" (86). The beginning and end of the assembled *L. agilis* La3 molecule were aligned against each other using BLAST (87–89) to determine if the molecule could be circularized. Seqret (EMBOSS version 6.6.0.0) was used to reformat the circularized fasta file to appropriate fasta format. Raw PacBio reads were aligned using blasr version 5.2.b99b47c (pitchfork release, 6 October 2016) (85) and output in .bam format to confirm correct circularization and polish the genome assembly. The aligned .bam file was sorted and ordered using the "sort" command in SAMtools, version 1.3.1 (90). The sorted alignment .bam file was indexed using pbindex version 0.5.0 (pitchfork release, 6 October 2016). Mapped reads were then visualized on Tablet (version 1.14.04.10) (91). Indexing of the *L. agilis* La3 genome file was done using the "faidx" command from SAMtools, version 1.3.1. The genome file was then polished using the "arrow" command from variantCaller, version 2.0.0 (pitchfork release, 6 October 2016) (92). To improve the accuracy of the genome, the MiSeq data were integrated into the polished genome for short-read error correction. An index of the genome was created using the "index" command from bwa, version 0.7.12 (93). The MiSeq output fastq files were concatenated using the "cat" command from GNU coreutils, version 8.21, and then aligned to the genome using the "mem" option from bwa, version 0.7.12. The resulting sam file was converted to .bam format, sorted, and indexed using the "view", "sort", and "index" commands, respectively, from SAMtools, version 1.3.1. The indexed mapped reads were used as the input for Pilon version 1.18 (94) for short-read error correction of the *L. agilis* La3 genome.

Annotation. The complete genome sequence was submitted to GenBank for the Prokaryotic Genome Annotation Pipeline (95). The genome was also submitted to RAST for annotation and SEED subsystem information (96–98). BAGEL3 BLASTN was used to identify potential bacteriocins present in the *L. agilis* La3 genome (99). Prophage regions were identified using PHAST (100).

Comparison to other *L. agilis* strains. Sequences of *L. agilis* strains were downloaded from NCBI GenBank, and genome statistics were compared. BPGA (version 1.3) (101) was used to compare the core and pangenomes at 70% similarity, as typically used in other comparative genomics studies (102).

Codon usage. A codon usage table was generated using cusp (EMBOSS, version 6.6.0.0) after extracting the coding sequences (CDS) from the annotated *L. agilis* La3 genome.

RNA isolation from *L. agilis* La3. RNA was isolated from the mid-logarithmic-phase (OD_{600} , 3.5) and early-stationary-phase (OD_{600} , 5.7) cultures to identify constitutively expressed genes. All reagents for RNA isolation and purification were made using diethyl pyrocarbonate (DEPC)-treated water to ensure no RNases would be active. *L. agilis* La3 was grown anaerobically at 42°C in a Whitley A35 anaerobic workstation. One milliliter of culture was taken, and two volumes of RNeasy lysis buffer (Qiagen) were added to sample and then vortexed for 5 s. The tubes were then centrifuged for 10 min at 5,000 $\times g$ and the supernatant discarded. A volume of 100 μ l lysozyme solution (1% 2-mercaptoethanol, 10 mM Tris-Cl, 1 mM EDTA, 15 mg/ml lysozyme, pH 8.0) was added and vortexed for 10 s and then incubated at room temperature for 1 h. Buffer RLT (Qiagen) was added (700 μ l) and vortexed for 10 s, and then the suspension was added to 2-ml screw-cap tubes containing 50 mg 0.1-mm zirconia/silica beads (Daintree Scientific). Samples were homogenized at maximum speed for 5 min in the Precellys 24 lysis and homogenization instrument (Bertin Technologies), and then samples were centrifuged at 14,000 $\times g$ for 10 s. Supernatant was kept, and 440 μ l 100% ethanol was added. The RNeasy minikit (Qiagen) was used to purify RNA per the manufacturer's instructions. Quality was determined using a NanoDrop ND1000 (ThermoScientific).

cDNA synthesis and library preparation. cDNA was prepared using 3.35 nmol random pentadecamer primers (103) and SuperScript II (400 U) (Invitrogen), and NEBNext second-strand synthesis enzyme mix (NEB) was used for second-strand synthesis per the manufacturer's instructions. Primers and small fragments were removed using the MinElute reaction cleanup kit (Qiagen). The Nextera XT DNA sample preparation kit (Illumina) was used per the manufacturer's instructions, with the following exceptions. The initial amount of DNA was halved, and during PCR cleanup, 25 μ l AMPure XP beads was added to size select fragment sizes of 500 to 700 bp, confirmed by analysis on the 2100 Bioanalyzer (Agilent) using the high-sensitivity DNA quick kit (Agilent) per the manufacturer's instructions.

RNA sequencing and analysis. Logarithmic and stationary-phase libraries were sequenced (2 $\times$ 300 bp) on a MiSeq (Illumina) with v3 sequencing chemistry per the manufacturer's instructions (104). Two million reads were taken as a subsample from the output fastq files and quality trimmed using FastqMcf, version 1.04.636, from ea-utils (105) with the following options: "-l 50 -q 15 -w 5 -H -X -homopolymer-pct 50 -low-complex-pct 70." The build command from Bowtie2 version 2.2.3 was used to build a reference genome index file and map the trimmed reads to the reference genome. SAMtools, version 0.1.19, was used to

convert to .bam, sort, and index files. Artemis (106) was used to visualize the mapped reads against the genome and calculate reads per kilobase of transcript per million mapped reads (RPKM). Numbers of transcripts per million reads (TPM) (107, 108) were calculated using the following formula: $TPM = (RPKM \text{ of feature} / \text{total RPKM}) \times 10^6$.

Fluorescent quantification of CreiLOV expression. Freshly transformed *E. coli* JM109 and *L. agilis* La3 were grown to mid-logarithmic phase (OD_{600} 0.4 and 3.5, respectively) in biological triplicate. Cell density was normalized in $1 \times$ phosphate-buffered saline (PBS) to an OD_{600} of 0.3 and 0.1, respectively. Whole-cell lysate from 5-ml cultures of *E. coli* and *L. agilis* La3 was obtained by resuspending cells in 1 ml $1 \times$ PBS and bead-beating in 2-ml screw-cap microtubes with 250 μm^3 0.1-mm zirconia/silica beads (BioSpec) at 6.5 m/s for 60 s using a FastPrep-24 classic instrument (MP Biomedicals). Lysed cells were then centrifuged at $2,000 \times g$ for 10 s. Supernatant (200 μl) was transferred to a 96-well black CellStar plate (Greiner), and CreiLOV expression levels were quantified using relative fluorescent units (RFUs) in technical triplicates. Fluorescence intensity was determined using a POLARstar Omega plate reader spectrophotometer and 360- to 10-nm excitation and 490- to 10-nm emission filters. The gain was set on the sample with highest fluorescence and $1 \times$ PBS was used as a blank. Wild-type *E. coli* JM109 and *L. agilis* La3 were used as negative controls. Data were then analyzed using GraphPad Prism (version 7.02). Numerical RFU values were analyzed using one-way analysis of variance (ANOVA), and Dunnett's test was done to correct for multiple comparisons. Linear regression analysis was used to correlate number of RFUs to TPM. Hedge's g was calculated using <https://www.polyu.edu.hk/mm/sizeeffectcalculator/calculator.html>.

Quantification of rNetB expression. Freshly transformed *L. agilis* La3 was grown to mid-logarithmic phase (OD_{600} 3.5) in biological triplicate, and then cell density was normalized in $1 \times$ PBS to an OD_{600} of 0.1. Whole-cell lysate was electrophoresed on 4% to 20% Novex Wedgewell Tris-glycine gels (ThermoFisher Scientific) per the manufacturer's instructions. Gels were either directly stained with Coomassie blue or used for Western blotting. Protein was transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were washed with 40 ml blocking solution (50 mM Tris base, 150 mM NaCl [pH 7.5], 0.05% Tween 20, 5%, wt/vol, skim milk powder). To preabsorb nonspecific, non-anti-rNetB antibodies, a primary antibody solution was prepared by adding 50 μl *L. agilis* La3 wild-type and *E. coli* JM109 wild-type whole-cell lysates to fresh antibody solution (1/500 dilution of rabbit polyclonal IgG antibody serum [anti-rNetB] in blocking solution). This was incubated at room temperature for 1 h and then centrifuged at $3,500 \times g$ for 10 min and the supernatant kept. The membrane was probed with this solution. The membrane was washed 3 times for 10 min in 40 ml TBST (50 mM Tris base, 150 mM NaCl [pH 7.5], 0.05% Tween 20) and then incubated with the secondary antibody solution (1/8,000 dilution of goat polyclonal antibody serum [anti-rabbit IgG]-AP [ThermoFisher Scientific]) in blocking solution. The membrane was then washed 3 times for 10 min in 40 ml TBS (50 mM Tris base, 150 mM NaCl [pH 7.5]) and the blot developed with 1.5 ml Western blue (Promega). When the desired level of development had been achieved, the blot was washed with deionized water. To determine stability of rNetB expression, *L. agilis* La3 was passaged for ≥ 100 generations without selection, and then Western blots were performed as described above. To determine protein concentration, rNetB production was semi-quantitatively compared to dilutions of known quantities of purified rNetB via Western blotting.

Promoter identification. Promoters were identified from the most abundant transcripts via visual comparison to a *Lactobacillus* promoter consensus sequence (52, 109) by searching upstream of identified genes.

Data availability. All data utilized in this study are found in the supplemental material. GenBank accession numbers can be found in Table S3. Briefly, the assembly accession numbers are the following: chromosomal DNA of *L. agilis* La3, [CP016766.1](#); pLa3_1, [CP034226](#); pLa3_2, [CP034227](#); pLa3_3, [CP034228](#); and pLa3_4, [CP034229](#). The DNA sequencing reads can be found with accession numbers [SRX4927791](#) and [SRX4927790](#) for PacBio RS II and 2×300 MiSeq, respectively. RNA sequencing data can be found with accession numbers [SRX4927788](#) and [SRX4927789](#) for logarithmic and stationary phases, respectively. DNA methylation data and motifs generated from PacBio reads can be found at <http://rebase.neb.com/cgi-bin/onumget?25455>. Synthetic plasmid and CreiLOV sequences can be found at https://figshare.com/articles/Genbank_files_of_Lactobacillus_agilis_La3_genetic_tools/12111015.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.2 MB.

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