

upp-Based Counterselective Genetic Cloning to Create Rotavirus Vaccine
Constructs Using *Lactobacillus acidophilus* Platform

Honors Thesis

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Abstract

Rotavirus is a major public health burden that causes severe gastroenteritis and kills more than 200,000 infants per year. Live attenuated vaccines have reduced effectiveness in low- and middle-income countries, which is correlated to gut microbiota composition. To combat this, we are developing an orally administered vaccine using the probiotic *Lactobacillus acidophilus* as a vector. Here, we used up-based genetic cloning to create multiple strains of *L. acidophilus* that express rotavirus' VP7 glycoprotein behind the highly expressed metabolic enzyme enolase. This counterselective genetic cloning process uses a temperature-sensitive helper plasmid and recombination-directing vector plasmid to integrate target sequences into the bacterial genome and remove selectable markers. Vaccine constructs were evaluated for recombination using genetic analysis and immunoassays. The vaccines are being tested in murine and porcine models to evaluate their ability to protect hosts against rotavirus.

Introduction

Rotavirus

Rotavirus infection causes acute gastroenteritis and is responsible for more than 200,000 deaths annually in children under the age of five¹. The disease is characterized by dehydration and severe diarrhea, resulting from viral damage to small intestine enterocytes². It spreads via fecal-oral transmission³. Causing 37% of all diarrheal-related deaths in the world, rotavirus is a major public health burden¹. While this virus is endemic worldwide, low- and middle-income countries have elevated mortality rates (Figure 1)². Almost half of all rotavirus deaths occur in 4 developing countries: India, Nigeria, Pakistan, and the Democratic Republic of the Congo¹.

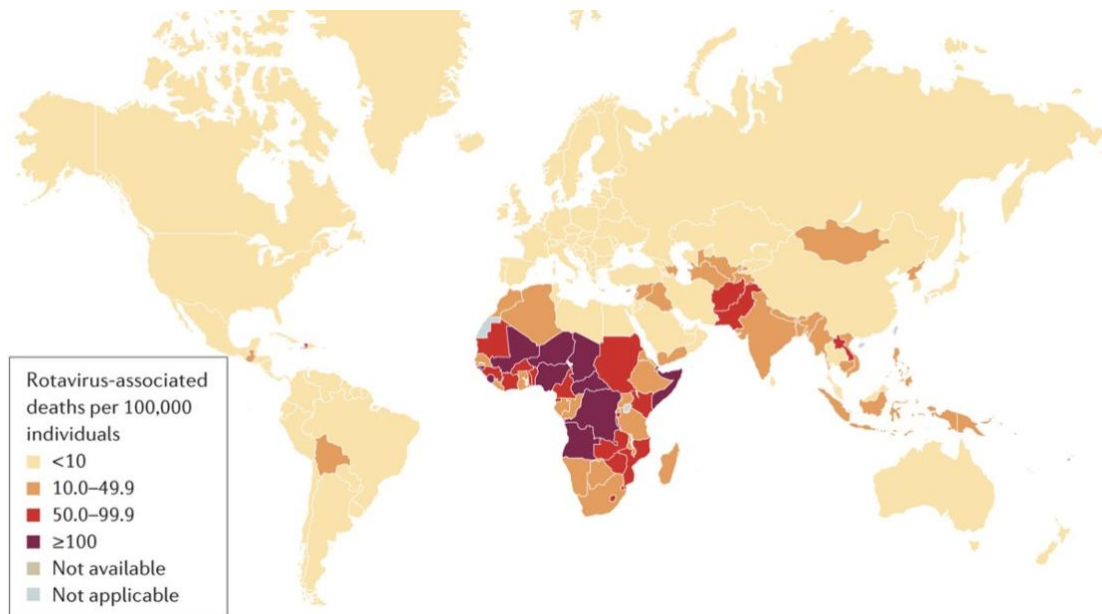


Figure 1: Distribution of rotavirus-associated deaths worldwide. Adapted from Crawford S. E. *et al.* (2017) Rotavirus infection. *Nat. Rev. Dis. Primers*. Developing countries, especially in sub-Saharan Africa, have high mortality rates in rotavirus infections. High-income countries have a reduced burden of rotavirus-associated deaths.

Rotavirus is a member of the virus family *Reoviridae*³. As such, it has a double stranded RNA virus genome. The genome is split into 11 segments and encodes 12 proteins (six structural, six non-structural), one from each segment except for the final segment that encodes two². Rotavirus does not have an envelope, but while most viruses have a capsid with one layer to protect genetic material, rotaviruses have a three-layered capsid (Figure 2)³. This stable casing confers protection for the enteric pathogen to traverse the gastrointestinal tract without being harmed by acid or digestive enzymes.

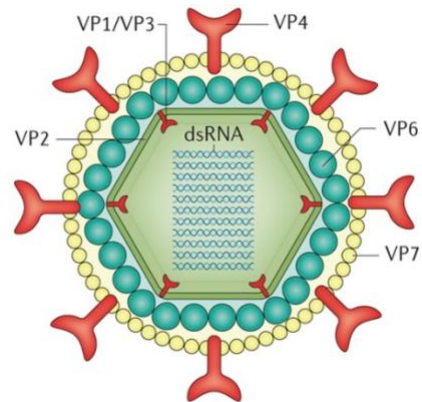


Figure 2: Rotavirus structure. Adapted from Crawford S. E. *et al.* (2017) Rotavirus infection. *Nat. Rev. Dis. Primers*. Rotavirus' three capsids are made of different structural proteins. VP4 and VP7 are on the outer capsid layer, VP6 is in the middle capsid layer, and VP2 is in the inner capsid layer. The core houses rotavirus' segmented double stranded RNA genome with the aid of VP1 and VP3.

Rotavirus' outermost proteins, VP7 and VP4, are immunologically relevant for vaccine development³. VP7 (G) is a glycoprotein that forms the shell of the outer capsid layer⁴. VP4 (P) is a protease-cleaved spike protein that extends from the capsid and attaches to host cells to initiate infection⁴. VP4 is cleaved into VP5 and VP8 domains to expose binding sites and facilitate entry into host cells, which is led by VP8 binding². Due to their accessibility and function in initiating viral infection, VP7 and VP4 are targets of neutralizing antibodies³. Consequently, these proteins are the foundation for protective vaccines.

Current Rotavirus Vaccines

While there are four WHO pre-approved live-attenuated, oral rotavirus vaccines, there are two, RotaTeq (2006) and Rotarix (2008), that are predominantly administered worldwide^{2,4}.

Administration of these vaccines has significantly reduced rotavirus-associated deaths. In 2000,

there were more than 500,000 deaths every year from rotavirus, and this was reduced to 215,000 in 2013¹. RotaTeq is pentavalent, containing five rotavirus strains, while Rotarix is monovalent and only has one². However, both contain VP7 and VP4 antigens from the most common rotavirus strains in order to provide broad protection^{2,3}. These vaccines are safe and licensed for administration in more than 100 countries³. RotaTeq and Rotarix exhibit high effectiveness in high-income countries but have reduced effectiveness in low- and middle-income countries (LMIC). For example, clinical trials of RotaTeq in developed countries showed that the vaccine reduced severe gastroenteritis cases by 98%³. However, this dropped to 48.3% and 39.3% when administered in Asia and sub-Saharan Africa respectively. Similarly, Rotarix effectiveness decreases from 84% to 57% when comparing developed countries and LMIC⁴. There is a need for continued research to address this gap in vaccine efficacy.

While it not fully understood why licensed vaccines are less effective in LMIC, there is a correlation between microbiota composition in the gastrointestinal tract and vaccine effectiveness. One study compared microbiomes in Dutch and Pakistani infants to antibody production after Rotarix vaccine administration⁵. They found that Dutch and Pakistani infants who responded well to the vaccine had similar microbiome compositions, notably with a 2.6-fold higher ratio of Gram-negative bacteria to Gram-positive bacteria compared to Pakistani non-responders. Responders tended to have increased levels of Firmicutes and Proteobacteria, particularly *Clostridium* as well as members of the Enterobacteriaceae family, such as *Serratia* spp. and *Escherichia coli*. In a similar study comparing Dutch and Ghanian infants, vaccine responders had a larger relative population of Bacilli, especially *Streptococcus bovis*, and a smaller population of *Bacteroides* and *Prevotella* compared to non responders⁶. It was

hypothesized this was due to Bacteroidetes having a less immunogenic lipopolysaccharide (LPS) than Enterobacteriaceae. Despite correlations in microbial populations, neither of these studies found a significant influence of nutrition status on vaccine response^{5,6}.

Lactic Acid Bacteria

Lactic acid bacteria (LAB) possess many intrinsic qualities that allow them to effectively colonize gastrointestinal tracts and support their hosts. LAB, including *Lactobacillus* spp., *Lactococcus* spp., and *Streptococcus* spp., are Gram-positive, non-spore forming bacteria that are commensal to the gut microflora⁷. They are considered by the Food and Drug Administration to be generally regarded as safe (GRAS) due to their probiotic properties that confer health benefits to the consumer⁷. Probiotics aid their hosts through diverse mechanisms, including secreting antimicrobial compounds, improving epithelial barrier integrity, and supporting intestinal immunity⁸. LAB are well adapted to host environments as shown by their acid-tolerance and mucoadhesive properties^{7,9}.

Lactobacillus spp. probiotics have been studied in their protection against rotavirus infection and enhancement of rotavirus vaccine efficacy. It was demonstrated in cell culture experiments that *L. casei* secretes a soluble factor that increases host surface expression of galactose, which blocks rotavirus infection¹⁰. Probiotics can also mitigate damage from rotavirus infections. Coculture of *L. rhamnosus* GG with rotavirus-infected epithelial cells showed decreased production of reactive oxygen species (ROS) and IL-6, a pro-inflammatory cytokine, both of which can increase inflammation and disrupt cell junctions¹¹. Treatment with probiotics also improves acute diarrhea symptoms in infected children, reducing duration of symptoms by 1-2

days^{2,3}. These benefits also translate to improved responses against rotavirus vaccines. Pigs colonized with *L. acidophilus* and *L. reuteri* experienced increased levels of interferon-gamma producing CD4⁺ T cells, which enhanced antigen-specific B and T cells responses¹². In India, infants that received *L. rhamnosus* GG in tandem with the Rotarix vaccine had modestly higher seroconversion rates, especially when additionally supplemented with zinc¹³. This was also seen in Finnish infants with administration of a candidate rotavirus vaccine¹³. In this way, *Lactobacillus* spp. are a natural adjuvant that help protect hosts against rotavirus and other enteric pathogens.

Due to *Lactobacillus* spp.'s innate protective and immunomodulating properties, they are emerging as viable rotavirus vaccine platforms. It is easy to modify *Lactobacillus* spp. genomes to express rotavirus antigens that stimulate a continuous, mucosal immune response as the bacteria traverse through and colonize the gastrointestinal tract⁹. Vaccines made in this way have robust safety profiles, are inexpensive, and have simple methods of administration and storage (there is no cold chain requirement)¹⁴. *Lactobacillus* spp. can effectively be harnessed as vaccine vectors for targeted immune induction to address microbiota-related deficiencies of current rotavirus vaccines.

Vaccine Construction

The goal of this project was to develop a series of recombinant *Lactobacillus acidophilus* bacteria strains that express either a rotavirus antigen or the model immune-stimulating protein, ovalbumin. These constructs can be used to evaluate *L. acidophilus* as a carrier of immunogenic proteins. Genetic construction was accomplished using the established *upp*-based

counterselective cloning procedure that allows for integration of genes at targeted locations in bacterial genomes¹⁵. This method, compared to plasmid-based protein expression, facilitates high gene stability and mitigates the needs for a continuous antibiotic-derived selective pressure¹⁶.

Genetic Cloning Mechanism

The system for *upp*-based counterselection for targeted genome integration used in this project was derived from Goh *et al.*¹⁵, which conducted a knock-out of S-Layer Protein in *Lactobacillus acidophilus* and will be the default reference for describing this mechanism of genetic cloning. It is worth noting that this system has been used by other groups to study integration of rotavirus genes at metabolic loci in *Lactobacillus casei*¹⁷.

This system of genetic recombination relies on the non-essential gene *upp*, which is a common counterselectable marker in bacterial cloning. *upp* encodes uracil phosphoribosyltransferase (UPRTase), an enzyme that converts uracil to uridine monophosphate (UMP) for nucleotide synthesis pathways. However, UPRTase can also recognize the base analog 5-fluorouracil (5-FU) and convert it to 5-fluoro-UMP. Downstream, this creates 5-fluorodeoxy-UMP (5-FdUMP), which inhibits thymidylate synthase, a key enzyme in DNA synthesis pathways¹⁷. Because of this, UPRTase is toxic to bacteria in the presence of 5-FU.

To effectively counterselect for nonfunctional UPRTases, the baseline *L. acidophilus* used in this cloning project encode a mutant Δupp that creates a nonfunctional UPRTase. These bacteria also contain a helper plasmid, which encodes *repA*. RepA is a protein used in pORI-based plasmid replication, but the pORI vector plasmid containing recombination sequences is *repA-*.

Therefore, it requires a *trans* acting RepA to undergo replication, provided for by the appropriately named helper plasmid. In this way, replication of the vector plasmid is conditional based on the presence of the helper plasmid. Additionally, the helper plasmid is temperature sensitive and will be cured from host bacteria at 42°C. This plasmid is maintained in bacteria using the antibiotic chloramphenicol as a selective pressure.

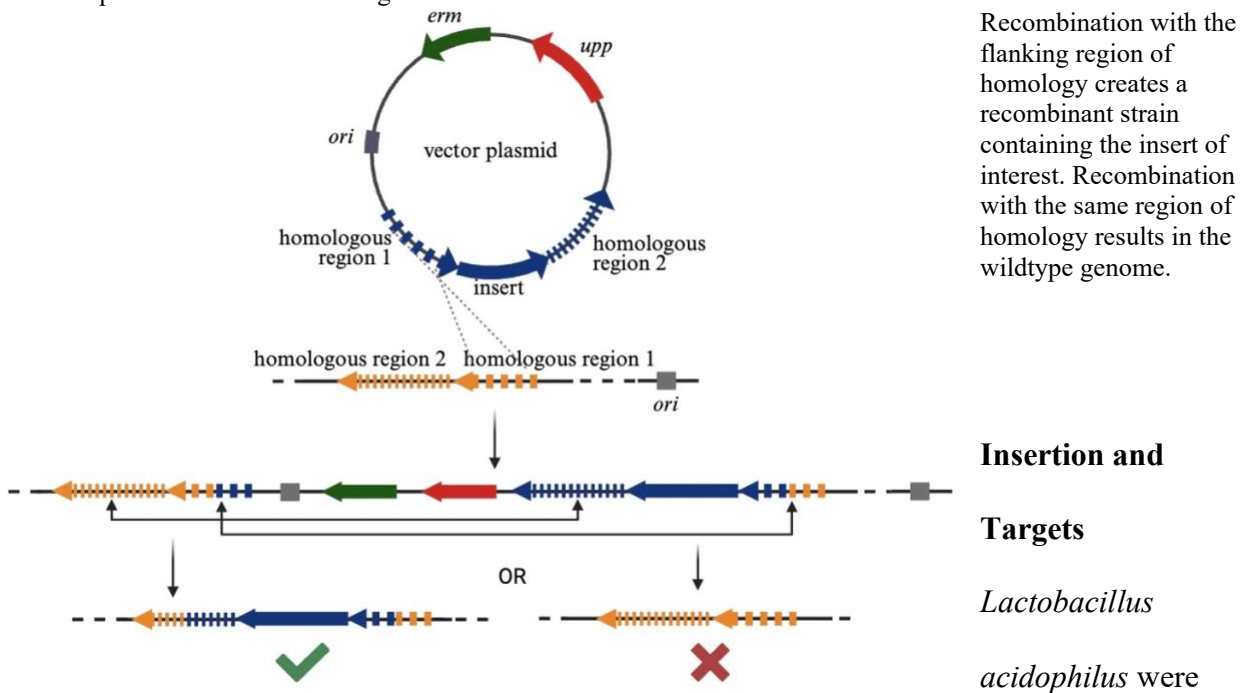
The vector plasmid contains the recombination sequence, which is constructed by amplifying the flanking regions (800-1300 bp) of the chromosomal integration site with the sequence of interest in the middle. This is accomplished with triple ligation using restriction enzymes. The vector plasmid also contains a non-mutated *upp* and a selectable marker for erythromycin resistance.

The vector plasmid is transformed into the *L. acidophilus* bacteria containing the helper plasmid. After stabilization, the bacteria are grown at impermissible temperatures, so the helper plasmid is cured from the bacteria. The loss of RepA induces chromosomal integration of the vector plasmid using one of its regions of homology in a single crossover event. These recombinant bacteria are erythromycin resistant, sensitive to 5-fluorouracil, and contain the sequence of interest in their genomes. They also have two origins of replication, which creates genomic instability that can be resolved with a double crossover event.

A double crossover event occurs when homologous regions within the chromosome recombine (Figure 3). There are two possible outcomes for recombination. If the same region of homology employed during the single crossover event recombines, then the plasmid is excised from the chromosome and the bacteria displays a wildtype phenotype. However, if the second region of

homology recombines, then the backbone of the integrated plasmid is removed, leaving the recombinant sequences of interest in the genome. When any double crossover event occurs, the selectable antibiotic resistance marker is removed. However, recombinant bacteria can be identified through counterselection using the *upp* marker. *Lactobacillus* are grown on plates that contain 5-fluorouracil, so any colonies that grow do not have a functional *upp* gene (i.e., undergone a double crossover event) because otherwise the 5-FU would be toxic to them due to UPRTase. PCR screening is performed on colonies to distinguish which kind of double crossover took place. This method of targeted gene replacement cloning produces markerless recombinant bacteria ready for experimentation.

Figure 3: Double crossover recombination mechanism. In a single crossover event, homologous recombination induces plasmid insertion into the genome. Double crossover recombination can result in two outcomes.



recombined using *upp*-based counterselection to express fragments of either the rotavirus antigen VP7 or the model immune-stimulating protein ovalbumin. As described above, VP7 is part of rotavirus' outer capsid layer, making it an ideal target for neutralizing antibodies. Ovalbumin (OVA), derived from chicken egg whites, is used as a model antigen to study MHC-II

presentation and CD4+ T cell activation; its peptide sequence 323-339

(ISQAVHAAHAEINEAGR) is a very immunogenic epitope and binds MHC-II very strongly¹⁸.

Constructs made with VP7 can be used to study *L. acidophilus* as a rotavirus vaccine vector while OVA constructs can evaluate *L. acidophilus* immunogenicity in a model system.

These constructs were also made with two different chromosomal targets, enolase and S-layer protein A. Enolase is a metabolic enzyme in glycolytic pathways and converts 2-phosphoglycerate into phosphoenolpyruvate¹⁷. While primarily found in the cytoplasm, enolase also localizes to the surface of *Lactobacilli*, especially in low pH environments¹⁹. It is “anchorless” due to the lack of a sorting domain but binds many extracellular matrix proteins including fibronectins, collagens, and mucins^{19,20}. Enolase is constitutively highly expressed being “one of the most abundantly expressed cytoplasmic proteins in many organisms”^{17,20}. In *Lactobacillus casei*, it is found in the same operon as GAPDH, a common housekeeping gene known for its consistently high expression¹⁹. This high expression profile substitutes the normally high expression seen from multicopy plasmids¹⁷. It is highly conserved across *Lactobacillus* spp., which makes it an ideal target for broadly applicable gene editing¹⁷. By inserting antigens immediately downstream of enolase, we can harness its strong promoter while not disrupting enolase’s functions.

The other target for gene editing conducted in this project was S-layer protein A (SlpA). S-layers are glycoprotein lattices that surround bacterial surfaces and are important for epithelial adhesion as well as cell protection, both of which are critical for bacterial survival and colonization of the GI tract¹⁵. SlpA is the primary S-layer protein found in *L. acidophilus*, although SlpB and SlpX

are also components of the S-layer²¹. SlpA binds uromodulin on M cells, which facilitates delivery to dendritic cells (DCs)²². Additionally, SlpA is a ligand of DC-SIGN (DC-specific ICAM-3-grabbing nonintegrin) and can modulate IL-4 production and T regulatory cell differentiation²³. While genetic insertions were performed behind the enolase gene, recombination was done in the middle of SlpA. This maximizes stable surface expression of the epitope for recognition by the immune system, particularly dendritic cells. In this way, short antigen sequences are better for insertion into SlpA, while longer antigen sequences are more suited for insertion behind enolase.

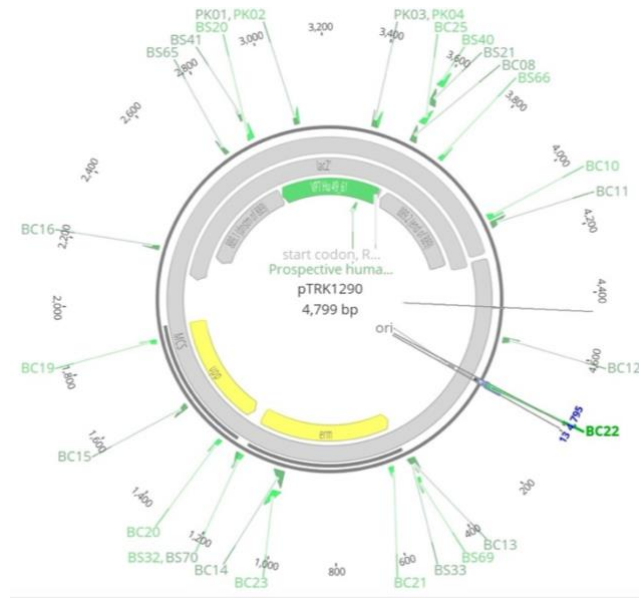
Additionally, adjuvants were added to the recombinant *L. acidophilus* strains to enhance immunogenicity. These were flagellin (FliC) and a fimbrial, adhesion protein (FimH), which are TLR5 and TLR4 agonists respectively^{5,24}. Their activation of the innate immune system has been shown to aid in host defenses against rotavirus⁵.

The reduced efficacy of rotavirus vaccines in LMIC is a pressing issue that may be addressed with lactic acid bacteria vaccines. To study immune induction of recombinant *L. acidophilus*, these vaccine constructs must be created. This project outlines the process of constructing and evaluating these vaccines.

Materials and Methods

Strains and Plasmids

Using *upp*-based counterselection genetic cloning, four vaccine constructs were created (Table 1). For each construct, a vector plasmid contains the insertion sequence with homologous regions (889.1 and 889.2) of the targets on either side (Figure 4). These vector plasmids are



transformed into *L. acidophilus* NCFM that have Δupp and harbor pTRK669. This is the temperature sensitive helper plasmid that is RepA⁺ and has resistance to chloramphenicol.

Figure 4: pTRK1290 plasmid map. An example plasmid map shows the insert, homologous regions, *upp*, erythromycin resistance gene, and the origin of replication found in all the vector plasmids.

Construct	Target	Insert	RepA ⁻ , Erm ^R vector plasmid	Starting LA NCFM strain with pTRK669 (RepA ⁺ , Cm ^R)
GAD310	Enolase (<i>eno</i>)	Human rotavirus VP7 50-265aa (648 bp)	pTRK1290 (extracted from <i>E. coli</i> EC101 NCK2792) provided by Sarah O'Flaherty, North Carolina State University	NCK200
GAD313	Enolase (<i>eno</i>)	Mouse rotavirus VP7 63-300aa (714 bp)	pTRK1291 (extracted from <i>E. coli</i> EC101 NCK2793) provided by Sarah O'Flaherty, North Carolina State University	NCK200
GAD316	Enolase (<i>eno</i>)	Mouse rotavirus VP7 50-265aa (648 bp)	pTRK1292 (extracted from <i>E. coli</i> EC101 NCK2794) provided by Sarah O'Flaherty, North Carolina State University	NCK200
GAD390	S layer protein A (<i>SlpA</i>)	Ovalbumin 323-339aa (51 bp)	pTRK1053 (extracted from <i>E. coli</i> EC101 AVB10)	GAD90 (<i>SlpA</i> -VP8_10aa_FimH-Nterm), Kan ^R

Table 1: Genetic construct information. Four vaccine constructs were made, with the target of either enolase or *SlpA*. Rotavirus VP7 or ovalbumin sequences were inserted. Vector plasmids and bacterial strains are included for

each construct. Antibiotic resistance is noted with the R superscript. Erm is erythromycin, Cm is chloramphenicol, Kan is kanamycin.

Escherichia coli EC101 harboring the vector plasmid of interest were grown from freezer stocks. Cultures were grown overnight in LB broth (EMD Millipore 1.10285.0500) in shaking conditions (Excelsa E24, New Brunswick Scientific) at 37°C. Broth was supplemented with appropriate antibiotics (150 µg/mL Erm (Fisher Biotech BP290-25) for NCK2792, NCK2793, NCK2794 and 100 µg/mL Erm, 40 µg/mL Kan for AVB10). Once grown, plasmids were extracted from *E. coli* cultures using Plasmid Miniprep Kit (NEB T1010S). Briefly, bacteria were pelleted with ten-minute centrifugation at 3286 x g (Allegra X-15R, Beckman Coulter) and resuspended in buffer B1. Bacteria were lysed with the alkaline buffer B2, and DNA was precipitated out with addition of the neutralizing buffer B3. DNA was spun down in a column with wash buffers 1 and 2 to remove cellular debris and salts. The final elution was conducted with nuclease free water (Biologix 19-6018-25). Briefly, Concentration and purity were measured with a spectrophotometer (NanoPhotometer, Implen), and plasmids were stored at -20°C.

Transformation by electroporation

L. acidophilus NCFM cultures were grown statically in MRS (De Man-Rogosa-Sharpe) (BD 288130) broth overnight. One mL of culture was transferred to 49 mL prewarmed MRS broth. Once the OD600 value, measured with SmartSpec™ 3000 (Bio-Rad 269BR), reached between 0.5 and 0.8, cultures were spun down for ten minutes at 4°C. This range of OD600 values is indicative of exponential growth, when optimal antigen and adjuvant expression is observed. The supernatant was poured off, and the bacterial pellet was thoroughly resuspended in 20 mL ice-cold 3.5X Sucrose Magnesium Electroporation Buffer (SMEB). The buffer was made with 1M

OmniPur® sucrose (Calbiochem 8510-500GM) and 3.5 mM MgCl₂ (Fisher Scientific M35-500) dissolved in ultrapure water (MilliQ Q-Pod CDUFBIOA1). Buffer was filtered with a 0.2 µm filter (Thermo Scientific 566-0020).

Bacteria were washed with 1X PBS (Corning 21-040-CV) three times with 20-minute centrifugation steps then gently resuspended in 1 mL 3.5X SMEB. 200 µL of bacteria were added to 1.5 mL microfuge tubes containing 500 or 1000 ng of the vector plasmid. The mixture was chilled on ice for 2 minutes and then transferred to 2 mm electroporation cuvette (Bulldog Bio 12358-346), careful to ensure that no bubbles were pipetted out. The cuvette was capped and quickly placed in the electroporation chamber of a gene pulser (Bio-Rad) set to 2.45 kV, 25 µFD, and 200 Ω. The electrical current was applied for 2-3 seconds. 0.8 mL pre-warmed MRS was added to the cuvette and all contents were pipetted into 2 mL pre-warmed MRS. Bacteria were incubated overnight at 37°C and then plated undiluted on MRS agar Difco™ (BD 214530) containing 5 µg/mL erythromycin and 2 µg/mL chloramphenicol. These were grown in anaerobic chambers with Pouch Anaero (Mitsubishi R682001) and evaluated for plasmid uptake after 48 hours.

Screening for plasmid uptake and double crossover

Colonies were screened for plasmid uptake. Each PCR reaction consisted of 12.5 µL Accustart II SuperMix (Quantabio 95136-500), 11 µL nuclease-free water, and 0.25 µL of both forward and reverse primers at 20 µM (Table 3, Appendix Table 1), all mixed in a PCR tube (Greiner Bio-One 608281). A sterile toothpick was used to transfer colonies from the plate to the PCR mixture. Temperature settings were inputted into C1000 Touch™ Thermal Cycler (Bio-Rad)

following manufacturer recommendations (Table 2). 5 μ L PCR product was mixed with EZVision dye (Amresco N472), and 4 μ L 1kilobase ladder (GoldBio D010-500 or NEB N0552S) was mixed with EZVision dye. The mixed products were loaded into 1% agarose gel (Invitrogen 16500500) in 1X TAE (G Biosciences R023) and ran at 115V for 30 minutes

(Pharmaia Biotech). Gels were visualized with ChemiDoc™

MP Imaging System (Bio-Rad 734BR-5115) and annotated

with Image Lab™ (Bio-Rad v6.1.0 build 7 standard edition).

94°C	3 min
94°C	30 sec
Annealing temp	30 sec
72°C	30 sec
72°C	2 min
12°C	Infinite

Table 2: Thermocycler conditions. Conditions for amplification using Accustart II SuperMix with annealing temperature varying based on primer sets used. Refer to Table 3 for annealing temperatures for specific primer sets.

Construct	Test	Forward primer	Reverse primer	Length (bp)	Annealing temperature
GAD310	pTRK1290 uptake	BS41 (889.1)	BS40 (889.2/plasmid)	727	51°C
	Double crossover	BS63 (genome)	BS64 (genome)	1995	50°C
GAD313	pTRK1291 uptake	BC16 (plasmid backbone)	BS04 (mouse VP7)	1028	57°C
	Double crossover	BS63 (genome)	BS64 (genome)	2040	50°C
GAD316	Double crossover	BS63 (genome)	BS64 (genome)	1978	50°C
GAD390	pTRK053 uptake	AV13 (plasmid backbone)	AV92 (ovalbumin)	508	52°C
	Double crossover	AK62 (genome)	AV92 (ovalbumin)	1133	50°C
	pTRK1034 uptake	AV01 (plasmid backbone)	1168R (FliC)	1502	53°C

Table 3: Primer sets. PCR reactions were used to screen colonies for plasmid uptake and double crossover. For each test, primers pairs, annealing location, expected product length, and annealing temperature are provided. Refer to Appendix Table 1 for primer sequences.

Colonies that had taken up the vector plasmid were grown in MRS broth. 100 μ L of the growth was passaged into 5 mL prewarmed MRS with 5 μ g/mL erythromycin at 42°C. Growing bacteria

at this temperature cures the bacteria of pTRK669 because this plasmid is temperature sensitive. Passaging was performed three times before bacteria were plated on MRS agar plates containing 5 µg/mL erythromycin and grown anaerobically at 37°C. Colonies were picked and grid plated such that the same colony was spread onto two agar plates, one containing 5 µg/mL erythromycin only and one containing 5 µg/mL erythromycin and 2 µg/mL chloramphenicol. Colonies that grew only the erythromycin plates were selected for further passaging because growth on agar containing chloramphenicol suggested resistance, which is provided from pTRK669 and should not present in bacteria.

Bacteria were passaged three times in MRS broth and grown anaerobically at 37°C. They were grown on semi-defined media (SDM)²⁵ agar plates containing 5-fluorouracil (Sigma Aldrich F6627-1G). SDM agar was made with the following ingredients: 20 g/L dextrose Difco™ (BD 215530), 1 g/L Tween80 (BD 231181), 2 g/L ammonium citrate (Sigma-Aldrich 09833-100G), 5 g/L sodium acetate (Molecular Sigma Biology S-2889), 0.1 g/L MgSO₄ 7H₂O (Amresco 0662-500G , provided by Dr. Brian Foy), 0.05 g/L MnSO₄ (Sigma-Aldrich M789-500G), 2 g/L K₂HPO₄ (Sigma-Aldrich P-5655), 5 g/L yeast nitrogen base (Difco™ 239210), 10 g/L Bacto™ Casitone (BD 225930), and 20 g/L agarose. Additionally, 5-fluorouracil was dissolved in DMSO (ATCC 4-X) to a concentration of 40 mg/mL. The solution was added to the SDM agar mixture for a final concentration of 100 µg/mL. 5-fluorouracil is light sensitive, so bottles and plates containing 5-fluorouracil were wrapped in aluminum foil.

Colonies that grew on the SDM agar plates were not sensitive to 5-fluorouracil, indicating they had undergone a double crossover event. They were grid plated on MRS agar containing 5µg/mL

erythromycin and MRS agar containing no antibiotics to check for removal of erythromycin resistance. Colonies without resistance were screened using the same PCR mixture and settings described previously with primer sets found in Table 3. Bands were visualized with a UV light wand (UVP UVM-57) and removed with an X-Tracta™ Agarose Gel Extraction tool (USA Scientific 5454-2500). DNA was purified using a gel extraction kit (NEB T1020S). The gel fragment was added to the Gel Dissolving Buffer, collected in spin column with a wash buffer, and eluted with nuclease-free water. DNA was sent for Sanger sequencing (Genewiz) and was used to determine accuracy of sequence insertion. Sequencing results were visualized and analyzed with Geneious Prime (v2024.0.5).

Flow cytometry to evaluate insert expression

Flow cytometry was performed to test for expression of surface antigens and adjuvants (Table 4). *L. acidophilus* cultures were started in MRS broth with the appropriate antibiotics. They were grown statically overnight in anaerobic conditions. One mL of the growth was passaged into 49 mL pre-warmed MRS. When cultures reached exponential growth (as determined by doubled OD600 value), they were centrifuged for ten minutes at 3186 x g and washed three times with 1X PBS. 1×10^7 colony forming units (CFUs) were added to 100µL staining buffer, made with 1% BSA (Equitech-Bio BAH65) and 1:1000 Kathon (SupelCo 5-00135). It also contained the primary antibody and was mixed in a V-bottom plate well (Medical Caplugs 333-8001-0IV). The plate was incubated on ice for 20 minutes with gentle rocking (Orbital Shaker AROS 160™ Thermolyne). 100µL staining buffer was added to the wells, and the plate was spun down for 5 minutes. Supernatants were flicked off and bacterial pellets were resuspended in 100µL staining buffer containing the appropriate secondary antibody. The plate was incubated for 20 minutes on

ice with gentle rocking and a cover to minimize light exposure fluorophore-conjugated antibodies. The plate was washed twice with staining buffer. Bacteria were resuspended in 100 μ L staining buffer to prepare for running through the flow cytometer. Data were collected on a three laser Cytex Aurora flow cytometer (Cytex Biosciences) and analyzed with FlowJo™ (v10.8.1).

Construct	Target	Order	Antibody	Catalog information	Dilution
GAD390	Ovalbumin (323-339)	Primary	OVA323-339 rabbit	Alpha Diagnostic OVA3231-A	1:100
		Secondary	Donkey anti-rabbit FITC	Biolegend 406403	1:100
	FliC	Primary	FliC mouse	Biolegend 629702	1:200
		Secondary	Goat anti-mouse PE	Invitrogen P21129	1:100
	FimH	Primary	FimH mAB824	Gifted from Dr. Evgeni V. Sokurenko (University of Washington, Seattle)	1:1000
		Secondary	Goat anti-mouse PE	Invitrogen P21129	1:100

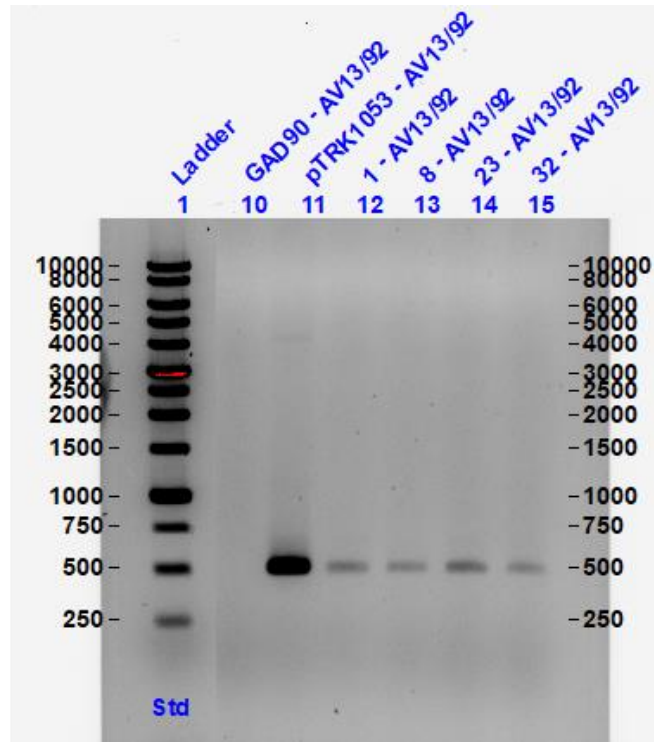
Table 4: Antibodies for flow cytometry. Antibodies were used to detect epitope expression in recombinant constructs. Information about antibody use and targets are provided.

Results

Using *upp*-based counterselection, four recombinant *L. acidophilus* strains were created to express either the model antigen ovalbumin or the rotavirus antigen VP7. The first step in this genetic cloning procedure was to use electroporation to transform vector plasmids into the bacterial host, which was evaluated in two ways. First, transformed plasmids conferred host bacteria with resistance to erythromycin. Bacteria were grown on MRS plates containing erythromycin to select for CFUs that had undergone a successful transformation. Second, PCR screening was performed to test plasmid uptake (Figure 5, Supplemental Figure 1A, 1B, 1C).

Figure 5: Gel depicting successful uptake of pTRK1053 by GAD390. PCR products amplifying the ovalbumin sequence in the vector plasmid were examined on a 1% agarose gel (Table 3). Bands at the anticipated ovalbumin sequence size (508 bp) were observed in all lanes except the negative control. It is noteworthy that this gel was cropped to exclude lanes 2-9 because this showed data from an irrelevant primer pair. The complete gel can be found as Supplemental Figure 1C.

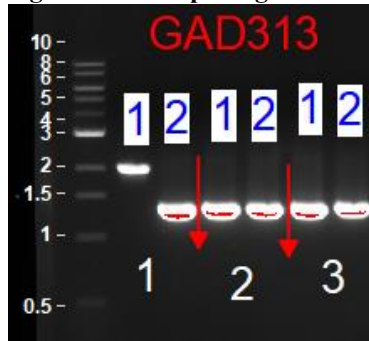
Recombination via double crossover was assessed in three ways. First, growth on SDM agar containing 5-fluorouracil counterselected for bacteria that had undergone double crossover



recombination due to this process' removal of *upp*. Second, bacteria were grown on MRS agar and MRS agar containing 5 µg/mL erythromycin to ensure bacteria did not harbor resistance to erythromycin. Only colonies without resistance were screened with PCR. Recombination via double crossover can result in two outcomes, a wildtype genome that does not contain sequences derived from the vector plasmid, or a recombinant genome with inserted antigen sequences. For the third method of evaluation, PCR screening was used to differentiate these outcomes. Primers annealed to genomic sequences and amplified the region of insertion between them. In this way, a PCR product was expected regardless of double crossover outcome, but the band location depended on recombination outcome. Recombination that inserted the antigen sequence created a product around 2000 bp long while the wildtype had approximately a 1300 bp long sequence, with some product length variation depending on construct specifications. This binary outcome of double crossover recombination can be seen in PCR screening results (Figure 6, Supplemental

Figure 2A, 2B, 2C). All constructs had at least one colony that underwent successful double crossover recombination that inserted the antigen sequence of interest into the bacterial genome.

Figure 6: Gel depicting successful VP7 sequence double crossover recombination in GAD313. PCR products amplifying the insertion site in the genome were examined, with blue text referring to colony number and white text referring to plate number. A band was seen at 2040 bp, indicating successful integration of the truncated mouse rotavirus VP7 sequence, was observed in colony 1 from plate 1. All other bands were observed at 1310 bp, the expected result if no insertion took place.



Sanger sequencing was employed to ensure accurate sequence integration. It was found that all extracted DNA contained the

expected sequences, showing precise insertion of antigen sequences (Figure 7, Supplemental Figures 3A, 3B, 3C). The sequencing reads spanned all regions of interest at least twice over.

Minor discrepancies in base calls were attributed to reduced accuracy at the beginning and end of Sanger sequencing reads.

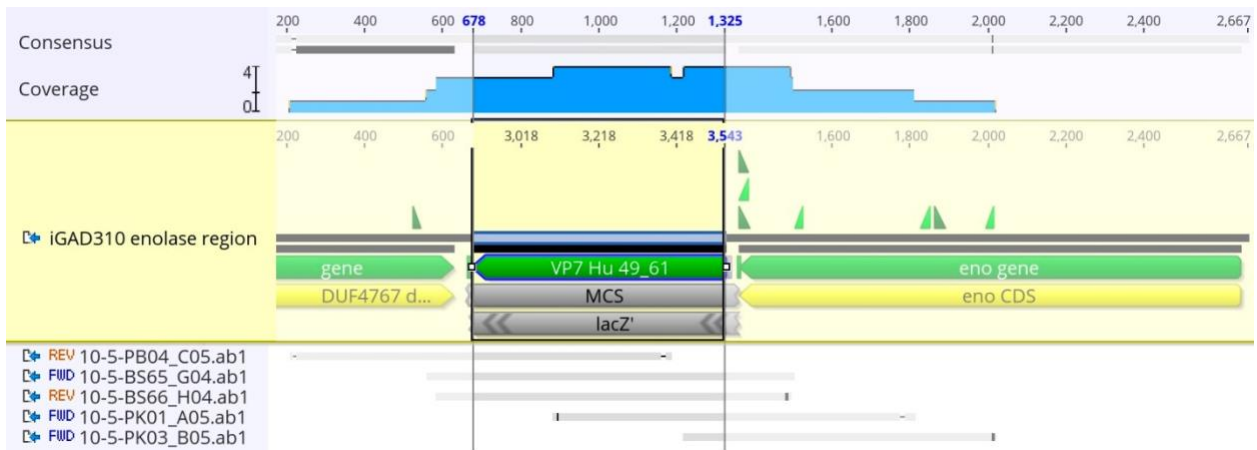


Figure 7: Alignment showing accurate human rotavirus VP7 sequence insertion in GAD310. Sanger sequencing reads of the insertion site of GAD310 were aligned to the predicted recombinant genome. Consensus alignment reads matched the genome, indicating the human rotavirus VP7 sequence was inserted into the genome without mutations.

The adjuvant flagellin (FliC) was derived from a plasmid, which was transformed into bacteria after genomic integration. Uptake of this plasmid was evaluated in two ways. First, the plasmid confers its host with erythromycin resistance. Colonies that grew on MRS agar plates containing erythromycin were identified as containing the plasmid. Also, genetic presence of the plasmid was determined using PCR with primers amplifying plasmid backbone and adjuvant sequences (Figure 8). It was found that bacteria readily took up this plasmid.

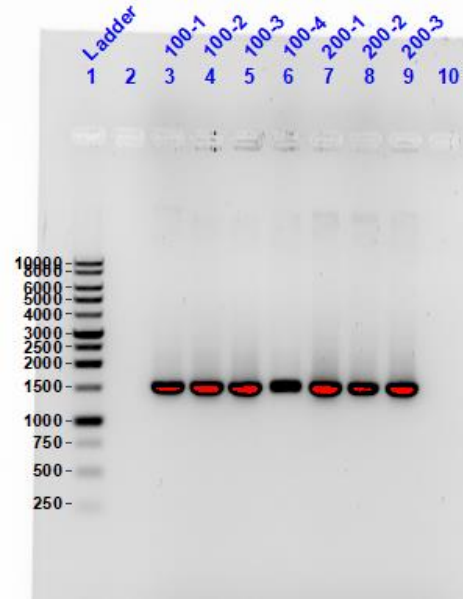


Figure 8: Gel depicting successful uptake of adjuvant FliC-expressing plasmid. PCR products amplifying the backbone of a FliC-expressing plasmid was examined on 1% agarose gel. All colonies were observed to have taken up plasmid as shown by the band at 1502 bp.

Flow cytometry was used to assess protein expression of inserted antigens and adjuvants. Only GAD390 proteins were tested with flow cytometry due to their localization to the surface. The results showed all antigens and adjuvants were expressed at high levels in GAD390 (Figure 9).

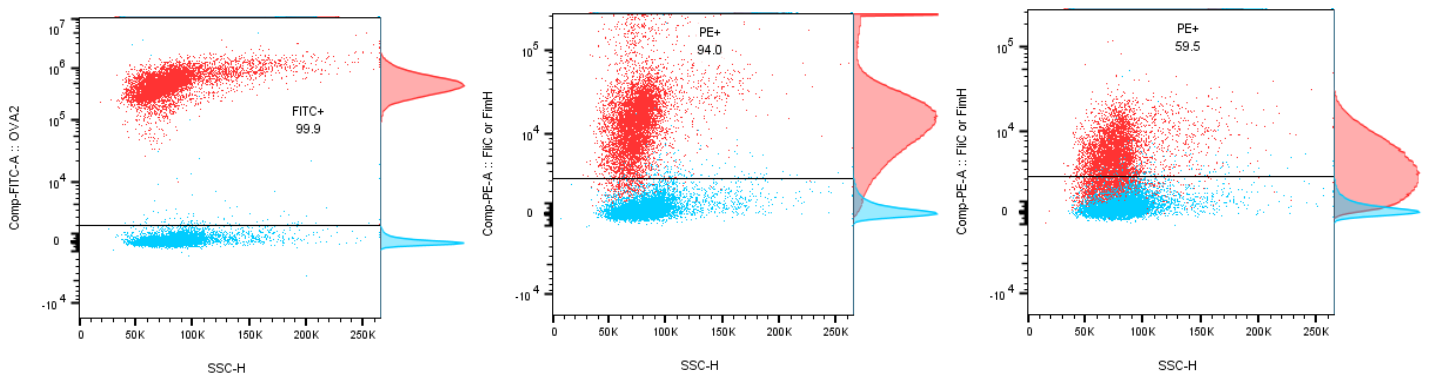


Figure 9: GAD390 highly expresses all expected antigens and adjuvants. Bacteria were stained with fluorophore-conjugated antibodies to mark antigen and adjuvant expression for flow cytometry analysis. It was determined that GAD390 (red) bacteria had 99.9%, 94.0% and 59.5% expression for ovalbumin (FITC), FliC (PE), and FimH (PE) respectively. $>6.5 \times 10^5$ cells were collected for analysis. Gates were set such that wildtype *L. acidophilus* (blue) was $<2\%$ positive.

Discussion

In this project, four novel recombinant strains of *L. acidophilus* were generated using *upp*-based counterselection. Two different sites, *enolase* and *SlpA*, were targeted for insertion of four different antigens. Antibiotic selection, PCR screening, gene sequencing, and flow cytometry were used to evaluate constructs for accurate insertion and expression, and it was determined that all antigens were integrated into precise locations in the genome seamlessly. These constructs were carefully designed to study immune activation by antigens carried by the oral vaccine vector *L. acidophilus*.

Through insertion of sequences of the outer capsid rotavirus protein VP7, GAD310, GAD313, and GAD316 were constructed as viable vaccines that can protect against rotavirus. These constructs are being evaluated in murine and porcine models to examine antibody production and protection against rotavirus. GAD310 is being tested in porcine models and GAD316 in murine models. GAD316 was modified to add a flagellin (FliC) adjuvant to become GAD318 and it was administered in conjunction with another *L. acidophilus* construct containing rotavirus' VP8 spike protein²⁶. This allows for maximal protection against rotavirus. This provides valuable insight into optimization of which rotavirus antigens (administered independently or simultaneously) are the best at eliciting protective immune responses. This can inform creation of future *L. acidophilus* vaccine constructs to precisely address deficiencies in current rotavirus vaccines.

Ovalbumin is a model antigen that induces robust immune stimulation, and, by adding this to the S-layer protein of *L. acidophilus*, can be used to study how the intrinsic properties of the

bacterial vector contribute to immune induction. For example, it is unknown how lactate secreted from *L. acidophilus* contributes to the immune response to the probiotic-based vaccine. GAD390 can be used as a model platform to study lactate secretion because it builds on the foundation of the well-characterized antigen ovalbumin to evaluate changes in immune induction. GAD390 can be used in both animal models and cell culture to examine response to the vaccine. In animal models, large scale responses can be examined, such as metabolism on a tissue-level and antibody production. In cell culture, GAD390 can be used to study individual cell activation, particularly CD4⁺ T cells because the ovalbumin 323-339 sequence is MHC class II restricted. In this way, GAD390 can be tested on a tissue- and cell-level to evaluate *L. acidophilus* as a vaccine vector.

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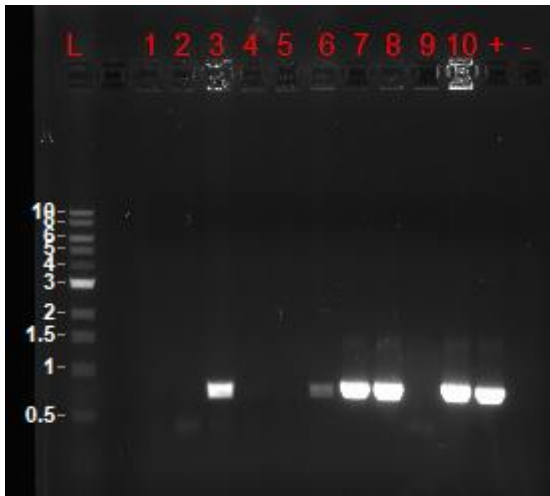
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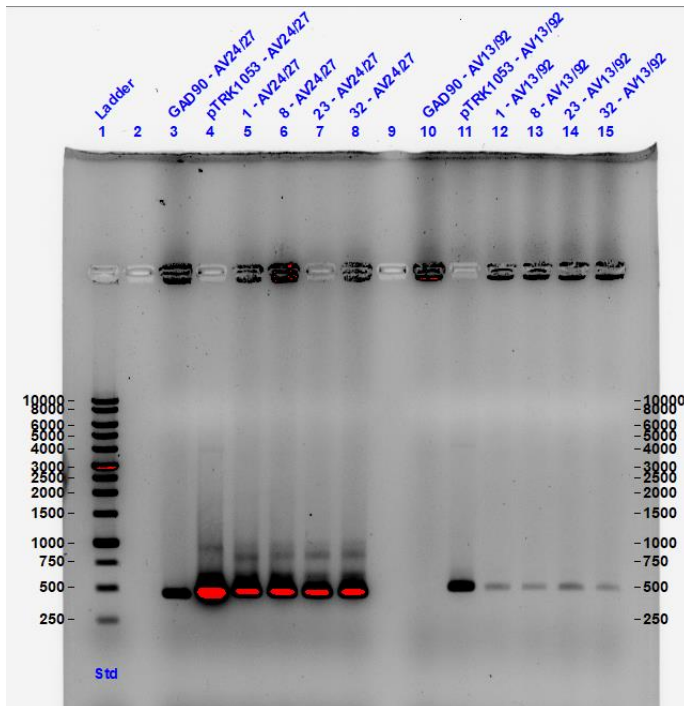
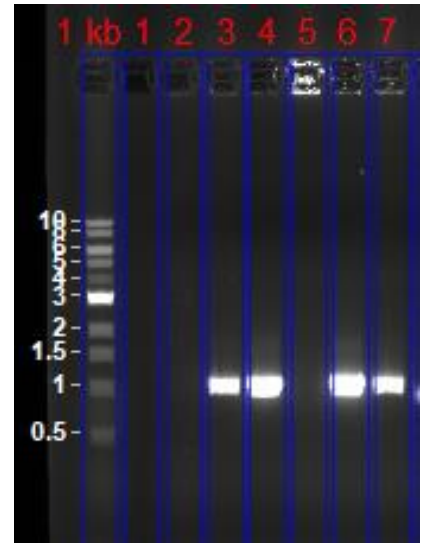
Supplemental Figures

Supplemental Figure 1 shows PCR screenings evaluating vector plasmid uptake.



Supplemental Figure 1A: Gel showing successful uptake of pTRK1290 by GAD310. PCR products amplifying pTRK1290 plasmid backbone were examined on 1% agarose gel. Bands at the expected 727 bp were observed in the plasmid positive control and colonies 3, 6, 7, 8, and 10.

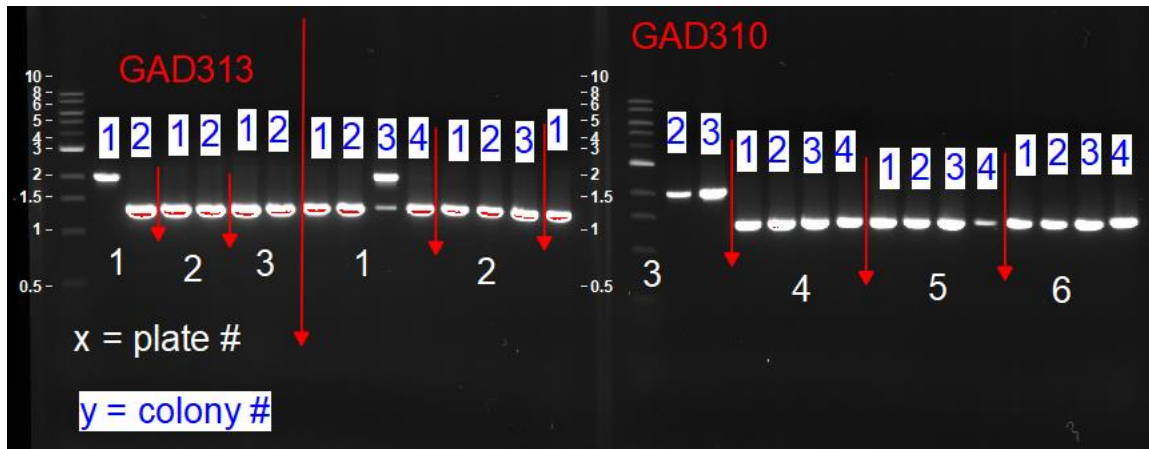
Supplemental Figure 1B: Gel showing successful uptake of pTRK1291 by GAD313. PCR products amplifying pTRK1291 plasmid backbone were examined on 1% agarose gel. Bands at the expected 1028 bp were observed in the plasmid positive control and colonies 3, 4, 6, and 7.



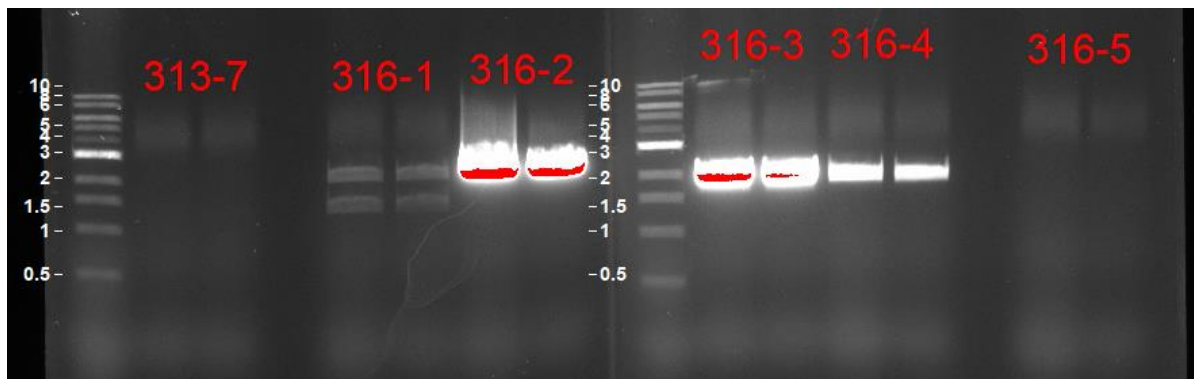
Supplemental Figure 1C: Unedited gel depicting successful uptake of pTRK1053 by GAD390. PCR products amplifying the plasmid ovalbumin sequence were examined on 1% gel in lanes 10-15. Bands at the expected 508 bp were seen in all colonies and the plasmid positive control. Lanes 3-8 contain the same colonies amplified with a different primer set that is not relevant for evaluating pTRK1053 uptake.

Supplemental Figure 2 shows PCR screenings evaluating double crossover recombination.

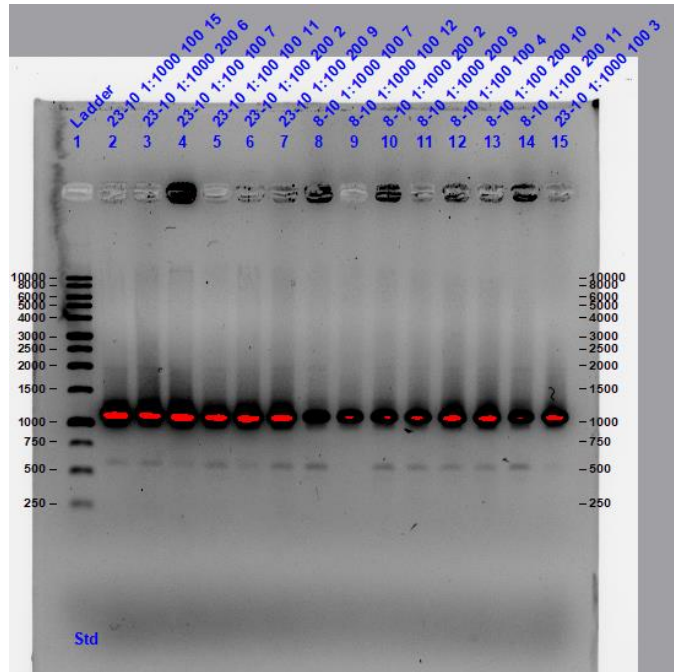
Supplemental Figure 2A: Gel showing successful double crossover recombination inserted antigens into GAD313 and GAD310 genome. PCR products amplifying the genomic region around the N terminal of enolase were visualized on 1% agarose gel. Bands at 2040 bp or 1995 bp for GAD313 and GAD310 respectively indicated successful antigen insertion by double crossover recombination. Bands at 1310 bp indicated the bacteria had the wildtype genome. Colonies that inserted the antigen of interest were 1-1 for GAD313 and 3-2 and 3-1 for GAD310. The double band seen by GAD310 1-3 indicated the colony harbored sequences from both double crossover recombination outcomes.



Supplemental Figure 2B: Gel depicting successful antigen sequence insertion of mouse rotavirus VP7 into GAD316 genome. PCR products amplifying the genomic region around the N terminal of enolase were visualized on 1% agarose gel. Bands at 1978 bp were observed in GAD316-2, 3, and 4, indicating successful insertion of the antigen sequence. An additional band at 1310 bp observed in GAD316-1 indicated impurity of recombinant colonies. No bands were observed by GAD316-5, likely due to incorrect colony transfer to PCR mixture. GAD313-7 gel results are shown but are not relevant to GAD316 double crossover recombination.

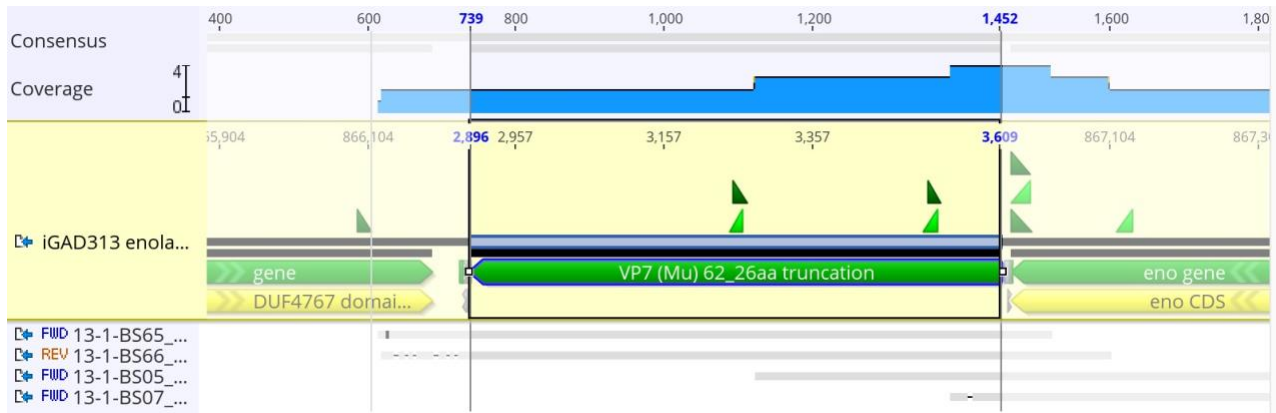


Supplemental Figure 2C: Gel showing successful insertion of ovalbumin 323-339 into GAD390 SlpA. PCR products amplifying genome-inserted ovalbumin were visualized on 1% agarose gel. The expected band product of 1133 bp was seen in all colonies, though only colony 9 exhibited this band exclusively. All others contained bands around 600 bp and 2000 bp and were not used for further study.

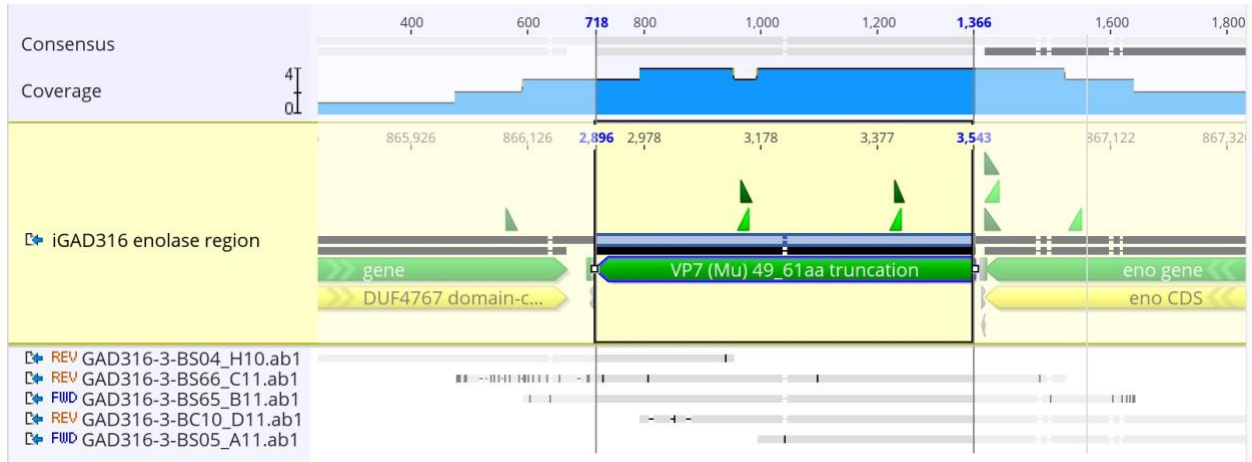


Supplemental Figure 3 shows Sanger sequencing results evaluating antigen sequence insertions.

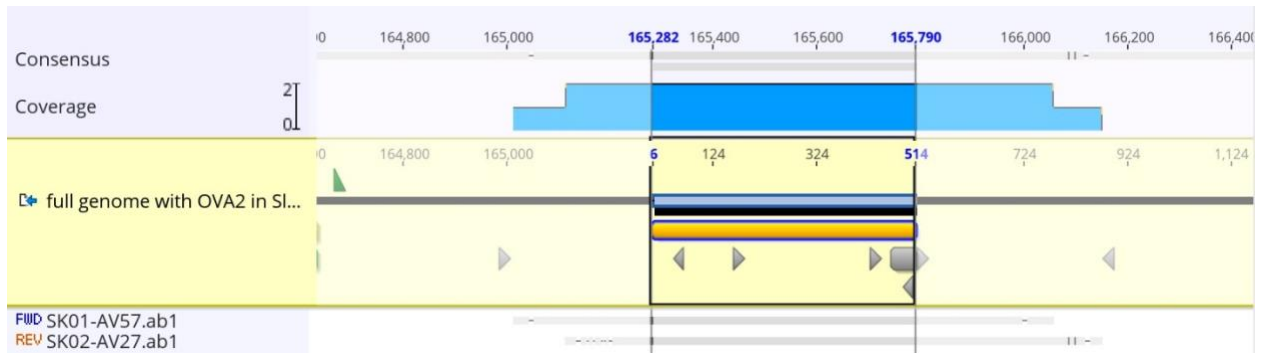
Supplemental Figure 3A: Sanger sequencing confirmation of successful mouse rotavirus VP7_62_26aa insertion at the N terminal of enolase of GAD313. Sanger sequencing reads of the insertion site of GAD313 were aligned to the predicted recombinant genome. Consensus alignment reads matched the genome, indicating the mouse rotavirus 26 amino acid VP7 sequence was inserted into the genome behind enolase without mutations.



Supplemental Figure 3B: Sanger sequencing confirmation of successful mouse rotavirus VP7_49_61aa insertion at the N terminal of enolase of GAD316. Sanger sequencing reads of the insertion site of GAD316 were aligned to the predicted recombinant genome. Consensus alignment reads matched the genome, indicating the mouse rotavirus 61 amino acid VP7 sequence was inserted into the genome behind enolase without mutations.



Supplemental Figure 3C: Sanger sequencing confirmation of successful ovalbumin 323-339 insertion in SIpA of GAD390. Sanger sequencing reads of the insertion site of GAD390 were aligned to the predicted recombinant genome. Consensus alignment reads matched the genome, indicating ovalbumin inserted into the genome in the middle of SIpA without mutations.



Appendix

Primer	Orientation	Sequence
1168R	Reverse	5' TGTGACCTTCGGCTTACTTGCAG 3'
AK62	Forward	5' GACGAATAAAACTCTAATGAG 3'
AV01	Forward	5' CACGGTCAAAGTGAATGGAAC 3'
AV13	Forward	5' ATGAAGAAAAATTTAAGAATCGTT 3'
AV27	Reverse	5' AGTATGAGGTGCTTTGAAGTAAC 3'
AV57	Forward	5' GTAATGCTTGTGGGGGTAAGC 3'
AV92	Reverse	5' CTCGTTAATTTTCAGCATG 3'
BC10	Reverse	5' GAATTAGCTGCATACTACGACAAGC 3'
BC16	Forward	5' ATTCAGGCTGCGCAACTGT 3'
BS04	Reverse	5' CGATGAGGCCAACAAATGG 3'
BS05	Forward	5' GTTGGCCTCATCGGTTTG 3'
BS07	Forward	5' GTCCTTGATCTCGGTTGC 3'
BS40	Reverse	5' GATGTTGACCACGATTAATG 3'
BS41	Forward	5' GAAATTGTACTCGGTGC 3'
BS63	Forward	5' CATGAGGATGAGATGAAG 3'
BS64	Reverse	5' GAAGAACTCAGAAGAAGC 3'
BS65	Forward	5' CAGTCAAACGAAATAAGC 3'
BS66	Reverse	5' CATCGCTGACTTGGCAGTTG 3'
PK01	Forward	5' GACAACCTATCCCTAACG 3'
PK03	Forward	5' CCATCATTGATTTGAGTACTTG 3'
PK04	Reverse	5' CTGAAGCAAGTACTCAAATC 3'

Table 1: Primer sequences. Polarity and sequence are provided for primers.