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Developing a vector for expressing serratiopeptidase in Lactococcus lactis

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Developing a vector for expressing serratiopeptidase in *Lactococcus lactis*

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Chronic inflammation stems from the inability of the body to resolve acute inflammation. Chronic inflammation is a growing health concern, as nearly 60% of Americans reported coping with at least one chronic condition (as of 2014). On a world scale, three fifths of deaths result from a chronic inflammation disease. Treatments for chronic inflammation include lifestyle changes (diet and exercise) and a variety of conventional drugs. Beyond these treatments, supplementing the body with systemic enzymes may attenuate inflammation. One promising enzyme is serratiopeptidase. Serratiopeptidase is a serine protease expressed by *Serratia marcescens* which has demonstrated anti-inflammatory, anti-edemic, and analgesic activity in laboratory studies and pre-clinical trials. Clinical trial data is less convincing, likely because the enzyme delivered is not bioavailable to the body after it passes through the stomach. To circumvent this issue, we proposed developing a plasmid vector that, when transformed into the probiotic *Lactococcus lactis*, would allow for bioactive serratiopeptidase production within the gut; thus, increasing bioavailability. The vector designed was delivered from the synthesis company in two fragments (they failed to assemble to full vector). After successfully, assembling the vector, the selection system in the commercial *L. lactis* strain we chose did not work as advertised in our hands. We introduced antibiotic resistance into the vector to facilitate the selection process after transformation. Unfortunately, the vector with antibiotic failed to transform *L. lactis* indicating some unknown problem with the vector / host system.

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"Struggle is the flame which forges the soul into steel"

-Unknown

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INTRODUCTION

Inflammation

Inflammation was first characterized by the Roman doctor Cornelius Celsus as "*Rubor et tumor cum calore et dolore"*, which means 'redness and swelling with heat and pain' (MEDZHITOV 2010). Loss of physiological function and vasodilation have since been added to this characterization (OWEN *et al.* 2013). Inflammation has two categories: acute (short-term) and chronic (long-term; unresolved acute inflammation). Although inflammation can have a negative connotation, it is important to recognize that the immune processes involving acute inflammation are normal and necessary for proper healing.

Acute inflammation is initiated in response to a harmful stimulus to help facilitate removal and subsequent healing of damaged tissues (OWEN *et al.* 2013; CHEN *et al.* 2017). Acute inflammation has four primary goals: reduce pathogen load at the infection site, prevent the spread of infection, repair tissue damage, and innate and adaptive inflammatory mediators. The most common triggers of acute inflammation are pathogens (e.g. bacteria, viruses, fungi) and external injury (e.g. tissue damage, foreign materials). The successful outcome for acute inflammation is healing. However, when acute inflammation fails to resolve it often progresses to chronic inflammatory disease.

Chronic inflammation is the body's attempt at clearing the initial acute stimulus, by inducing a stronger inflammatory response. This pro-inflammatory response is driven by increased output of cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which are involved with the receptor-mediated activation of intracellular inflammatory pathways (CHEN *et al.* 2017). There is new evidence emerging which suggests a major link

between chronic inflammation and autoimmune disease generally, and with diseases within the gut specifically, such as irritable bowel disorder (COHEN *et al.* 2019).

Traditionally, autoimmune disease was a blanket statement characterized by the body attacking its tissues; currently, autoimmune disease is split into autoinflammatory diseases (innate dysfunction) and autoimmune disease (adaptive dysfunction). Autoimmune disease occurs when the body cannot distinguish self (cell surface markers, etc.) from non-self (pathogens, etc.) and attacks healthy tissues. This classification only applies to some autoimmune diseases. Autoimmune disease classification is not exact and work is ongoing to improve their characterization. The exact etiology of autoinflammatory and autoimmune diseases is unknown; however, the gut microbiome plays a strong role in maintaining balanced immune responses (KIRSCHMAN AND MILLIGAN-MYHRE 2019).

The human gut microbiome is comprised of mutualistic bacteria that interface with the immune system to aid in defending against pathogens (KIRSCHMAN AND MILLIGAN-MYHRE 2019). A healthy microbiome consists of trillions of microbes representing over 1000 bacterial species working in concert; therefore, dysregulation of this system can lead to gut dysbiosis and chronic inflammation (CHEN 2018; KIRSCHMAN AND MILLIGAN-MYHRE 2019). We recognize that the bacteria in the gut are involved in regulating processes (such as inflammation) for the host and it is for this reason that there is interest in applying anti-inflammatory probiotic therapies to the gut (LIN *et al.* 2021; WICKRAMASURIYA *et al.* 2021; YAO *et al.* 2021).

Anti-Inflammatory Therapies

The most commonly used anti-inflammatory drugs are non-steroidal anti-inflammatory drugs (NSAIDs) or corticosteroids. These are drugs like naproxen, ibuprofen, and aspirin, which

inhibit cyclooxygenase (COX) enzymes and cause reduced inflammation and pain (BERTOLINI *et al.* 2002). Metformin, another NSAID, is used to treat low-grade inflammation in type II diabetes by reducing circulating TNF-α & IL-1β (DE OLIVEIRA *et al.* 2019). Corticosteroids sometimes are used for more severe inflammatory disorders (e.g. rheumatoid arthritis). Corticosteroid drugs mimic cortisol. Cortisol is a steroid hormone that is released in response to stress and has major implications in metabolism, inflammation, blood pressure regulation, and bone formation (VOET *et al.* 2015). Corticosteroids work by suppressing the immune system to decrease inflammation. Prolonged use of NSAIDs can lead to a variety of harmful conditions including ulcers, gastrointestinal bleeding, acute renal failure, strokes, heart attacks, and blood clots (MARCUM AND HANLON 2010; NAILWAL AND DOSHI 2021). Similarly, corticosteroid use can have harmful side effects including peptic ulcer disease, gastrointestinal bleeding, pancreatitis, adrenal suppression, and diabetes (ORAY *et al.* 2016; CAPLAN *et al.* 2017). Thus, side effects from these more common therapies have precipitated an interest in alternative therapies.

Enzyme-based therapies have been successful at treating conditions with underlying inflammation. Enzymes as a treatment for inflammation were first introduced into modern medicine in the early 1950s when intravenous trypsin delivery successfully treated post-surgical swelling and bruising following sports injury (BERTOLINI *et al.* 2002). This success led to further exploration using the proteolytic enzymes trypsin, chymotrypsin, bromelain, and papain. It was determined that combining these enzymes offered anti-inflammatory synergy in rat paw edema (WOOD *et al.* 1997). Further studies concluded that oral therapy with proteolytic enzymes produced some analgesic and anti-inflammatory effects, with inconsistent results (LEIPNER *et al.* 2001). Oral combinations of proteolytic enzymes such as trypsin: chymotrypsin have shown both efficacy and safety in facilitating tissue repair from accidental injuries, surgical and orthopedic

injuries, and burns (CHANDANWALE *ET AL.* 2017; SHAH AND MITAL 2018). Moving beyond trypsin and chymotrypsin, one enzyme that has shown some promise as an anti-inflammatory therapy is serratiopeptidase (TIWARI 2017).

Serratiopeptidase

Serratiopeptidase (Synonyms: serralysin, serrapeptidase) is a proteolytic enzyme produced by the commensal bacterium *Serratia marcescens* within the gut of the silkworm (*Bombyx mori*) (NAKAHAMA *et al.* 1986). The moth uses the enzyme to digest its cocoon before emerging as an adult. Serratiopeptidase displays anti-inflammatory (SELAN *et al.* 2017; TIWARI 2017) and anti-edemic properties (JAISWAL AND MISHRA 2018). Additionally, serratiopeptidase is involved with the recruitment of neutrophils and other lymphocytes to the site of inflammation (CHAPPI D *et al.* 2015). In clinical trials, patients with conditions for which edema was observed reported pain reduction following treatment with serratiopeptidase (BHAGAT *et al.* 2013). It is surmised that the reduction in inflammation and concomitant reduction of edema is the reason for pain reduction.

Serratiopeptidase is a serine protease, which makes sense because insect silk protein is very serine-rich. In addition to cleaving serine-rich proteins, serine proteases have a high affinity for cyclooxygenase enzymes I and II (BERTOLINI *et al.* 2002). Cyclooxygenases are necessary for increasing eicosanoid production during a proper inflammatory response (VANE 1998). Cyclooxygenase II (COX-2) is the enzyme that produces downstream mediators of inflammation such as prostaglandins and leukotrienes (NØRREGAARD *et al.* 2015). Thus, the inhibition of COX-2 is beneficial in reducing pain and inflammation, which is the sole premise of NSAIDs (BERTOLINI *et al.* 2002). While the mode of action for serratiopeptidase is unclear, it is proposed to act by some unknown regulation of COX-2, reduction of capillary permeability induced by

histamine and bradykinin, and by solubilizing non-living tissues such as mucous and blood clots (DOSHI *et al.* 2020).

Serratiopeptidase was characterized as being structurally similar to matrix metalloproteinases (JAISWAL AND MISHRA 2018). Metalloproteinases (MMPs) are proteolytic enzymes with metal ions in their catalytic site that directly influence their target substrate (KARABENCHEVA-CHRISTOVA *et al.* 2017). They are secreted as proenzymes (zymogens) and require extracellular activation, often by other MMPs. They are collectively responsible for clearing all components of the extracellular matrix (ECM) proteins and basement membrane, concerning tissue remodeling and maintenance (LÖFFEK *et al.* 2011; KARABENCHEVA-CHRISTOVA *et al.* 2017). Serratiopeptidase is most similar to MMP-8 (aka Neutrophil collagenase), a proenzyme found in human neutrophils and macrophages (JAISWAL AND MISHRA 2018). MMP-8 possesses tumor-suppressive properties (DECOCK *et al.* 2015) and cleaves various chemokines involved in inflammation (THIRKETTLE *et al.* 2013). Additionally, Thirkettle et al. (2013) found that MMP-8 upregulates interleukins 6 and 8 (IL-6; IL-8). IL-6 is the chief stimulator of most acute phase inflammatory proteins (GABAY 2006). IL-8 is produced by macrophages/epithelial cells and is known as the neutrophil chemotactic factor, as it is responsible for attracting neutrophils to the site of damage or infection. Given serratiopeptidase's similarities to MMP-8, it may have similar effects in the immune response to reduce inflammation.

Currently, Serratiopeptidase is produced using biotechniques and is sold over the counter as a supplement. The protein is 487 amino acids long and weighs approximately 52 kDa (Uniprot: A0A0G3VN57). Fermentation is the primary method of serratiopeptidase production. The process involves culturing wild-type or mutant strains of *S. marcescens* and purifying the

enzyme from the culture medium using chemical precipitation, ultra-filtration, anion-exchange, and size-exclusion chromatography to obtain pure protein (TANEJA *et al.* 2017; VELEZ-GOMEZ *et al.* 2019). This approach is strain-dependent but overall, the yields are low (SRIVASTAVA *et al.* 2019). The fermentation-derived serratiopeptidase is active over a wide pH range (pH 6-10 > 50% activity after 1h incubation) (SRIVASTAVA *et al.* 2019). Similarly, enzyme sourced in this manner is stable over a wide temperature range (4-37 ℃) in *in vitro* assays (www.brenda-enzymes.org; EC 3.24.40). Higher temperatures progressively reduce activity, with complete inactivation occurring at 55 ℃ (www.brenda-enzymes.org; EC 3.24.40).

Due to the pathogenic nature of *S. marcescens* and the low yield of serratiopeptidase from *S. marcescens* fermentation, serratiopeptidase expression in alternate hosts has been explored. In general, *E. coli* is a viable host for SP expression. *E. coli* C43 cells did produce some serratiopeptidase, but the yield was very low, and the other strains tested failed to produce transformants (SRIVASTAVA *et al.* 2019). It is speculated that this is due to serratiopeptidase toxicity, but this has not been confirmed. In a recent study using *E. coli* BL21 cells, active refolded serratiopeptidase was produced under an inducible T7 promoter. Using *E. coli* BL21 cells [DE3] and the Champion pET SUMO vector, 86 mg L^{-1} serratiopeptidase was recovered from a 20 g L^{-1} wet weight pellet (DOSHI *et al.* 2020). This vector has a small ubiquitin-like modifier (SUMO) that increases solubility and allows for higher yield after purification of native proteins in *E. coli (*DOSHI *et al. 2020)*. As this vector is a low copy number plasmid, scaling this research up would require significant optimization. Serratiopeptidase has also been cloned into the yeast *Pichia pastoris* using a vector system. Using the genomic DNA from *S. marcescens* MTCC 8708, the serratiopeptidase gene was cloned into *P. pastoris* and optimal conditions (30 ℃; pH 8.0) were determined (KAVIYARASI AND SURYANARAYANA 2016). While these

approaches yield active enzymes, there is no way to deliver them without passing through the acidic environment of the GI tract. As such, expression in these alternate hosts fails to eliminate the problem of low bioavailability after oral delivery.

Orally delivered serratiopeptidase lacks bioavailability due to acidic conditions in the stomach, enzymatic breakdown in the GI, and poor absorption across the intestinal lining (SHINDE AND KANOJIYA 2014; BANERJEE *et al.* 2016). Additionally, tablet-based enzyme formulations contain reduced enzyme activity due to compression used in their formulation (RAJVAIDYA *et al.* 2007; SHARMA *et al.* 2014). Within the pharmaceutical world, direct compression is often used to create oral formulations. Direct compression uses heat and mechanical compression forces and is often problematic for sensitive enzymes. As an example, even with low compression, a preparation of pepsin in solid dosage form did not yield enough active enzyme to justify its cost (KRISTO AND PINTYE-HODI 2013). A variety of nanocarrier systems have been employed in an attempt to increase the bioavailability of orally-delivered serratiopeptidase (aquasomes, liposomes, and polymer barriers) with limited pre-clinical or clinical success (SHAH AND PARADKAR 2005; RAJVAIDYA *et al.* 2007; SANDHYA *et al.* 2008; RAWAT AND SARAF 2009; UMASHANKAR *et al.* 2010).

Non-oral Serratiopeptidase Delivery Routes

Because orally-delivering serratiopeptidase is such a challenge, other methods of delivery that circumvent the oral route have been developed. Hydrogel delivery systems loaded with serratiopeptidase have been tested for transdermal delivery. Hydrogels are natural or synthetic polymeric networks that have very high water-absorption capacity and are often used for drug loading and tissue regeneration (TOH AND LOH 2014). Hydrogels containing serratiopeptidase and gentamycin microspheres promoted wound-healing by debridement and antibiofilm activity

(SINGH AND SINGH 2012). The effectiveness of gentamycin, in this case, was enhanced by the presence of serratiopeptidase (SINGH AND SINGH 2012). When formulated as a niosomal (non-ionic surfactant-based vesicles) hydrogel, serratiopeptidase offered prolonged inhibition against rat hind-paw edema inflammation induced by carrageenan injection (SHINDE AND KANOJIYA 2014). When serratiopeptidase was used in a thermosensitive hydrogel suspension, enhanced anti-biofilm activity against *Staphylococcus epidermidis* was observed but the serratiopeptidase formulation did not provide a sustained release profile as with the controls (SARIGOL *et al.* 2018). While transdermal serratiopeptidase delivery is possible, delivery is limited to local external injury and therefore not viable for treating inflammation situated deep within the body.

Serratiopeptidase has been studied in chemically active, magnetic nanoparticle drug delivery systems. Serratiopeptidase was immobilized on magnetic nanoparticles (MNPs) using chitosan (KUMAR *et al.* 2013) and carboxyl groups (KUMAR *et al.* 2014) to help crosslink active serratiopeptidase to the solid cores. This led to a maximum protein and enzyme loading capacity of 264 mg/g and 325 U/g MNPs, respectively. In the case of the carboxyl-linked serratiopeptidase, a maximum protein and enzyme loading capacity of 115.8 mg/g and 168.32 U/g were determined (KUMAR *et al.* 2014). In both cases, the serratiopeptidase-linked MNPs possessed increased membrane permeability *in vitro*. In the carrageenan-induced rate paw edema model, the magnetic nanoparticles were delivered locally to the injury site using an external magnetic field and increased anti-inflammatory effects were observed when compared to free enzyme (KUMAR *et al.* 2014). While these serratiopeptidase-linked MNPs work well for targets on or just below the skin, MNPs with serratiopeptidase were unable to reach deep tissues (MCBAIN *et al.* 2008).

An aerosol-based serratiopeptidase delivery system has been examined. Aerosol delivery of liposomal Levofloxacin-serratiopeptidase caused decreased microbial load, decreased cytokine levels, and decreased inflammatory mRNA markers in the lungs of *Staphylococcus aureus*-infected rats (GUPTA *et al.* 2017). This method is fast-acting and provides the rapid intra-pulmonary introduction of antibiotics. However, aerosol-based formulations are quickly cleared from the lungs and thus would require frequent and long-term applications to be a successful approach (GUPTA *et al.* 2017). Additionally, repetitive and long-term applications could lead to drug overdosing (HILL *et al.* 2015). Another concern is that the high concentrations needed can trigger respiratory side-effects (ZAROGOULIDIS *et al.* 2013). Research is ongoing to reduce these side effects and increase the efficacy of aerosol serratiopeptidase delivery.

Serratiopeptidase in Clinical Trials

Serratiopeptidase has been used post-surgery to decrease inflammation and swelling. In a multi-center study (n=174), buccal swelling was significantly reduced in the serratiopeptidase (One 10 mg tablet daily) control group with no reported side-effects (TACHIBANA *et al.* 1984). The study was randomized, double-blind, and placebo-controlled. Additionally, swelling following the repair of the upper ankle joint due to sports injury was reduced by 50% three days post-operation with all pain gone after 10 days in the serratiopeptidase treatment group (ESCH *et al.* 1989). These results match an earlier report using oral serratiopeptidase in post-operative therapy (n=295) where serratiopeptidase was tested against the anti-swelling drug A-4700. In this study, both serratiopeptidase and the drug decreased swelling, thereby decreasing painful edema (TSUYAMA *et al.* 1977). In both studies the treatment dose was unspecified, and the length of the treatment was not mentioned.

Serratiopeptidase has been used in various ear, nose, and throat (ENT) studies. In a multicenter study (n=376), respiratory disease patients were split into three groups with serratiopeptidase and Seaprose S (similar supplement reported to have anti-inflammatory properties) treatment groups. There was no significant change in either treatment group compared to the placebo for improving sputum expectorant (NAGAOKA *et al.* 1979). A smaller study was performed looking at chronic respiratory disease but specifically non-bronchial asthma. The randomized study (n=40) compared serratiopeptidase to various mucolytic agents and observed that serratiopeptidase reduced sputum viscoelasticity (SHIMURA *et al.* 1983). In both respiratory studies, the serratiopeptidase dose was the same (30 mg daily) but the smaller study used a single application compared to three 10 mg applications in the larger study. In another study, serratiopeptidase decreased mucus viscosity but not elasticity in chronic sinusitis using the same dosage as the previous studies (MAJIMA *et al.* 1988).

After these studies, a more standardized approach was taken for ENT disorders with 3 doses of 10 mg daily in another multicenter study (n=193) (MAZZONE *et al.* 1990). This study was double-blind, randomized, and contained a placebo. Serratiopeptidase efficacy was confirmed for reducing pain and mucus secretions, increasing ability to swallow and decreasing nasal congestion (MAZZONE *et al.* 1990). Subsequently, Nakamura et al. (2003) examined the characteristics of the sputum following serratiopeptidase treatment in chronic airway disease. Serratiopeptidase decreased viscoelasticity, the weight of solids, neutrophil counts, and observable symptoms (NAKAMURA *et al.* 2003). Unfortunately, this study had a low sample size (n=29), no treatment control group, and was an open-label trial. Taken together, there is evidence to support further research on using serratiopeptidase for ENT disorders.

Serratiopeptidase increases the effectiveness of antibiotics in combinatory therapies. Serratiopeptidase increased the penetration of Sulbenicillin into fluid exudates within osteoarticular infections (OKUMURA *et al.* 1977). Similarly, it increased the local concentrations of Cefotiam in lung tissue for lung cancer patients following thoracotomy (KOYAMA *et al.* 1986).

There has been some interest in using serratiopeptidase in dentistry. Serratiopeptidase reduced swelling and pain following third molar removal in a combinatorial therapy with paracetamol (AL-KHATEEB AND NUSAIR 2008) and alone against ibuprofen, paracetamol, and betamethasone (CHOPRA *et al.* 2009). Patients in both trials using serratiopeptidase exhibited reduced pain and swelling, but results were not significantly different when compared to the other treatments in the Chopra et al. study (2009).

Serratiopeptidase has been tested in clinical trials for its efficacy against a variety of disorders. The existing clinical studies lack the efficacy and safety needed to support claims of the therapeutic use of serratiopeptidase due to their variable quality (BHAGAT *et al.* 2013). Despite the current challenges, serratiopeptidase continues to show promise as a anti-inflammatory therapy. The lack of clinical efficacy likely stems from the low bioavailability of the orally-delivered lyophilized enzyme. Here I propose developing a food-grade plasmid vector for serratiopeptidase expression within the gut by a food-grade strain of *Lactococcus lactis*.

The construction of a lactic-acid bacterium (LAB) food-grade vector system must contain proper selection measures. Lactic-acid bacteria are a large group of $Gram^{+}$ bacteria that are considered generally recognized as safe (GRAS) as they ferment carbohydrates to produce lactic acid, most often in the dairy industry. A selectable marker must be used, and must apply to GRAS organisms as approved by the FDA (https://www.fda.gov/food/generally-recognized-safe-gras/microorganisms-microbial-derived-ingredients-used-food-partial-list). Achieving GRAS

status often is achieved via sugar fermentation phenotypes (such as lactose or melibiose) (GU *et al.* 2014). One example of a useful selectable marker is α-Galactosidase (EC 3.2.1.22) due to its ability to hydrolyze melibiose, as some LAB and other microbes cannot normally utilize melibiose (GU *et al.* 2014). If a non-food-grade selection measure is required, it must be easily removable (TAGLIAVIA AND NICOSIA 2019).

Objectives

- Design a food-grade vector for serratiopeptidase expression in *L. lactis*
- Confirm that the vector will transform *L. lactis*
- Confirm that serratiopeptidase is expressed in *L. lactis*

MATERIALS AND METHODS

Vector Design

The plasmid vector requirements are as follows: it must be food-grade (no antibiotic resistance genes), it must express serratiopeptidase, and the serratiopeptidase must be secreted by the bacteria. Additionally, the enzyme must also be active. The sequence for the serratiopeptidase gene was obtained from GenBank (Accession number: KR014114.1; https://www.ncbi.nlm.nih.gov/genbank/). A codon-optimized food-grade vector with serratiopeptidase under the control of a strong inducible promoter and secretion signal was designed (pEWU1, 4685bp) by the EWU biology 2017-2018 Biotech student cohort (T. Mauzy was part of this cohort). The vector sequence was genetically watermarked at the 3'UTR of the serratiopeptidase gene reading "EAGLEALCHEMISTS" when translated (Appendix A).

The vector was designed such that it could be easily manipulated for current and future experiments. The circular vector contains a single EcoRI site. The EcoRI site allows for linearization and recircularization, and insertion into another vector such as pUC57 for delivery from the synthesis company and propagation in *E. coli*. Two NheI sites flank the serratiopeptidase gene. These sites allow for easy removal of serratiopeptidase and subsequent ligation to yield the no-gene control. Additionally, these sites could be used in the future to insert other genes for expression. The PlacA promoter was chosen as it is a strong promoter inducible by lactose. In this way, we could regulate gene expression during in vitro and in eventual in vivo studies by regulating the presence/absence of lactose. Therefore, beyond its use with serratiopeptidase, this vector could function as a platform for testing other therapeutic enzymes in *L. lactis*. A full map of the vector is presented in figure 1. The codon-optimized vector sequence is presented in Appendix A.

Vector Synthesis

Vector synthesis was contracted to GenScript (http://www.genscript.com). The codon-optimized sequence ordered was 4691 bp. As mentioned above, the vector had a single EcoRI site so that it could easily be inserted into the pUC57 cloning vector at GenScript and easily removed from pUC57. The final product promised by GenScript was to be 4 µg of pUC57 + pEWU1 plasmid, with sequencing to verify proper construction.

GenScript failed to assemble and deliver the complete vector and instead delivered two fragments (A and B) which we then had to assemble ourselves.

Escherichia coli Transformation

All plasmids were manipulated *in E. coli*. Transformations were done into chemically competent or electrocompetent (EC) DH5α or MC1061 *E. coli*. Commercially available chemically competent DH5α cells were used (Cat. #: C404010; https://www.thermofisher.com/). Electrocompetent MC1061 were prepared using a standard protocol. In brief, cells were grown at 37 ℃ until an OD600 of 0.4-0.7 and then subjected to a series of washes before being resuspended in 10% glycerol (See Appendix B for full details).

Chemically competent cells were transformed as follows: TOP10 cells were thawed, and 1-5 µL plasmid DNA was added to each reaction. Cells were mixed gently by flicking before incubation on ice. Cells were then heat shocked (30 seconds; 42 ℃), placed on ice, and then SOC medium was added for recovery (1 hour; 37 ℃) before being plated on selective media (See Appendix B for full details).

Electrocompetent cells were transformed as follows: 50 µL EC MC1061 cells were mixed on ice with 1-5 µL of the respective plasmid DNA. The mixture was transferred into a 0.2 cm electroporation cuvette (Cat. #1652082; www.bio-rad.com) and pulsed once in a BioRad Pulser II using the standard Ec2 *E. coli* setting. The electroporated cells were plated on LB medium plates containing 60 µg/mL ampicillin or 60 µg/mL carbenicillin (See Appendix B for full details).

Plasmid isolation

Transformed *E. coli* cells were cultured in LB broth with the appropriate selection at 37°C overnight. The following day, plasmid minipreps were conducted using PureLink™ Quick Plasmid Miniprep Kit (Cat. #: K210010; www.thermofisher.com) following standard

manufacturer's protocols to yield ample supply of pUC57+A and pUC57+B DNA (See Appendix B for details). The plasmid DNA concentrations were determined using a NanoDrop™ Lite spectrophotometer.

Vector Assembly Attempt I – NEBuilder HiFi DNA Assembly

Our first attempt at assembling the two fragments relied on a Gibson assembly process using the NEBuilder HiFi DNA assembly master mix (Cat. #: E2621S; https://www.neb.com/). This was chosen because the two fragments delivered had complementary overlapping ends (required for Gibson assembly homologous recombination reaction).

The purified plasmids were used as templates in PCR reactions using custom primers so that each fragment was amplified from the insert in the respective pUC57 vector backbone with very specific 5' and 3' ends (in preparation for the Gibson assembly process). Fragment A was amplified with AF/AR primers (Table1) and fragment B was amplified with the BF/BR primers (Table 1). After PCR amplification, the 5' end of fragment A would contain the designed EcoRI site, the 3' end of fragment A and the 5' end of fragment B had the requisite amount of overlap necessary for the Gibson ligation, and the 3' end of Fragment B had the designed EcoRI site.

Fragment A (1768bp) was amplified using a basic PCR master mix from Promega (Cat. # PRM7502; www.fishersci.com). PCR using the Elongase® Enzyme Mix (Cat. #10480-010; www.fishersci.com) was used to amplify the longer B fragment (2935bp). Reactions with appropriate elongation times (2 and 3 minutes, respectively for A and B) and temperature gradients for the annealing step were conducted following manufacturer's standard protocols in 25µl reaction volumes (1 µL of template DNA, 1 µL each forward primer, 1 µL of reverse

primer, 12.5 µL of master mix, and 9.5 µL of water; see Appendix B). Each primer was used at a 10 µM working concentration. The Elongase® PCR reactions were conducted following the manufacturer's recommendations with a final magnesium concentration. As per the manufacturer, magnesium concentrations ranging from 1.0 to 2.0mM were tested. The 1.0 mM concentration was used.

Gel-purified PCR amplicons were used in the HiFi DNA assembly process. Briefly, the amplicons were placed into a microfuge tube containing NEBuilder HiFi assembly master mix at a total concentration of 0.15 pmol in a ratio of 1:1 Fragment A: Fragment B following the manufacture's recommendations. Samples were incubated in a thermocycler at 50 °C for 15 minutes and then placed on ice. The assembled product was then used as the template in Elongase® PCR as above (using the AF and BR primers; Table 1), followed by gel electrophoresis (1% agarose gel in 1X TAE 1 µL of GreenGlo™ http://www.thomassci.com) and imaged (see Appendix B).

Vector Assembly Attempt II – Restriction Digestion and Ligation

As the Gibson ligation failed to assemble the fragments, an alternate restriction digestion and ligation scheme was devised that would allow for assembling pEWU1 in the pUC57 backbone (Figure 2). Subsequently, pEWU1 could be removed from pUC57 using the two flanking EcoRI sites and then circularized by ligation before transformation procedures.

Upon plasmid purification (as above), pUC57 + A and pUC57 + B plasmids were independently digested with HpaI and ApaI following standard manufacturer's protocols (Appendix B and see Figure 2 for the respective restriction enzyme site locations). The resulting digestion products were electrophoresed (as above, see appendix B). The gel was visualized

under blue light and the bands containing linear pUC57 + fragment A or linear fragment B were excised and purified using the PureLink™ Quick Gel Extraction and PCR Purification Combo Kit as per the manufacturer's recommendations (Cat. #: K220001; www.thermofisher.com).

The DNA fragment containing pUC57 + A was dephosphorylated using Alkaline Phosphatase as per the manufacturer's recommendation (Cat. #: M0290; www.neb.com). This dephosphorylated DNA and fragment B were then ligated using the Quick Ligation™ Kit as per the manufacturer's recommendation (Cat. #: M2200S; www.neb.com) to yield pUC57 + pEWU1 (Figure 2).

The serratiopeptidase gene was removed from the pUC57 + pEWU1 construct to generate pUC57 + pEWU1^{-SP} (no gene control). This was done by digesting the circular pUC57 + pEWU1 vector with NheI (two sites flanking the serratiopeptidase gene) and electrophoresing the resulting digestion products (as above). The resulting band was purified from the agarose gel and ligated (as described above) to produce pUC57 + pEWU1^{-SP}. Correct vector assembly was confirmed by restriction digestion analysis. Properly assembled vectors with (Figure 3A) and without (Figure 4A) serratiopeptidase will produce predictable restriction patterns (Figure 3B and 4B, respectively).

pEWU1 and pEWU1^{-SP} were separated from their respective pUC57 backbones by digestion with EcoRI followed by electrophoresis, gel purification, and ligation, as described above, in preparation for transformation into *L. lactis*.

Vector Sequence Check by Sanger Sequencing

The assembled pUC57 + pEWU1 vector was sequenced using a custom set of primers (Table 2) to confirm the desired sequence remained after all of the assembly manipulations. Paid sequencing services were provided by Genewiz (www.genewiz.com).

The sequence delivered by Genewiz was compared to the expected pEWU1 sequence using BLASTn and BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the "align two or more sequences" feature.

Both the expected sequence and the sequence delivered by Genewiz were submitted to the Protein Homology/Analogy Recognition Engine V2.0 (Phyre^2) for 3D structural analysis.

Lactococcus lactis **Transformation**

Plasmids were transformed into *L. lactis* (http://www.mobitech.com; food-grade NZ3000 strain). This auxotrophic strain has the lac operon chromosomally integrated with the *lacF* gene deleted (*lacF*-). This makes the strain unable to grow using lactose as a carbon source unless it possesses the *lacF* gene on a plasmid. pEWU1 has the *lacF* gene.

Electrocompetent *L. lactis* NZ3000 cells were prepared following the manufacturer's guidelines (Appendix B). Untransformed bacteria were grown in M17 media containing 0.5% w/v glucose (G-M17). All incubations were done at 30°C and were checked after 18-20 hours.

We transformed the EC NZ3000 cells with pEWU1 or pEWU1^{-SP}. As indicated above, each vector was digested out of the pUC57 backbone using EcoRI, gel purified, and ligated. The respective ligation reactions were used for the transformations into *L. lactis*.

Colony PCR was used to determine whether any colonies possessed the desired plasmid. Briefly, colonies were swirled into 20 µL of TE (10 mM Tris, 1 mM EDTA, pH 8.0) with 0.1%

Triton X-100) and then placed into boiling water for 5 min. The samples were removed from the boiling water and centrifuged at 13,000 x g for 10 minutes. For each sample, 2 µL of the resulting supernatant was added into a pre-mixed 13 µL PCR solution (1 µL each forward primer, 1 µL of reverse primer, 12.5 µL of master mix, and 8.5 µL of water; see Appendix B) to reach a 25 µL final volume. The primers used for these steps were: pEWU1 [M13F (-41) and SeraSeq_511R] and pEWU1^{-SP} (SP_Control_FP with SP_Control_RP; Table 2).

In an alternate experiment [after erythromycin (Erm) resistance was introduced into the vectors; see next section], the ligations reactions where Erm was introduced into the vectors (generating pUC57 + pEWU1/Erm^{+} or pUC57 + pEWU1^{-SP}/Erm^{+}) were used for *L. lactis* transformations, as above. "No Ligase" controls were included for the respective transformations using ligation products. These transformation reactions were plated on agar containing 5 µg/mL of erythromycin.

Additionally, pUC57 + pEWU1^{-SP}/Erm^{+} supercoiled plasmid DNA generated in MC1061 cells, for propagation and to confirm that the Erm gene was functioning properly, was used to transform the electrocompetent NZ3000 cells.

The pGh9:ISS1 (Erm-containing) plasmid was used as a positive control in each of the experiments using erythromycin selection. For each sample, 1 µL of a 50 ng/µL plasmid solution was used for transformation. Transformed cells were grown on G-M17 plates containing 5 µg/mL of erythromycin (See Appendix B). A no DNA control was used as the negative control.

Generally, electrocompetent NZ3000 cells were transformed as follows: 50 µL of cells were pipetted gently into pre-chilled (on ice) 2mm cuvettes (see Appendix B for full details). This was followed by the addition of 1-5 µL of plasmid DNA to achieve 50ng of the plasmid.

Cuvettes were placed into a BioRad Gene Pulser II and pulsed with the following settings: 2000 V, 25 µF, 200Ω. After pulsing, 950 µL of recovery broth (G-M17B + 20 mM $MgCl_2$ + 2 mM $CaCl_2$) was added to each cuvette. Cuvettes were incubated on ice for 5 minutes. Subsequently, the cells recovered for 1 hour at 30 ℃ without shaking. pEWU1-transformed cells were plated onto Lactose-M17 agar. No DNA controls were plated onto the L-M17 agar. No-DNA positive controls were grown on G-M17 agar. After recovery in the recovery media (with glucose), only transformed cells should continue growing in the Lactose-only plates.

Erythromycin Resistance Gene Insertion

As initial transformation attempts in *L. lactis* failed due to inconsistencies in the lactose selection process, we decided to introduce antibiotic resistance into pEWU1. Although this would eliminate the "food grade" characteristic of the plasmid it should allow for testing whether the plasmid could be successfully transformed into *L. lactis*.

Adenine methylase (*ermAM* aka *ErmR*) has been previously characterized and confers erythromycin resistance to *Streptococcus lactis* (GenBank: AAB96789.1; https://www.ncbi.nlm.nih.gov/protein/2773394). Shuttle cloning vectors (e.g pTRKH2) have been successfully developed and introduced in both high and low copy number using this gene and work well in both *L. lactis* and *E. coli* (O'SULLIVAN AND KLAENHAMMER 1993).

Thus, a sequence containing *ErmR* and the necessary restriction sites was designed. The unique SnaBI (upstream) and SpeI (downstream) sequences allowed for inserting *ErmR* into pUC57 + pEWU1 by ligation (as described above in vector assembly; see Appendix A).

RESULTS

Vector Synthesis

Vector synthesis was contracted to GenScript (www.genscript.com). GenScript failed to deliver a fully synthesized vector. Upon no information beyond "our standard procedures" failed to assemble the last two fragments, GenScript delivered the two fragments ("A" and "B") cloned individually into pUC57 (Figure 2). Thus, the task of final assembly was left to us.

Vector Assembly Attempt I - NEBuilder HiFi DNA Assembly

Fragment A was successfully PCR amplified from the pUC57 + A vector template furnished to us by GenScript (1768 bp amplicon, Figure 5B). Amplification of fragment B using the Elongase® enzyme mix produced the desired DNA band upon gel electrophoresis (2497 bp amplicon, Figure 6B). However, these reactions contained large amounts of non-target background DNA (Figure 6B). Various attempts to eliminate this non-target amplification by titrating temperature, magnesium, and template concentration were unsuccessful (data not shown). Thus, the band of interest was excised, and the DNA was purified from the agarose.

Following the excision and purification of the amplicons for fragments A and B, the NEBuilder HiFi master mix was used to combine these PCR-amplified fragments. Using the HiFI reaction product as the template, Elongase® PCR using the primers (Table 1) for the 5' end of fragment A and the 3' end of fragment B failed to amplify pEWU1 in its entirety. As such, we were unable to proceed further and moved to an alternate assembly method.

Vector Assembly Attempt II – Restriction Enzyme Assembly

The pUC57 plasmids containing the A or B fragments were successfully restricted with HpaI and ApaI and assembled by ligation to become pUC57 + pEWU1. This plasmid was propagated DH5α *E. coli*. This was confirmed by restriction digestion. Plasmids extracted from

transformants exhibited the predicted (Figure 3) restriction digestion patterns corresponding to the correct assembly of pUC57 + pEWU1 (Figure 8).

The serratiopeptidase gene was removed from this construct to produced pUC57 + pEWU1^{-SP}. This was achieved by digesting the construct at the purposefully designed NheI sites flanking the serratiopeptidase gene. This product was ligated and transformed into *E. coli* cells. A colony containing the pUC57 + pEWU1^{-SP} plasmid was identified by colony PCR (Figure 9). After propagation and plasmid purification, the plasmid was digested. The predicted digestion pattern (Figure 4) and the actual digestion pattern were identical (Figure 10), confirming the successful production of pUC57 + pEWU1^{-SP}.

Vector Sequencing

The pUC57 + pEWU1 was sent to Genewiz for sequencing to confirm that the pEWU1 sequence remained correct after manipulation. The sequencing results indicated that a G to A substitution was present in the pEWU1 RepA gene (see Figure 1 for RepA location within pEWU1; Figure 11A**)**. Unfortunately, this error was present in the original DNA delivered by GenScript (their QC failed and we failed to recognize it) and was not generated during our manipulations.

Both the target sequence and the mutated sequence were examined using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to investigate the substitution. The change caused a non-synonymous substitution; the G to A altered the corresponding glycine residue to a glutamic acid residue (Figure 11A and Figure 11B).

To further investigate the effects of this change, the sequences were subjected to the Protein Homology/analogy Recognition Engine (PHYRE2) to analyze the structural impact of the missense variant (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The submission

showed no change at the end of the alpha-helix region which the missense mutation inhabits (Figure 12A and Figure 12B). Additionally, the 3D crystalline structures for both standard RepA and the mutated RepA showed no visible changes (Figure 12C and Figure12D).

Transformation of *L. lactis*

The transformations of *L. lactis* by pEWU1 and pEWU1^{-SP} produced by restriction and ligation did not yield Lac+ colonies using the Lac+/- selection scheme and selective media. The no-DNA control transformation (which should have had no colonies; Figure 13A) grown on plates with lactose had roughly as many colonies as the plates receiving cells putatively transformed with plasmid DNA. This indicates that the selection process did not work as these Lac- (untransformed cells) grew on plates lacking lactose. The no-DNA transformation cells plated onto plates containing glucose (Figure 13B) have lots of growth but this is not informative in light of the growth on the negative control plates. All three plated volumes (10, 100, and 900 µL) of pEWU1-transformed cells produced many colonies, suggestive of background growth. The plate receiving 10 µL of the transformation reaction (Figure 13C) had distinct colonies that could be picked. Similarly, all three plates containing the three volumes of the pEWU1^{-SP} transformation produced many colonies. Again, the plate receiving only 10 µL of the transformation reaction (Figure 13D) had some discernable colonies that could be picked.

Hundreds of colonies from the pEWU1 and pEWU1^{-SP} transformation plates were analyzed by colony PCR but none of them contained a plasmid (blank gel data not shown).

Erm selection was successfully integrated into this system [erythromycin resistance was confirmed in *E. coli* (data not shown)]. The no-DNA negative control

transformation using the Erm ligation reactions did not yield any colonies, confirming successful erythromycin selection (Figure 14A). Transformation reactions using pGH9:ISS1 (positive control plasmid with Erm) resulted in *L. lactis* colonies, indicating successful transformation and selection (Figure 14B). Transformations using pUC57 + pEWU1 + Erm (Figure 14C), or pUC57 + pEWU1^{-SP} + Erm (Figure 14D) ligation reactions did not produce colonies, indicating that the respective constructs either did not enter the cells, failed to replicate within the cells, or failed to yield gene expression within the cells.

Finally, supercoiled pEWU1^{-SP} + Erm plasmid DNA harvested from MC1061 cells, the pGH9:ISS1 positive control, and a no DNA control were used to transform *L. lactis.* The no-DNA negative control did not yield any colonies (Figure 15A), indicating proper erythromycin selection. The positive control had many colonies (Figure 15B), indicating successful transformation. The plate receiving the pEWU1^{-SP} + Erm transformation did not have any colonies, indicating that our plasmid did not successfully transform the cells (Figure 15C).

DISCUSSION

The challenges began when GenScript continuously delayed the delivery of our designed vector and then completely failed to deliver said vector. Instead, the eventual pEWU1 was delivered as two independent fragments cloned into pUC57. The reasons for this failure were never explained beyond stating that the fragments would not assemble with their "standard procedures". The genetic sequence within the region where fragments A and B overall is unremarkable: not G:C or A:T rich and does not have repetitive sequences. The "standard" cloning strain that the company uses may have been incapable of maintaining the vector once it was fully assembled. Although the vector was codon-optimized for *L. lactis*, some of the genes

may have been expressed in the *E. coli* strain being used leading to toxic effects that eliminated any colonies that might have had the assembled vector. It is well known that many microbial genes are toxic to *E. coli (KIMELMAN et al. 2012)*.

Given that GenScript delivered two fragments, we were left with two options: abandon the project or attempt the assembly. A Gibson ligation seemed the likeliest path to success; however, a restriction and ligation scheme eventually delivered the complete vector.

Gibson ligation via the NEBuilder HiFi DNA assembly failed to assemble the two pEWU1 fragments. The main challenge with this approach came from an inability to properly generate the B fragment via PCR. This approach required that we used very specific primers with little regard for how well the primer pairs would function as pairs during the PCR reactions. We had to put primers at specific locations. The only avenue available to us for controlling primer melting temperature then was the length of each primer. Additionally, this negated the ability to use a mispriming library in primer design. As such, it is not surprising that we observed lots of mispriming. The result was a large amount of background in the B-fragment PCR done using Elongase and a plasmid miniprep product that almost certainly included at least contamination level amounts of *E. coli* genomic DNA. While we were able to cut from the gel the desired band and purified it, it was almost certainly contaminated with non-target strands of DNA. Finally, the Elongase reaction attempted to amplify the complete vector after ligation shared the same problem: two mismatched primers forced to work together. The results being that the full amplicon was not generated.

Restriction enzyme assembly was used to successfully assemble pEWU1 using a pUC57 backbone. This approach was possible due to the presence of a mutual, unique HpaI restriction endonuclease site contained in the complementary overlap regions of fragments A and B

generated by GenScript. Additionally, this approach required a second unique restriction site: ApaI. This site is downstream of the A fragment in pUC57 and flanks the downstream end of the B fragment in the pUC57 vector containing fragment B. By digesting both of these vectors with HpaI and ApaI, we were able to generate pEWU1 successfully. This allowed us to then remove the serratiopeptidase gene easily by restriction to generate a no gene control. These results were confirmed by digesting pEWU1 and pEWU1-SP and obtaining the correct distinct restriction patterns. This progress allowed us to move to the next step of the project. Before moving on to transforming *L. lactis* with these vectors we sequenced pEWU1 to confirm that the sequence remained correct after much manipulation.

Sequencing revealed a G to A non-synonymous substitution leading to a glycine becoming a glutamic acid in the RepA protein. Further analysis revealed that this "change" was a synthesis error as the vector delivered by GenScript contained this difference. Structural analysis using the Phyre2 database suggested that this change did not affect the alpha helix where it resides or the overall 3D structure of the protein. Thus, we decided to proceed without fixing this substitution in pEWU1 *repA*.

A RepA hexamer regulates plasmid copy-number. It is possible that this substitution led to alteration of the secondary, tertiary, or quaternary structure of the protein very slightly and thus reduced plasmid number to the extent that it would not be maintained after transformation or increased plasmid number so drastically that it stressed successfully transformed cells to the point of death.

Once assembled, the pUC57 + pEWU1 vector was further manipulated in *E. coli*. The pEWU1^{-SP} no gene control was successfully generated by removing the serratiopeptidase gene from pUC57 + pEWU1. The no-gene control would have been used in eventual expression

experiments. Successful removal of the serratiopeptidase gene was confirmed by restriction analysis.

The initial *L. lactis* transformation using food-grade selection on L-M17 plates failed. After being excised from pUC57 using EcoRI, pEWU1 and pEWU1^{-SP} were ligated and the ligation reactions were used for transformation. The negative control transformation (without DNA) also produced colonies. This indicated that the selection system with the NZ3000 host cells is not sensitive enough for our purposes. This is likely because the media contained trace sugars that allow the bacteria to grow even in the absence of lactose. Lactose is the selection sugar and only transformants should have grown on the plates.

After initial food-grade selection in *L. lactis* failed, we successfully introduced erythromycin resistance to pEWU1 and pEWU1^{-SP}. The ErmR sequence was modified such that it could be inserted into our vectors by restriction and ligation using two unique restriction endonuclease sites which were available on both vectors: SpeI and SnaBI. As ErmR will work for selection in *E. coli*, this introduction was fairly straightforward as we kept the pEWU1 vectors in pUC57. Ligation reactions with pEWU1 + ErmR or pEWU1^{-SP} + ErmR were used to transform *L. lactis*. In these attempts, only the positive control plasmid successfully transformed the bacteria. As transformation efficiency is known to be low in this bacterial species, we hypothesized that the ligation reaction lacked enough ligation product for successful transformation. Thus, we decided to attempt transformation with supercoiled pUC57 + pEWU1^{-SP} + ERM.

Using pUC57 + pEWU1^{-SP} + ERM, we were unable to transform *L. lactis* by direct supercoiled plasmid transformation. The positive control, pGh9:ISS1, successfully transformed the cells indicating that the cells were competent and the procedure was done correctly. The no

DNA negative control confirmed that the G-M17 (5 μg/mL) plates were properly selective. Given that the empty vector failed to transform *L. lactis* it is unlikely that the full vector would have been able to do so. As such, these experiments were not undertaken.

The vector should have worked as designed but it has not. Our system has some as of yet undetermined flaw that prevented the successful transformation of empty (pEWU1^{-SP}) into *L. lactis*. Further experimentation could be undertaken to determine whether the G:A substitution is the culprit or whether the strong promoter in the vector produced a small toxic peptide in the empty vector that prevented cell growth. It is also possible that the addition of the watermark in the 3' untranslated region of the serratiopeptidase mRNA could have affected successful transcriptional termination. Unterminated transcription could stress the cells and prevent colony growth. However, the gene would not have been expressed in the absence of lactose. Therefore, this issue could not have been the cause of failure in the cells transformed with vectors containing the erythromycin resitance gene as these cells were grown in media with glucose and without lactose. As such, it is unlikely that that watermark was the cause of the failure to transform *L. lactis*.

Ninety percent of drugs in the biotech industry pipeline that seek approval from regulatory agencies, fail to gain said approval and never reach the marketplace. Many more candidate drugs are eliminated from the product pipeline before seeking approval. Beyond that, many ideas die after some experimentation is done in the laboratory. That is to say that biotech research is very challenging and research projects (even the many based on excellent ideas) often fail to yield positive results that will allow further progress along a specific line of experimentation. Thus, this attempt to develop a vector for expressing serratiopeptidase in *L. lactis* has excellent company.

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TABLES

Table 1. **Sequences of primer used to amplify target DNA in preparation for NEB HiFi Ligation assembly of pEWU1 A and B fragments.** The bold sequence indicates an integrated EcoRI recognition sequence corresponding to the EcoRI restriction site at the beginning of fragment A and end of fragment B, respectively. The underlined "primer tails" were added to facilitate restriction by EcoRI (after assembly) in preparation for the circularization of the complete pEWU1 vector by ligation.

Primer Name	**Primer Sequence**
Serrat-A-1768F (AF)	GAGAAA**GAATTC**TTCAAGGTAAAACAAACAATTTCAAAC
Serrat-A-1768R (AR)	AACGCTCAGGTCGGTCAC
Serrat-B-2935F (BF)	AGCAACGTGACCGACCT
Serrat-B-2935R (BR)	GAGAAA**GAATTC**AATATTATCTTTTATGATACAATTAAAAGA

Table 2. Sequences for primers used for Sanger sequencing for plasmid synthesis verification. Asterisks denote IDT-calculated melting temperatures as these were ReadyMade™ primers from the IDT website (https://www.idtdna.com/site/order/stock/index/readymade).

Name	Sequence	Melting Temp (°C)
	Forward Sequencing	
M13F (-41)	CGCCAGGGTTTTCCCAGTCACGAC	64.1*
SeraSeq_907F	AAGCAACGTTAAACACCCGG	59.3
SeraSeq_1593F	GTGCGAGCGACTGGATTC	58.6
SeraSeq_2208F	AAAGCAGCAGTTGATAAAGCAA	57.4
SeraSeq_2867F	CCCCATTAAGTGCCGAGTG	58.2
SeraSeq_3409F	GGCTGTCAGTCCTTTACACG	58.5
	Reverse Sequencing	
M13R (-27)	CAGGAAACAGCTATGAC	47.0*
SeraSeq_511R	GCCGTTAATTTGGATGCCGT	59.9
SeraSeq_3663R	GCGTCCTTTGATTCATGAGTCA	59.0
SeraSeq_4247R	AGTCATCTCTTCTCTGTTCACAA	57.2
	pEWU1 Colony PCR	
SP_Control_FP	CAGCCCCGTTGTCAGGTG	60.67
SP_Control_RP	AACATCTTCGCTGCAAAGCC	59.76

FIGURES

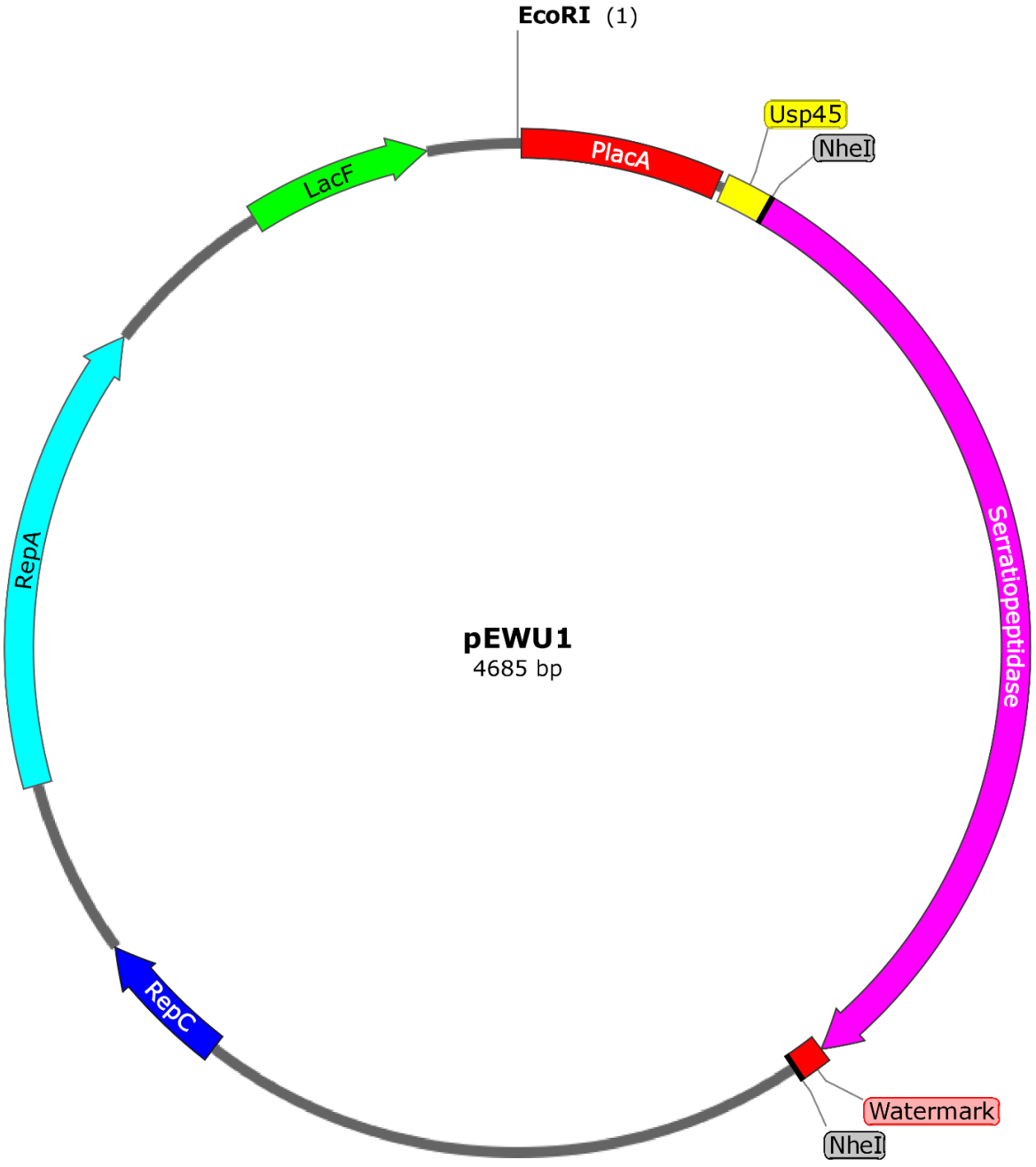


Figure 1. Food-grade pEWU1 vector map containing EcoRI and NheI restriction sites. The EcoRI site will allow for insertion/removal of the pEWU1 from a pUC57 backbone for manipulation in *E. coli*. *lacF* confers selection based on lactose. PlacA is the inducible

promoter, and Usp45 is a lactococcal extracellular secretion signal. *repA* and *repC* are required for plasmid replication within *L. lactis*.

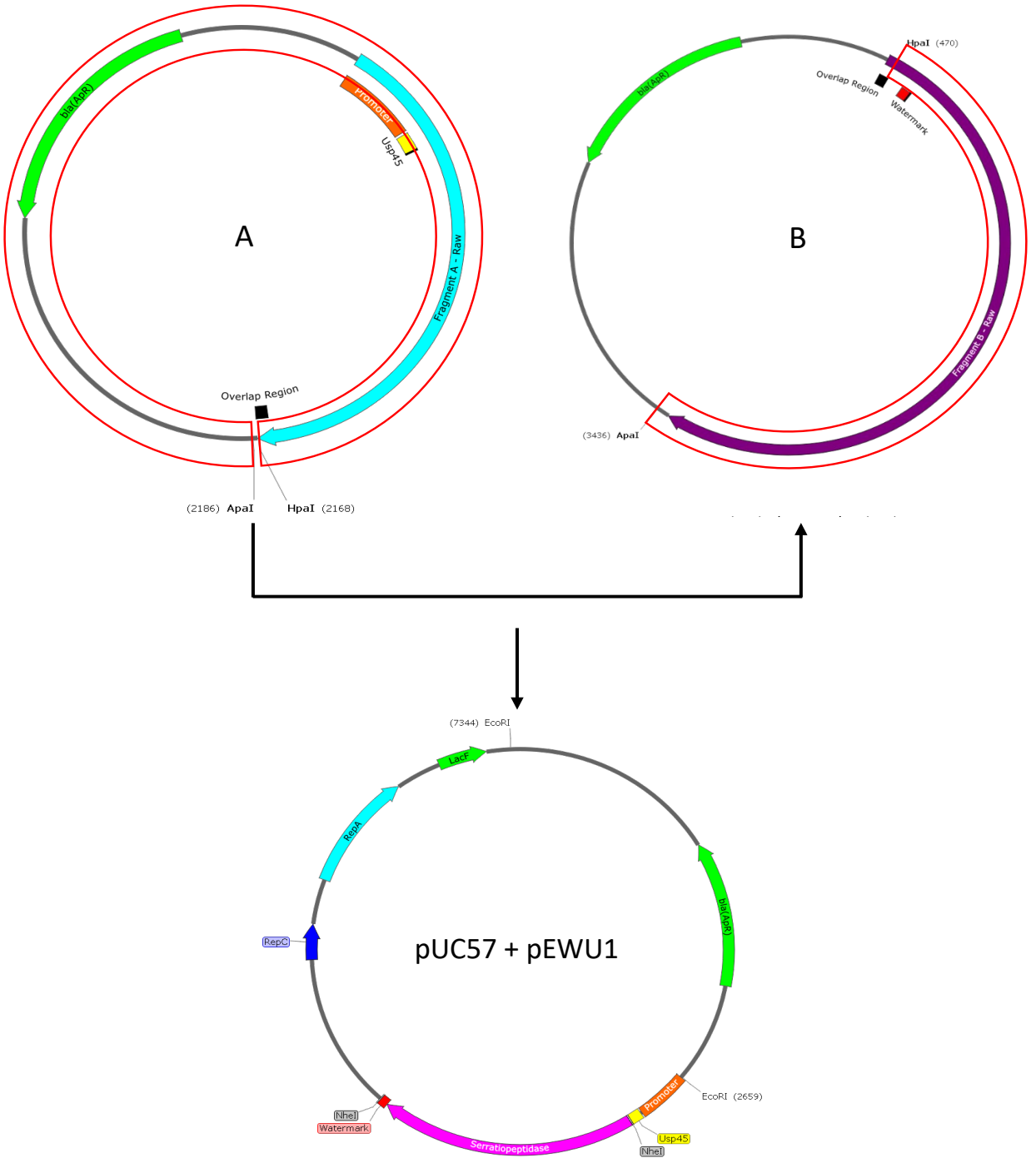


Figure 2. Assembly workflow for combining the pEWU1 fragment A with fragment B and fusing into pUC57. Vectors containing A/B were digested using HpaI and ApaI. The Vector + B was dephosphorylated before subsequent ligation with the A fragment.

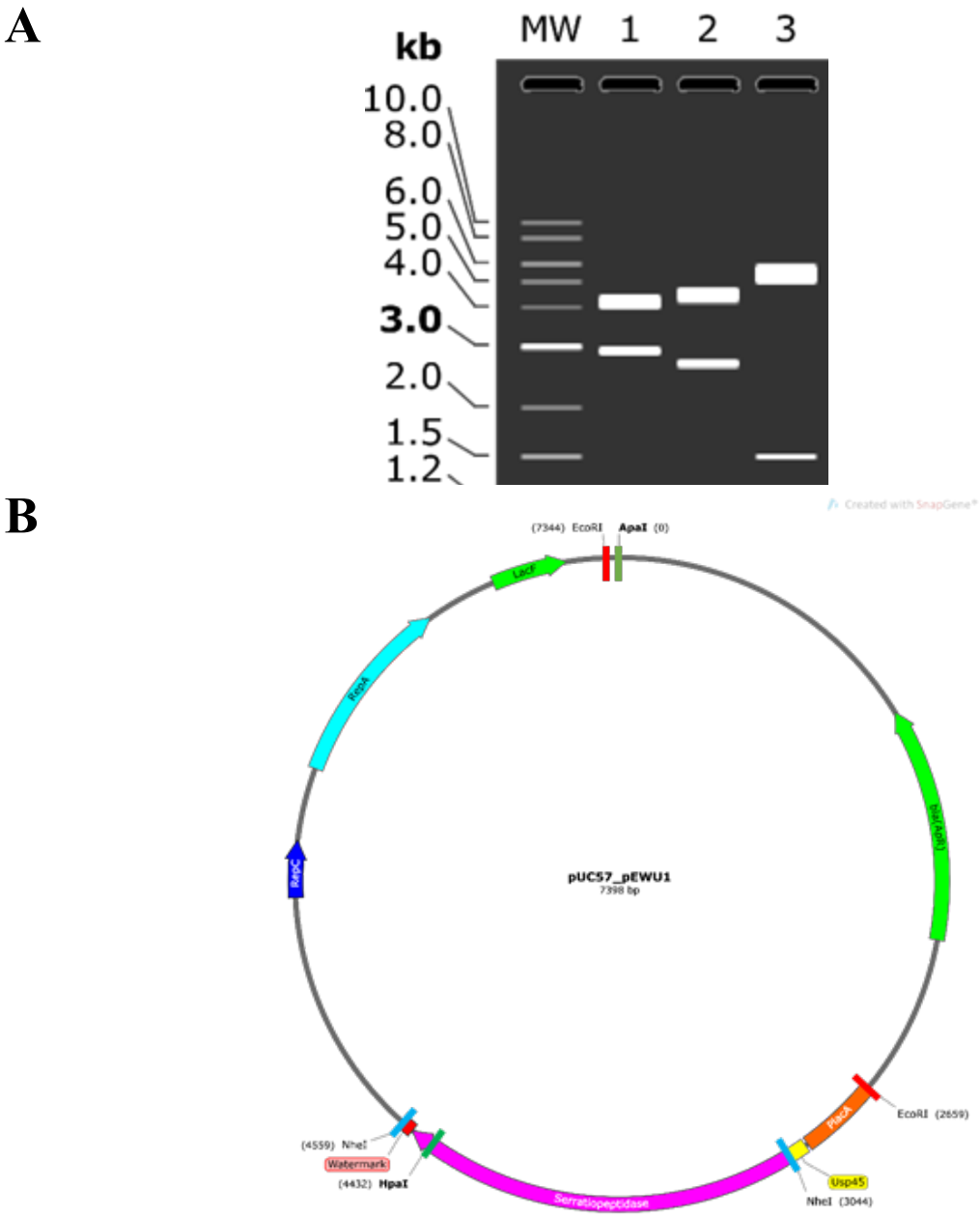
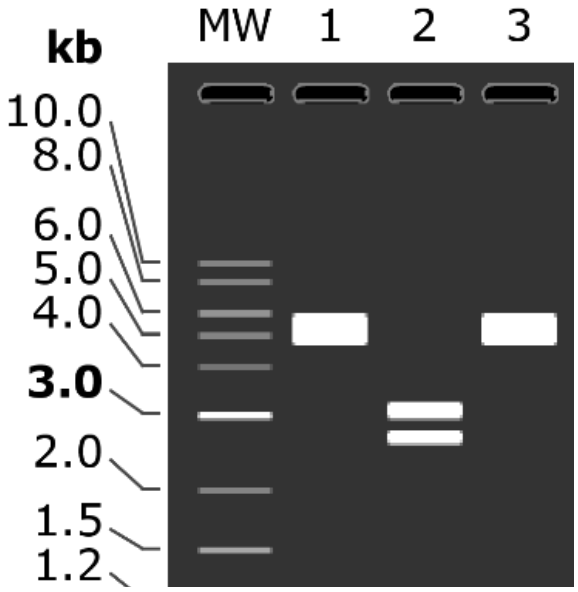


Figure 3. pUC57 + pEWU1 predicted digestion pattern (A) based on restriction respective enzyme site locations within the assembled pEWU1 within the pUC57 backbone plasmid map (B).
Restriction with HpaI/ApaI (lane 1 in A and GREEN cut sites in B) should produce a 4432 bp band and a 2966 bp band; digestion with EcoRI (lane 2 in A and RED cut sites in B) should produce a 4685 bp band (pEWU1) and a 2713 bp band (pUC57); digestion with NheI (lane 3 in A and BLUE cut sites in B) should yield a 1515 bp band (serratiopeptidase coding sequence) and a 5883 bp band [linear pUC57 + pEWU1 lacking serratiopeptidase; pEWU1^{-SP}]

A



B

Figure 4. pUC57 + pEWU1^{-SP} predicted digestion pattern (A) based on restriction at respective enzyme site locations within the assembled pEWU1 ^{SP} within the pUC57 backbone plasmid map (B). Restriction with ApaI (lane 1 in A and GREEN cut site in B) should produce a 5883 bp band (linear pUC57 + pEWU1 lacking serratiopeptidase; aka pEWU1^{-SP}); digestion with EcoRI (lane 2 in A and RED cut sites in B) should produce a 3170 bp band (pEWU1^{-SP}) and a 2713 bp band (pUC57); digestion with NheI (lane 3 in A and BLUE cut site in B) should yield a 5883 bp band (pEWU1^{-SP}).

A

B

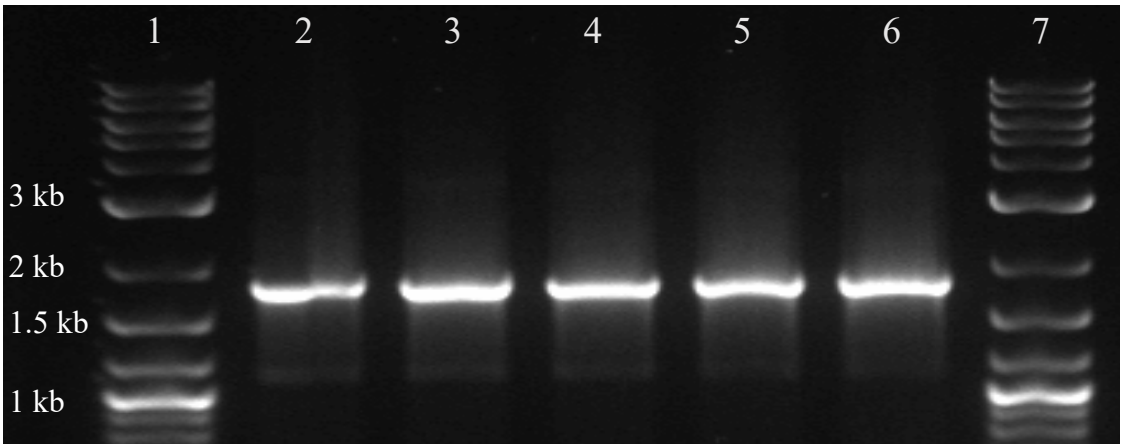


Figure 5. Linearized plasmid vector map for pUC57 + A fragment (A) for verification of fragment A gel electrophoresis after successful PCR amplification (B). (A) Forward (green) and reverse (red) primer (Table 1) locations are indicated that yield a 1768 bp amplicon containing the pEWU1 "A" fragment. (B) Gel electrophoresis image showing PCR amplification of the expected 1768 bp band. Lanes 1 and 7 = 2-log ladder. Lanes 2-6 = temperature gradient for samples being tested in increasing temperature gradient from left to right (55 ℃ to 59 ℃). All temperatures primed bands. Lane 6 was selected for use in assembly.

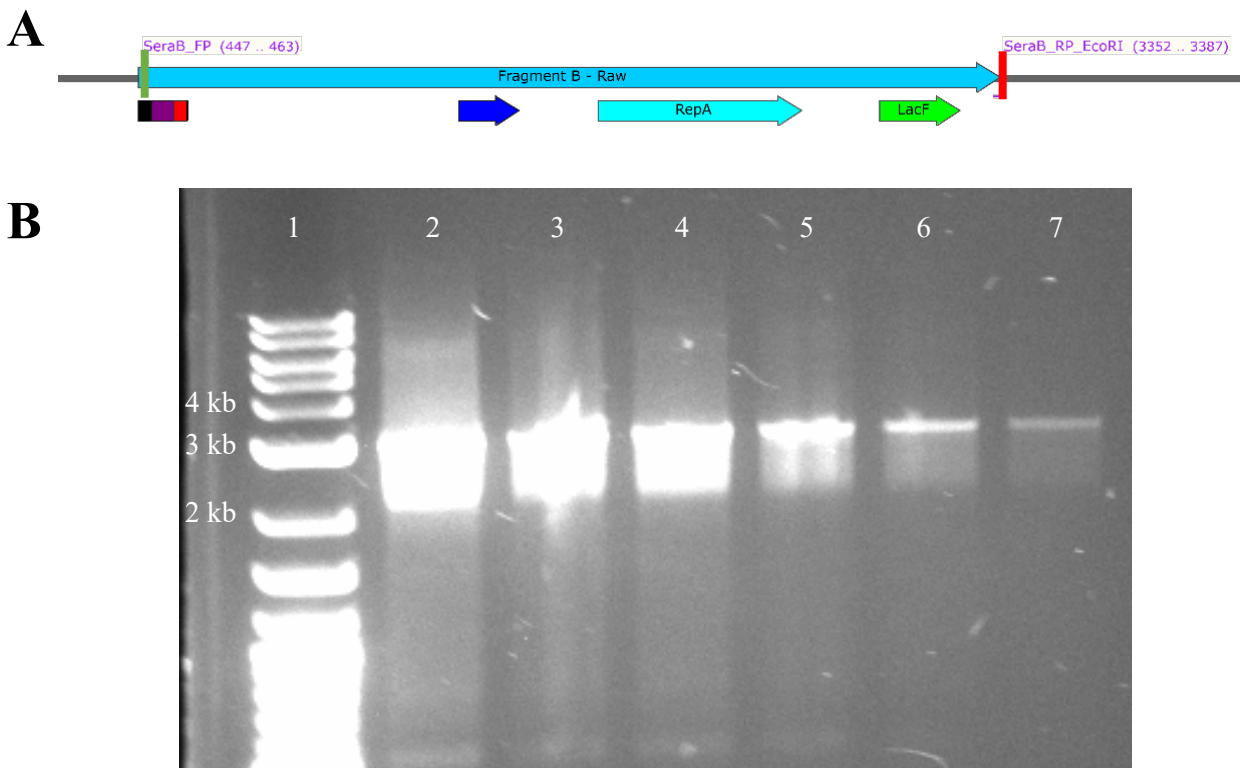


Figure 6. Linearized plasmid vector map for pUC57 + B fragment (A) for verification of fragment B gel electrophoresis after unsuccessful PCR amplification (B). (A) Genetic map showing the location of the forward primer (GREEN) and the reverse primer (RED) for amplifying the B fragment. The right primer had a 6 bp overhang that is unaccounted for in the image. The amplicon generated should be 2947 bp. (B) PCR amplification showing fragment B amplification. Lane 1 = 2-log ladder. Lanes 2-7 = temperature gradient ranging from 50-58.3 °C. The DNA in the lanes in the middle of the gel migrated more slowly than the DNA in the lanes at the edge of the gel giving the appearance that the amplicon is longer than expected.

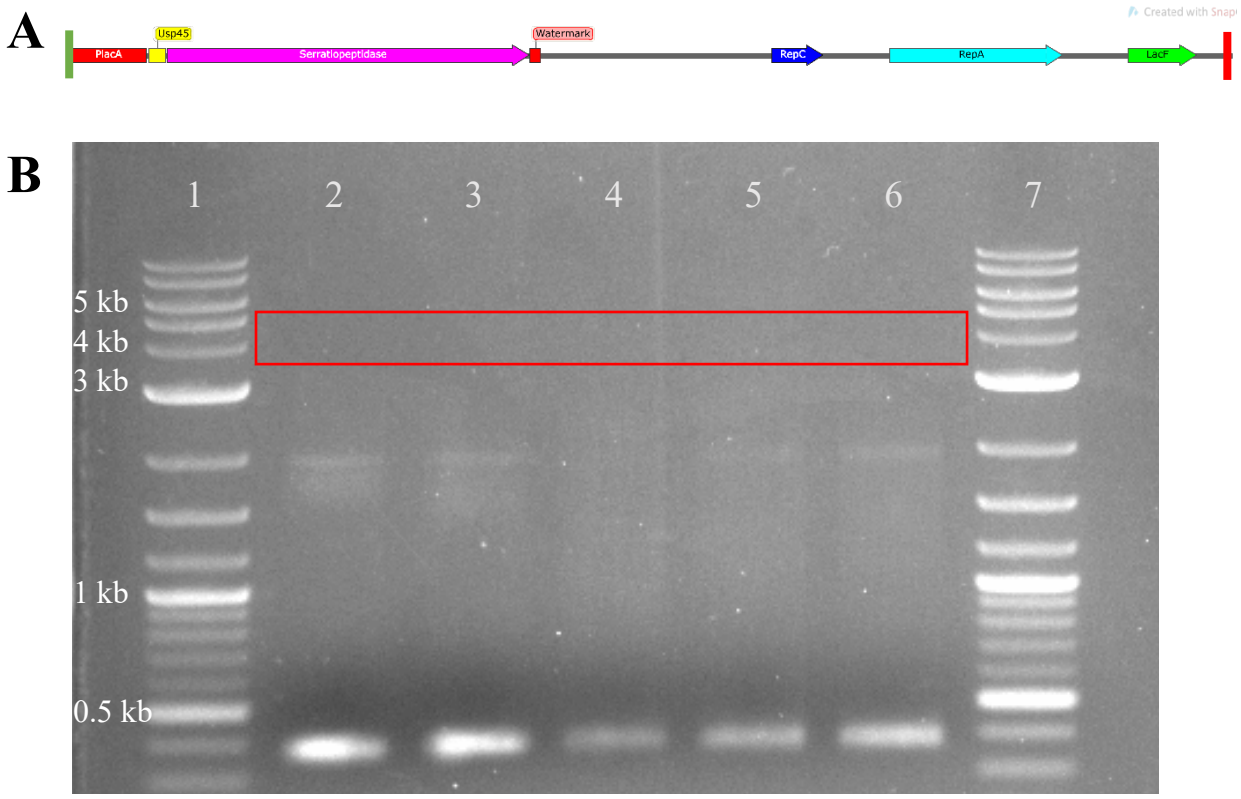


Figure 7. Linearized plasmid vector map for pEWU1 (A) used for unsuccessful verification of pEWU1 after failed Gibson assembly PCR (B). (A) AF primer (GREEN marker) and BR primer (RED marker) from table 1 were used to attempt the final Gibson assembly PCR amplification of pEWU1. (B) The red box indicates the failure to generate the final 4693 bp pEWU1 amplicon by Gibson assembly combination of pUC57 + A + B and subsequent PCR.

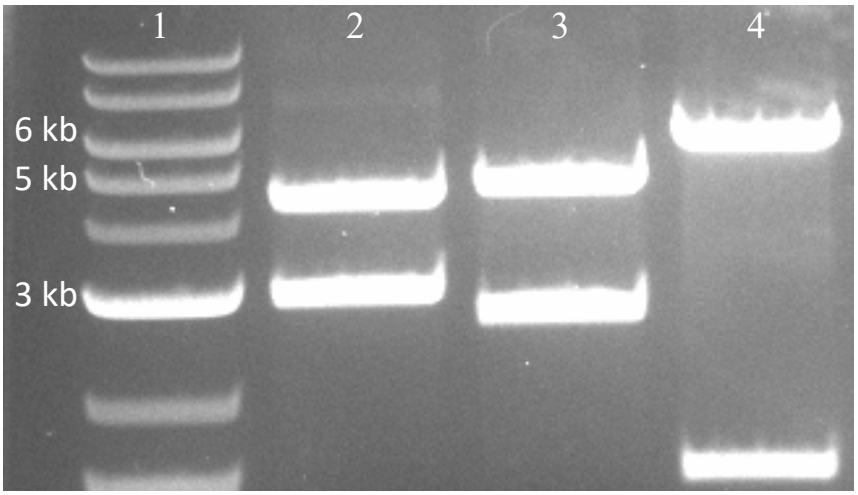


Figure 8. Restriction digestion patterns correctly identify the proper formation of the pUC57 + pEWU1 vector. Lane 1 = 2-log DNA ladder. The vector was digested with HpaI/ApaI (lane 2), EcoRI (lane 3), and NheI (lane 4). This result matches the predicted pattern from figure 3A.

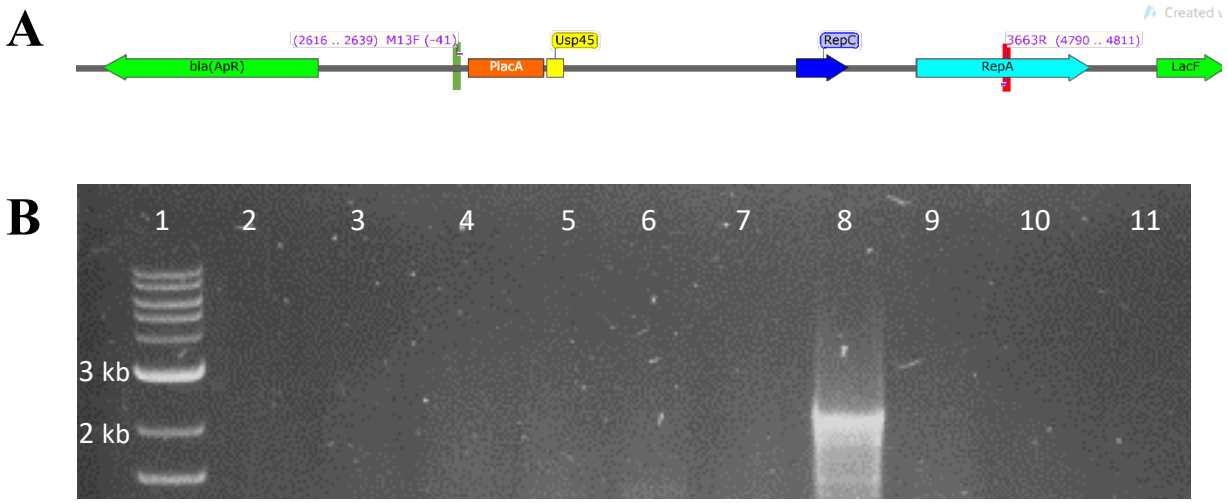


Figure 9. Linearized plasmid vector map for pUC57 + pEWU1^{-SP} vector (A) for verification of successful *E. coli* colony PCR indicating pUC57 + pEWU1^{-SP} transformant (B). (A) Genetic map showing the location of the forward primer (GREEN) and the reverse primer (RED) used to determine the success of *E. coli* colony PCR. (B) Using the M13F and 3663R primers (Table 1), colony 7 was determined to have the appropriate 2196 bp band indicating the successful removal of serratiopeptidase. All lanes correspond to isolated colonies following transformation. Lane 8 corresponds to the only colony that was positive for the correct band. Lanes 1 = 2-log ladder.

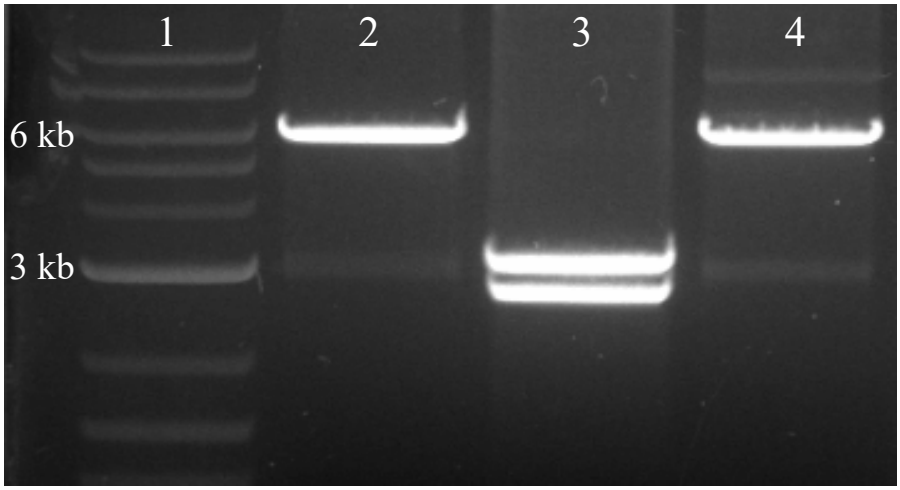


Figure 10. Restriction digestion patterns correctly identify proper formation of pUC57 + pEWU1 "empty" vector. Lane 1 = 2-log ladder. As with the predicted pattern, the middle lane 3 digested with EcoRI contains the 3170 bp band of interest (linear pEWU1 "empty"). Lane 2 = ApaI and lane 4 = NheI. Results match figure 4A.

A

Score	Expect	Identities	Gaps	Strand
1609 bits(871)	0.0	873/874(99%)	0/874(0%)	Plus/Plus

```
Query  3883  AAAGATATTGTTTCAACAAACTCTAGCGCCTTTAGATTATGGTTTGAGGGCAATTATCAG  3942
             ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  421   AAAGATATTGTTTCAACAAACTCTAGCGCCTTTAGATTATGGTTTGAGGACAATTATCAG  480
```

B

Sequence ID: Query_51843 Length: 232 Number of Matches: 1

Range 1: 1 to 232 Graphics ▼ Next Match ▲ P

Score	Expect	Method	Identities	Positives	Gaps
471 bits(1211)	3e-176	Compositional matrix adjust.	231/232(99%)	231/232(99%)	0/232(0%)

```
Query  1    MAIKNTKARNFGFLLYPDSIPNDWKEKLESLGVSMAVSPLHDMDEKKDKDTWNSSDVIRN  60
            MAIKNTKARNFGFLLYPDSIPNDWKEKLESLGVSMAVSPLHDMDEKKDKDTWNSSDVIRN
Sbjct  1    MAIKNTKARNFGFLLYPDSIPNDWKEKLESLGVSMAVSPLHDMDEKKDKDTWNSSDVIRN  60

Query  61   GKHYKKPHYHVIYIARNPVTIESVRNKIKRKLGNSSVAHVEILDYIKGSYEYLTHESKDA  120
            GKHYKKPHYHVIYIARNPVTIESVRNKIKRKLGNSSVAHVEILDYIKGSYEYLTHESKDA
Sbjct  61   GKHYKKPHYHVIYIARNPVTIESVRNKIKRKLGNSSVAHVEILDYIKGSYEYLTHESKDA  120

Query  121  IAKNKHIYDKKDILNINDFDIDRYITLDESQKRELKNLLLDIVDDYNLVNTKDLMAFIRL  180
            IAKNKHIYDKKDILNINDFDIDRYITLDESQKRELKNLLLDIVDDYNLVNTKDLMAFIRL
Sbjct  121  IAKNKHIYDKKDILNINDFDIDRYITLDESQKRELKNLLLDIVDDYNLVNTKDLMAFIRL  180

Query  181  RGAEFGILNTNDVKDIVSTNSSAFRLWFEGNYQCGYRASYAKVLDAETGEIK  232
            RGAEFGILNTNDVKDIVSTNSSAFRLWFE NYQCGYRASYAKVLDAETGEIK
Sbjct  181  RGAEFGILNTNDVKDIVSTNSSAFRLWFEDNYQCGYRASYAKVLDAETGEIK  232
```

Figure 11. Fragment B (sent from GenScript) contained a single base pair error that led to a non-synonymous substitution. A. Nucleotide BLASTn (compare two sequences) results showing G to A error generated during fragment synthesis. B. An amino acid alignment obviates the glycine (G) to glutamic acid (D) change resulting from the synthesis error.

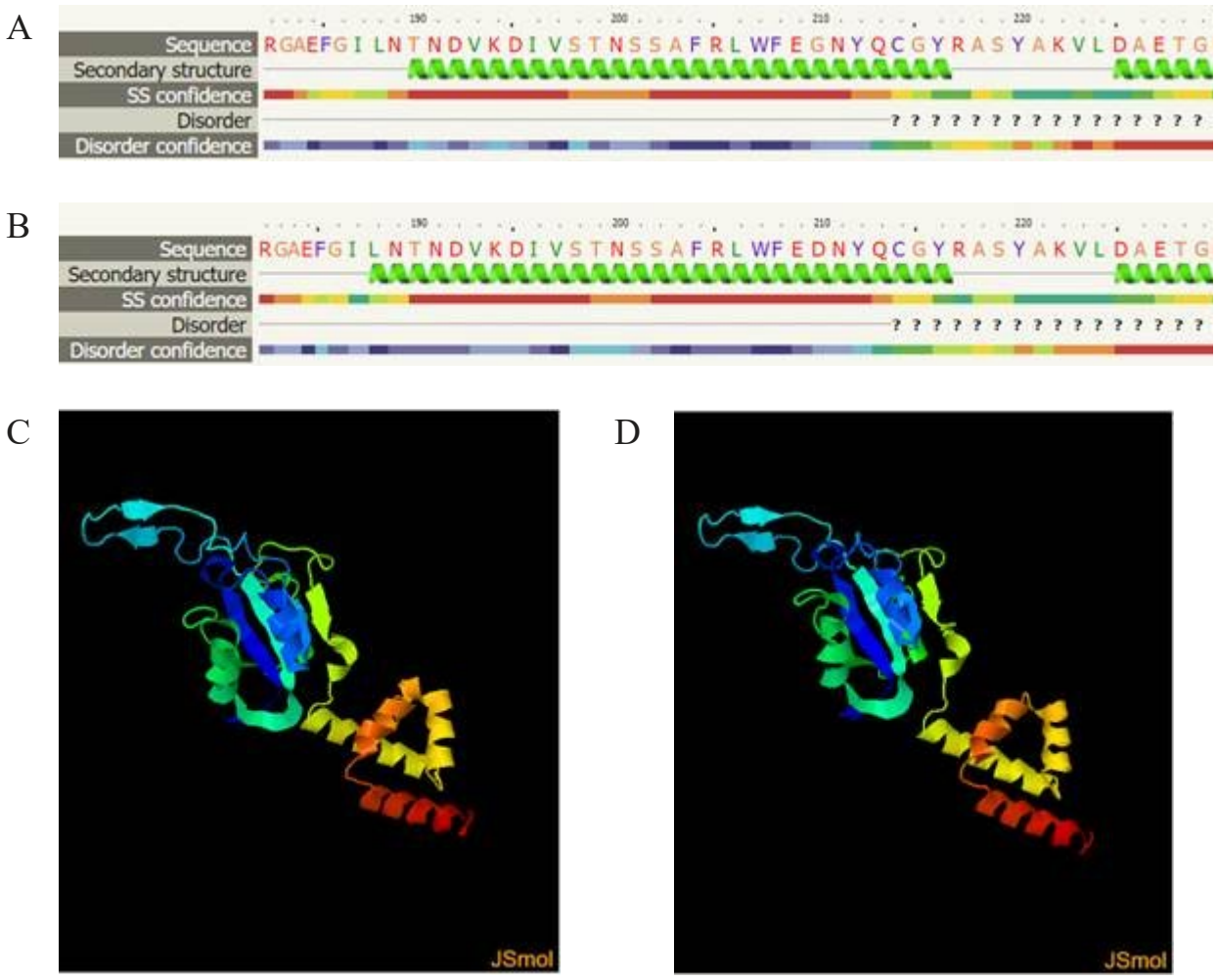
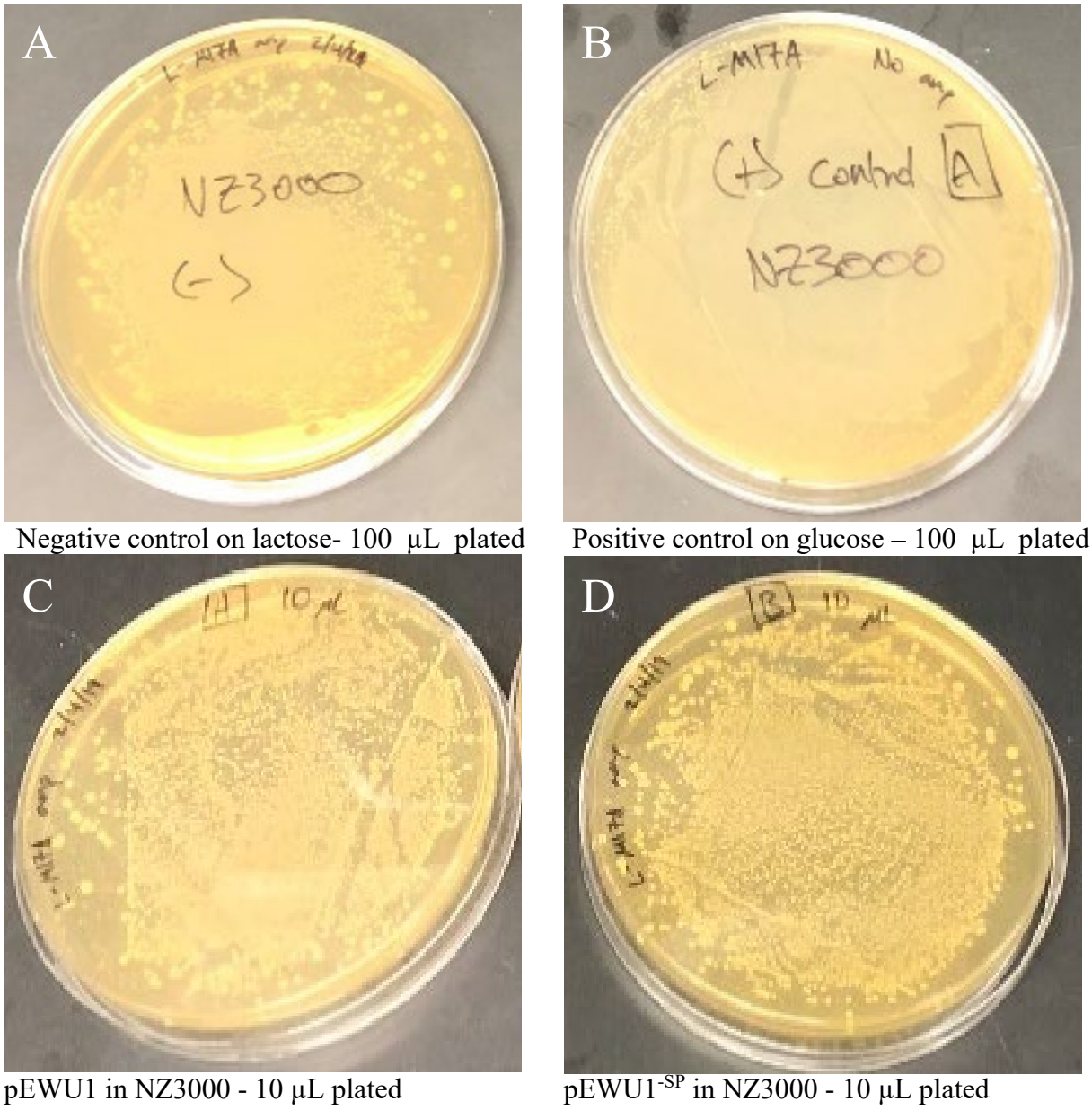


Figure 12. The non-synonymous substitution introduced by GenScript did not alter the structure of the RepA protein. A. The wildtype RepA sequence that was ordered produced a glycine (G) at position 210. B. The sequence delivered by GenScript produced an Aspartic Acid (D) at position 210 but did not disrupt the alpha helix. The crystal structure for wildtype RepA (C) and the structure including the non-synonymous substitution (D) are identical.



Negative control on lactose- 100 µL plated

Positive control on glucose – 100 µL plated

pEWU1 in NZ3000 - 10 µL plated

pEWU1^{-SP} in NZ3000 - 10 µL plated

Figure 13. Transformation of *Lactococcus lactis* NZ3000 using a no DNA negative control on lactose (A), a positive control on glucose (B), pEWU1 (C), and pEWU1^{-SP} (D). Volumes plated are listed below the plates. 100 µL and 900 µL were also plated for each transformation above but are not shown.

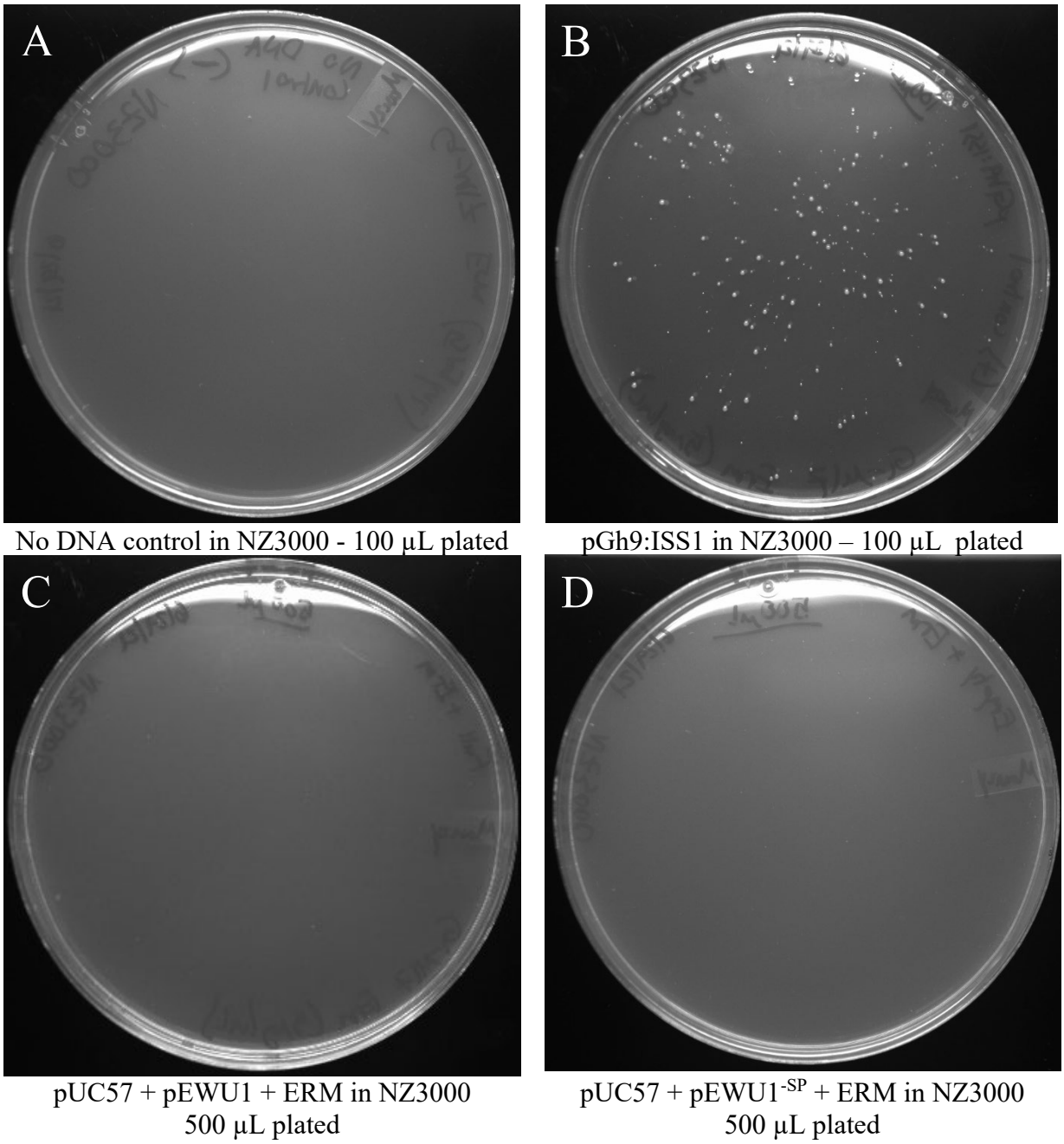
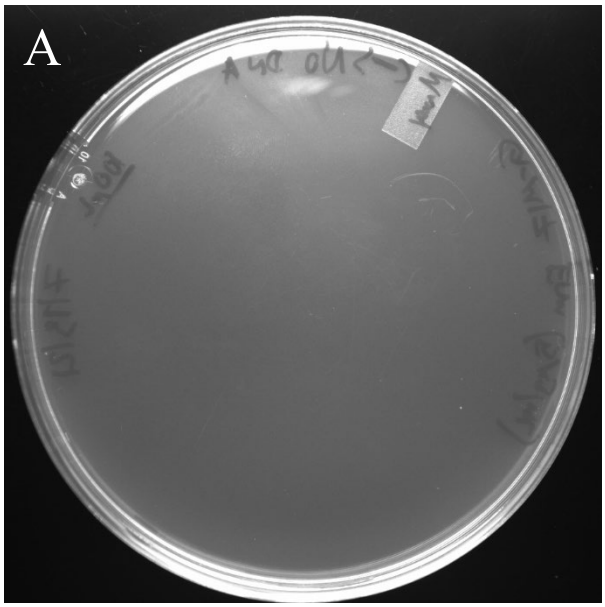
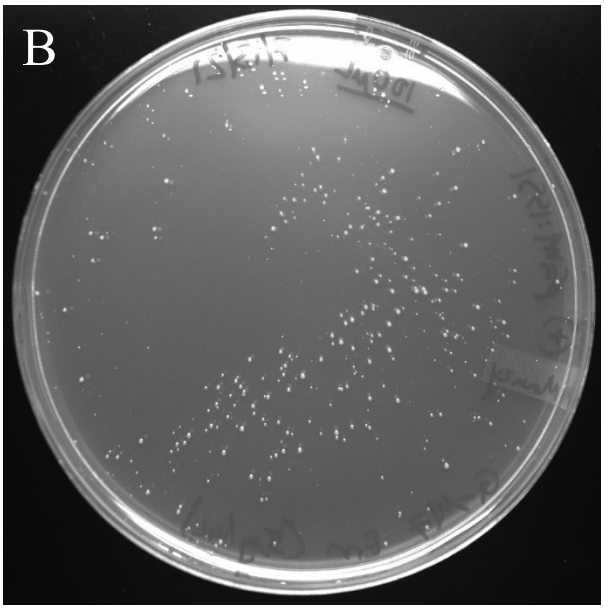


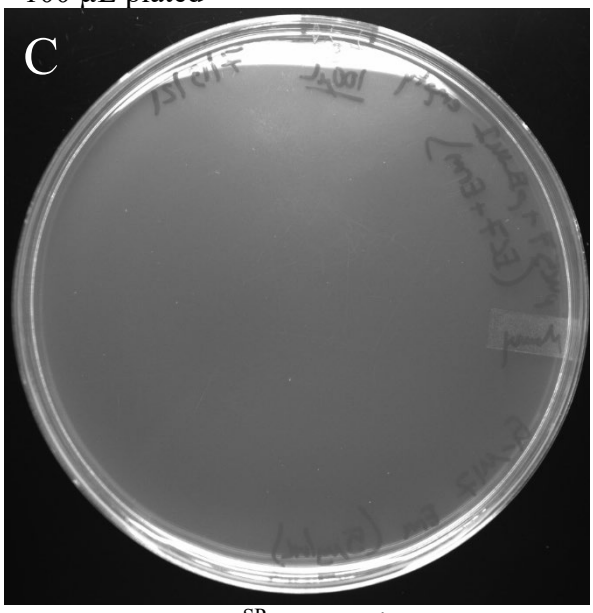
Figure 14. Transformation of *Lactococcus lactis* NZ3000 with ligation cloning using a no DNA negative control (A), pGh9:ISS1 positive control (B), pUC57 + pEWU1 + ERM (C), and pUC57 + pEWU1^{-SP} + ERM (D). No ligase controls were used for each transformation to ensure proper cloning techniques were followed. Volumes plated and plasmid are listed under each image. For C and D 100 µL were also plated for each transformation (plate images not shown). Additionally, all ligation reactions had respective no-ligase controls. As expected, none of those plates had colonies (plate images not shown).



No DNA negative control in NZ3000
- 100 µL plated



pGh9:ISS1 Positive control in NZ3000
- 100 µL plated



pUC57 + pEWU1^{-SP} + ERM in NZ3000- 100 µL plated

Figure 15. Transformation of *Lactococcus lactis* NZ3000 with supercoiled plasmid using a no DNA negative control (A), pGh9:ISS1 plasmid (Erm+) positive control (B), and pUC57 + pEWU1^{-SP} + Erm (C). Volumes plated and plasmid are listed under each image. Plasmids used were harvested from erythromycin-resistant (conferred from plasmid) mc1061 cells.

Appendix A: Sequences

pEWU1 Vector Sequence (Codon optimized for *L. lactis*)

[Start codon (ATG) and stop codon (TAA) bold/underlined for serratiopeptidase gene.]

GAATTCTTCAAGGTAAAACAAACAATTTCAAACAAAAACAAACGTTAGATGATGAAATAAGAAC

AGAGGATTGACGTATATTAGCTTAGGTCAGATTTTGTATAAGACGAAAATAAAGTAGGACCTCT

TAATCAGTAAGTTATAGAAAGTAAAAGACTTTTGTAATACCTGAATAGATATTTCACGTCCATT

TTGTGATGGATTAAATGAACAAAAATGAACAATAATTTAACGGTGTTATCTATTTTTTAAAAAA

ACAAATAAAAAAAAACAAAAAATTAACAAAAATAGTTGCGTTTTGTTTGAA**ATG**AAAAAAAAGA

TTATCTCAGCTATTTTAATGTCTACAGTGATCTTAAGTGCTGCAGCCCCGTTGTCAGGTGTTTA

CGCTAGCATGCAGAGCACCAAGAAAGCGATCGAGATTACCGAAAGCAGCCTGGCGGCGGCGACC

ACCGGTTACGACGCGGTGGACGATCTGCTGCACTATCACGAGCGTGGTAACGGCATCCAAATTA

ACGGCAAGGATAGCTTCAGCAACGAGCAGGCGGGCCTGTTTATCACCCGTGAAAACCAAACCTG

GAACGGTTACAAGGTGTTCGGCCAGCCGGTTAAACTGACCTTCAGCTTTCCGGACTATAAATTC

AGCAGCACCAACGTTGCGGGTGATACCGGCCTGAGCAAGTTTAGCGCGGAGCAGCAACAGCAAG

CGAAACTGAGCCTGCAGAGCTGGGCGGACGTGGCGAACATCACCTTCACCGAAGTTGCGGCGGG

TCAAAAGGCGAACATTACCTTTGGCAACTACAGCCAGGACCGTCCGGGTCACTACGATTATGGC

ACCCAAGCGTATGCGTTCCTGCCGAACACCATCTGGCAGGGTCAAGACCTGGGTGGCCAGACCT

GGTACAACGTGAACCAAAGCAACGTTAAACACCCGGCGACCGAGGATTATGGTCGTCAGACCTT

TACCCACGAAATTGGTCACGCGCTGGGCCTGAGCCACCCGGGTGACTACAACGCGGGCGAGGGC

AACCCGACCTACCGTGACGTGACCTATGCGGAAGATACCCGTCAGTTCAGCCTGATGAGCTACT

GGAGCGAAACCAACACCGGTGGCGATAACGGTGGCCACTATGCGGCGGCGCCGCTGCTGGACGA

TATCGCGGCGATTCAACACCTGTACGGTGCGAACCTGAGCACCCGTACCGGTGACACCGTGTAT

GGCTTCAACAGCAACACCGGCCGTGATTTTCTGAGCACCACCAGCAACAGCCAGAAGGTTATCT

TCGCGGCGTGGGACGCGGGTGGCAACGACACCTTCGATTTTAGCGGTTATACCGCGAACCAACG

TATTAACCTGAACGAGAAGTGGTTTAGCGATGTTGGTGGCCTGAAGGGTAACGTGAGCATCGCG

GCGGGCGTTACCATCGAAAACGCGATTGGTGGCAGCGGTAACGACGTGATTGTTGGCAACGCGG

CGAACAACGTGCTGAAGGGTGGCGCGGGTAACGACGTTCTGTTCGGTGGCGGTGGCGCGGATGA

ACTGTGGGGTGGCGCGGGTAAAGACATCTTCGTGTTTAGCGCGGCGAGCGATAGCGCGCCGGGT

GCGAGCGACTGGATTCGTGATTTCCAGAAGGGCATCGACAAAATTGATCTGAGCTTCTTTAACA

AAGAGGCGCAAAGCAGCGACTTCATCCACTTTGTTGATCACTTTAGCGGTACCGCGGGTGAAGC

GCTGCTGAGCTACAACGCGAGCAGCAACGTGACCGACCTGAGCGTTAACATTGGTGGCCACCAG

GCGCCGGATTTCCTGGTGAAAATCGTTGGTCAAGTGGACGTTGCGACCGATTTTATTGTG**TAA**G

AAGCGGGCCTGGAAGCGCTGTGCCATGAAATGATTAGCACCAGCGCTAGCTGATGTTTGACATC

ATATAAACAAAGAAATGATGAAAACGTTATCTTGAACATTTTGCAAAATATTTTCTACTTCTAC

GTAGCATTTCTTTTTAAAATTTAGGAGGTAGTCCAAATGGCTATTGTTGTTGGTGCAGATCCTC

TAGACAGCTGGGATCCGTCGACCCCGGGCTGCAGGCATGCGGTACCACTAGTTCTAGAGAGCTC

AAGCTTTCTTTGAACCAAAATTAGAAAACCAAGGCTTGAAACGTTCAATTGAAATGGCAATTAA

ACAAATTACAGCACGTGTTGCTTTGATTGATAGCCAAAAAGCAGCAGTTGATAAAGCAATTACT

GATATTGCTGAAAAATTGTAATTTATAAATAAAAATCACCTTTTAGAGGTGGTTTTTTTATTTA

TAAATTATTCGTTTGATTTCGCTTTCGATAGAACAATCAAAGCGAGAATAAGGAAGATAAATCC

CATAAGGGCGGGAGCAGAATGTCCGAGACTAATGTAAATTTGTCCACCAATTAAAGGACCGATA

ACGCGTCGAGTGCATATTTTCGGCAATCTTCTCAATGAGATGCTCTTCAGCATGTTCAATGATG

TCGATTTTTTATTAAAACGTCTCAAAATCGTTTCTGAGACGTTTTAGCGTTTATTTCGTTTAGT

TATCGGCATAATCGTTAAAACAGGCGTTATCGTAGCGTAAAAGCCCTTGAGCGTAGCGTGGCTT

TGCAGCGAAGATGTTGTCTGTTAGATTATGAAAGCCGATGACTGAATGAAATAATAAGCGCAGC

GTCCTTCTATTTCGGTTGGAGGAGGCTCAAGGGAGTTTGAGGGAATGAAATTCCCTCATGGGTT

TGATTTTAAAAATTGCTTGCAATTTTGCCGAGCGGTAGCGCTGGAAAATTTTTGAAAAAAATTT

GGAATTTGGAAAAAAATGGGGGGAAAGGAAGCGAATTTTGCTTCCGTACTACGACCCCCCATTA

AGTGCCGAGTGCCAATTTTTGTGCCAAAAACGCTCTATCCCAACTGGCTCAAGGGTTTGAGGGG

TTTTTCAATCGCCAACGAATCGCCAACGTTTTCGCCAACGTTTTTTATAAATCTATATTTAAGT

AGCTTTATTTTTGTTTTTATGATTACAAAGTGATACACTAATTTTATAAAATTATTTGATTGGA

GTTTTTTAAATGGTGATTTCAGAATCGAAAAAAAGAGTTATGATTTCTCTGACAAAAGAGCAAG

ATAAAAAATTAACAGATATGGCGAAACAAAAAGATTTTTCAAAATCTGCGGTTGCGGCGTTAGC

TATAGAAGAATATGCAAGAAAGGAATCAGAACAAAAAAAATAAGCGAAAGCTCGCGTTTTTAGA

AGGATACGAGTTTTCGCTACTTGTTTTTGATAAGGTAATTATATCATGGCTATTAAAAATACTA

AAGCTAGAAATTTTGGATTTTTATTATATCCTGACTCAATTCCTAATGATTGGAAAGAAAAATT

AGAGAGTTTGGGCGTATCTATGGCTGTCAGTCCTTTACACGATATGGACGAAAAAAAAGATAAA

GATACATGGAATAGTAGTGATGTTATACGAAATGGAAAGCACTATAAAAAACCACACTATCACG

TTATATATATTGCACGAAATCCTGTAACAATAGAAAGCGTTAGGAACAAGATTAAGCGAAAATT

GGGGAATAGTTCAGTTGCTCATGTTGAGATACTTGATTATATCAAAGGTTCATATGAATATTTG

ACTCATGAATCAAAGGACGCTATTGCTAAGAATAAACATATATACGACAAAAAAGATATTTTGA

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CAAACTCTAGCGCCTTTAGATTATGGTTTGAGGGCAATTATCAGTGTGGATATAGAGCAAGTTA

TGCAAAGGTTCTTGATGCTGAAACGGGGGAAATAAAATGACAAACAAAGAAAAAGAGTTATTTG

CTGAAAATGAGGAATTAAAAAAAGAAATTAAGGACTTAAAAGAGCGTATTGAAAGATACAGAGA

AATGGAAGTTGAATTAAGTACAACAATAGATTTATTGAGAGGAGGGATTATTGAATAAATAAAA

GCCCCCCTGACGAAAGTCGATCGACATGGACTGATAAAGTATAGTAAAAACATAAAACGGAGGA

TATTGTTGTGAACAGAGAAGAGATGACTCTCTTAGGGTTTGAAATTGTTGCTTATGCTGGAGAT

GCTCGCTCTAAGCTTTTAGAAGCGCTTAAAGCGGCTGAAAATGGTGATTTCGCTAAGGCAGATA

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TCGAGAAGCTTCTGGGGAGGAACTTCCATACAGTGTTACTATGATGCATGGTCAGGATCACTTG

ATGACTACGATCTTATTAAAAGATGTGATTCATCACCTCATCGAACTTTATAAAAGAGGAGCAA

AGTAATTAATGCATAAACTCATTGAACTTATTGAGAAAGGGAAACGACGGATCAGGATCTCAGA

ATTGTACCCCTTTCTTTCATAAAGTAATTTTTTTCCAAAGATAATTCTCTTTTAATTGTATCAT

AAAAGATAATATTGAATTC

Erythromycin Resistance Gene Sequence

TACGTAATGAACAAAAATATAAAATATTCTCAAAACTTTTTAACGAGTGAAAAAGTACTCAACC

AAATAATAAAACAATTGAATTTAAAAGAAACCGATACCGTTTACGAAATTGGAACAGGTAAAGG

GCATTTAACGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCTATTGAATTAGACAGTCAT

CTATTCAACTTATCGTCAGAAAAATTAAAACTGAATACTCGTGTCACTTTAATTCACCAAGATA

TTCTACAGTTTCAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAGTATTCCTTACCATTT

AAGCACACAAATTATTAAAAAAGTGGTTTTTGAAAGCCATGCGTCTGACATCTATCTGATTGTT

GAAGAAGGATTCTACAAGCGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTC

AAGTCTCGATTCAGCAATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAA

CAGTGTCTTAATAAAACTTACCCGCCATACCACAGATGTTCCAGATAAATATTGGAAGCTATAT

ATGTACTTTGTTTCAAAATGGGTCAATCGAGAATATCGTCAACTGTTTACTAAAAATCAGTTTC

ATCAAGCAATGAAACACGCCAAAGTAAACAATTTAAGTACCGTTACTTATGAGCAAGTATTGTC

TATTTTTAATAGTTATCTATTATTTAACGGGAGGAAATAAACTAGT

Appendix B: Protocols

Transformation – Chemically Competent DH5α *E. coli* (TOP10)

Before starting:

- Prepare water bath - 42 ℃
- Shaking incubator (37 ℃; 200 rpm) for the duration of the procedure
- Obtain ice bucket
- Allow S.O.C. medium to equilibrate with room temperature
- Warm LB plates containing 60 µg/ml ampicillin at 37 ℃ for ~30 min
- Thaw one tube of OneShot™ TOP10 (chemically competent) cells ON ICE
    - (If using your own CC *E. coli*, use equivalent 50uL aliquot)
- Untransformed *E. coli* – save some or use some from a streak plate for the negative control

Transformation Protocol

1. Add 1-5uL of target DNA (up to 50 ng) into each vial of CC *E. coli* and mix by gentle 'flicking' motion. Avoid mixing via pipetting.
2. Incubate tube(s) on ice for 5 min.
3. Place tube(s) with lid cap into a float. Place cells into a water bath. Heat shock for exactly 30 seconds without shaking/agitation.
4. Immediately transfer tube(s) to the ice following heat shock. Wipe water off tubes carefully if necessary.
5. Add 250 µL S.O.C medium (room temp) to tube(s).
6. Cap tube. Place tube horizontally into shaking incubator (37 ℃; 200 rpm) for 1 hour.
7. **At this stage, you may prepare your aseptic workstation for working with bacteria.
8. Once 1-hour incubation is over: obtain 4 plates for transformation. One will be negative control using untransformed *E. coli*.
9. With remaining plates, spread the following aliquots from tube(s) onto LB-ampicillin plates: 10uL, 100uL, and remaining volume.
10. Once done spreading, place plates into 37 ℃ the incubator with the gel side down and incubate for 30 minutes.
11. Once 30 min incubation has finished, flip the plates and leave them overnight at 37 ℃.
12. The following day select transformants for analysis.

Electrocompetent *E. coli* mc1061

Adapted from the Castillo lab
Makes approximately 20 reactions

Day (or multiple days) before making cells:

1. Autoclave 100mL LB, store at room temperature
2. Autoclave 500mL dH_20, store in the refrigerator (4ºC)
3. Autoclave 500mL flask
4. Autoclave test tubes with lids
5. Make sure you have sterile 50mL falcon tubes
6. NOTE: you can autoclave the media in the flask

The day before making cells:

1. Grow a 2mL overnight culture of chosen *E. coli* strain in LB medium at 37ºC with shaking (use a small sterile test tube with lid) (no selection, remember it doesn't have a plasmid yet).

Day of (perform manipulation with Bunsen burner where possible):

1. Use 1mL overnight culture of *E. coli* to inoculate 100mL of media (100mL media should be in 500mL flask)
2. Incubate for 2-3 hrs at 37ºC with shaking until the OD600 is between 0.4 and 0.7
3. Turn on the centrifuge in the biotech lab and set the temperature at 4ºC; it will cool down while your cultures grow.
4. SO THE CULTURE DOESN'T overgrow, check the OD600 at 2 hours
    - Remove 0.5 mL culture using sterile technique and place in a cuvette
    - Make a blank also, 0.5mL LB in a cuvette
    - Use biorad machine at OD600 to measure
5. If not between 0.4 and 0.7 return culture incubation for appropriate time based on OD600 (*E. coli* double OD600 every 20 minutes at 37ºC). You will need to check OD600 again after additional incubation.
6. When between 0.4-0.7, pour approximately 45mL of culture into each of 2, sterile 50mL falcon tubes. Make sure they are balance—both at 45mL (marks on side of the tube). STORE TUBES ON ICE. CELLS MUST be kept on ice at all times now.
7. Place tubes in centrifuge rotor across from each other—so they are balanced. Screw on rotor lid. Pellet the cells by centrifugation for 5 minutes at 6,000 RPM.
8. Remove promptly when done spinning and pour off supernatant (in the waste flask).
9. Add 45 mL of ice-cold sterile water to each pellet and vortex vigorously to resuspend.
10. Place tubes in centrifuge rotor across from each other—so they are balanced. Screw on rotor lid. Pellet the cells by centrifugation for 5 minutes at 6,000 RPM.
11. Remove promptly when done spinning and pour off supernatant (in the waste flask).
12. Add 45 mL of ice-cold sterile water to each pellet and vortex vigorously to resuspend.

13. Place tubes in centrifuge rotor across from each other—so they are balanced. Screw on rotor lid. Pellet the cells by centrifugation for 5 minutes at 6,000 RPM.
14. Remove promptly when done spinning and pour off supernatant (in the waste flask).
15. Add 25 mL of ice-cold sterile 10% glycerol (dilute 50% glycerol using ice-cold sterile water) to each pellet and vortex vigorously to resuspend.
16. Place tubes in centrifuge rotor across from each other—so they are balanced. Screw on rotor lid. Pellet the cells by centrifugation for 5 minutes at 6,000 RPM.
17. Remove promptly when done spinning and GENTLY pour off supernatant (in the waste flask).
18. Resuspend pellets in 1mL of ice-cold sterile 10% glycerol. Transfer both resuspended pellets to a microfuge/Falcon tube—this will make it easier to aliquot. Keep this tube on ice!
19. Aliquot 100ul of cells to microfuge tubes that have been labeled with strain name, Ecomp, and date (labeling tubes before aliquoting saves time).
20. Put your cells in the -80ºC
21. Note—you can check competency right away or future.

Transforming *E. coli* Cells by Electroporation to check transformation efficiency!

1. Thaw one aliquot of the electrocompetent cells on ice.
2. Put two electroporation cuvettes on ice (label pUC19 and no DNA)
3. Label two sterile microfuge tubes, 1 pUC19, 1 no DNA.
4. To the pUC19 tube, add 1ul of pUC19 (**note the concentration**, often is at 10pg/ul). Don't add DNA to the no DNA tube.
5. Now, add 50ul of electrocompetent cells to each tube—gently mix pUC19 tube by pipetting up and down a couple of times.
6. Pipette the mixtures into the correspondingly labeled chilled cuvettes, making sure that the mixture is at the bottom of the cuvette by gently tapping the cuvette on a flat surface.
    - Be sure to wipe any condensation off the sides of the cuvette before electroporation.
7. Turn on the biorad pulser and choose the Ec2 setting (for 2mm gap cuvettes).
8. Get your recovery media and tips out and ready.
9. Place the cuvette in the pulser and press the "Pulse" button.
10. VERY QUICKLY—as quickly as possible--after electroporation, add 950 µL of SOC (or other recovery media) to the cuvette to recover the cells. Once the cells are in recovery media—you don't need to hurry.
11. Transfer the mixture to a 1.5 mL microcentrifuge tube (with appropriate labels)
12. Incubate for ~60 minutes at 37°C in the heat block
13. Plate cells (50 µl) from each transformation on an LB plate containing the appropriate antibiotic (pUC19 is AMP)
14. Incubate overnight at 37°C for ~18 hrs.
15. Calculate transformation efficiency as transformants/ug DNA
16. The cells should be at least $1x10^8$ transformants/ug

Electrocompetent *L. lactis* NZ3000 – Cell preparation and transformation

Before starting:

- Prepare and filter (0.22 µm) 50 mL 10% glucose solution
- Autoclave flasks containing 400 mL, 50 mL, and 5 mL (use test tube here) of M17 broth
    - use the above glucose to achieve 0.5% glucose concentration here AFTER autoclaving media
- Autoclave glycerol stock (volume/concentration at your discretion; see below)
- Prepare and filter (0.22 µm) 550 mL 0.5 M sucrose + 10% glycerol (4 ℃)
- Prepare and filter (0.22 µm) 200 mL 0.5 M sucrose + 10% glycerol + 50 mM EDTA (4 ℃)

Cell preparation (use Bunsen burner where possible)

Day 1:

- Inoculate 5 mL G-M17B with frozen NZ3000 glycerol stock
- Grow at 30 ℃ overnight, without shaking

Day 2:

- Inoculate 50 mL G-M17 with a 1:100 dilution of the overnight culture from day 1
- Grow at 30 ℃ overnight, without shaking

Day 3:

- Add 50 mL overnight culture to flask containing 400 mL G-M17B
    - Grow until OD600 = 0.2-0.3 (Approx. 3 hours)
- Spin down cells - 20 minutes, 6000 x g, 4 ℃. Decant supernatant.
    - May need to divide cells up into multiple 50 mL Falcon tubes. Plan ahead.
- Resuspend and wash cells in 400 mL 0.5 M sucrose + 10% glycerol (4 ℃), spin down at 6000 x g (4 ℃) for 10 min. Decant supernatant.
- Resuspend and wash cells in 200 mL of 0.5 M sucrose + 10% glycerol + 50 mM EDTA (4 ℃), keep suspension on ice for 15 min, and then spin down as with above. Decant supernatant.
- Resuspend and wash cells with 100 mL 0.5 M sucrose + 10% glycerol (4 ℃), spin down at 6000 x g (4 ℃) for 10 min. Decant supernatant.
- Resuspend all cells into 4 mL 0.5 M sucrose + 10% glycerol (4 ℃).
- Dispense cells into desired aliquot volumes (50 µL for one reaction or 100 µL for 2 reactions).
- Store cells at -80 ℃ until transformations.

Electroporation

1. Pipette 50 µL electrocompetent cells into pre-chilled 2 mM electroporation cuvette with 1 µL DNA (50 ng/µL). Gently mix, being VERY CAREFUL not to introduce bubbles. Keep cuvette on ice.
    a. If bubbles are introduced: use an autoclave-sterilized toothpick to pop
2. Prepare a Bio-Rad Gene Pulser II (or a similar machine) with the following settings:
    a. 2000 V, 25 µF, 200 Ω
3. Pulse cells (a normal reading is approximately 4.5-5.5 ms)
    a. If it pops (arc), or the reading is not in this range, you will need to redo the transformation.
4. Quickly and carefully add 950 mL G-M17 + 20 mM $MgCl_2$ + 2 mM $CaCl_2$ recovery medium to cuvette. Set cuvette on ice for 5 min (you no longer need to hurry).
5. Transfer cells from the cuvette to a sterile microfuge tube. Incubate for 1 hour at 30 ℃, without shaking.
6. Plate 100 µL and 500 µL volumes for experimental transformations. For positive and negative controls, plate 100 µL.
7. Incubate plates overnight at 30 ℃. If no growth occurs, you may need to incubate an additional day.

Colony PCR – *Lactococcus lactis*

Materials: 2x master mix, upH_2O, forward primer, reverse primer, *L. lactis* colonies, pipette tips, PCR strips and lids (or individual tubes), microcentrifuge tubes, TE + 0.1% Triton X-100, corresponding primers

PCR Settings: COL PCR 1 (full; 35 sec elongation) or COL PCR 2 (Empty; 50 sec elongation)

Primers: pEWU1 primers = M13F, 511R; Empty = SP_Control FP/RP. See Table 2 for primer sequences.

Before starting: **Start run and pause it to allow the lid to heat up and start a water bath to get to boiling with a few boiling stones in the large beaker**)

1. Wipe counter down with 70% Ethanol or 0.05% bleach solution. You can spray your gloves if you feel they were contaminated at any time.
2. Prepare sub-master mix:

1x	**x.5 (calculate)**
12.5 µL 2x master mix	
1 µL Forward Primer (FP)	
1 µL Reverse Primer (RP)	
8.5 µL upH_20	

3. Once sub-MM has been prepared, vortex and quick spin. Pipette 23 µL into each labeled PCR tube.
4. Prepare TE + 0.1% Triton X-100 (if out). Pipette 20 µL into microcentrifuge tubes (1.5 mL) for each colony being testing INCLUDING the positive control. Negative control can be left out of this and the next steps.
5. Turn on Bunsen burner to work with bacteria.
6. Colony selection will be dependent on the screen performed.
7. For each colony: carefully use the edge of a pipette tip to obtain a small number of bacteria on the outside of the tip. Once you have it, swirl the tip in the appropriate pre-treatment tube containing TE + 0.1% Triton X-100.
8. Put tube caps on each microcentrifuge tube, and place it into a float. Place this into a boiling water bath for 5 min (Making sure tubes aren't going to touch the bottom of the beaker).
9. Remove and wipe the water off of the tubes. Centrifuge at 13,000x g for 10 min.
10. Pipette 2 µL of the supernatant from each pre-treatment tube into their corresponding PCR tube. This is the template that will contain the plasmid needed for the reactions to work.
11. Once each strip is finished, cap them. Be sure that each lid is on!
12. Vortex and quick spin each tube strip. Once all strips are ready, begin PCR using the same settings as found below, with the updated elongation times as seen at the beginning of this protocol.

Gel Electrophoresis

Prior to starting: prepare 1x TAE (40 mM Tris, 20 mM acetate, 1 mM EDTA) and gel electrophoresis equipment. Determine loading gel capacity for your electrophoresis apparatus and set up a small ice bath.

Cast Gel

1. Measure out 1g agarose per 100 mL 1x TAE running buffer.
2. Add agarose to a flask containing 1x TAE. Heat until agarose crystals are dissolved.
3. Place flask into the ice bath and swirl until cooled down enough to touch to hand.
4. Add 1 µL GreenGlo™ dye per 100 mL volume. Swirl to mix, avoiding bubble formation.
5. Pour the gel into the gel casting tray with the appropriate gel electrophoresis comb. Completely cool.
6. Remove comb and place gel into electrophoresis chamber. Fill the chamber with the same TAE used to cast the gel.

Load Gel

1. Determine max volume that can be loaded into wells, if necessary
2. Prepare samples with 6x loading due and TE (if applicable):
   a. For PCR sample (25 µL): add 5 µL DNA + 5 µL TE + 2 µL loading dye per sample into fresh microcentrifuge tubes. Mix and quick spin. Place on ice.
   b. For restriction digestions (RD): add 8 µL loading due to each 50 µL digestion sample. Mix and quick spin. Place on ice.
3. Load samples
   a. For PCR samples: load 10uL per well. If possible, leave space between samples if bands are to be excised using blue light.
   b. For RD samples: load as much of the 50uL digestion as wells can hold. If the gel wells are small, split each digestion into two lanes and load up to 25uL per lane. If possible, leave space between samples if bands are to be excised using blue light.

Restriction Digestions (50 µL reactions)

1) ApaI and HpaI

1. 1 ug DNA
2. 5 µL NEBuffer (10x)
3. 1 µL ApaI (After step 4)
4. upH2O to 50µL
5. Incubate at 25 °C for 120 min
6. Move to 37 °C. Add 1 µL HpaI (keeping at 37 °C)
7. Incubate an additional 120 min
8. Load straight into 0.7% gel

2) EcoRI

1. 1 ug DNA
2. 5 µL NEBuffer (10x)
3. 1 µL EcoRI-HF (After step 4)
4. Increase H2O to 50 µL
5. Incubate at 37 °C for 120 min
6. Load into 0.7% gel

3) NheI

1. 1 ug DNA
2. 5 µL NEBuffer 2.1 (10x)
3. 1 µL NheI (After step 4)
4. upH2O to 50µL
5. Incubate at 37 °C for 120 min

Before starting, pull all appropriate samples and reagents from the freezer onto the ice. Calculate volumes of DNA to be added for each digestion reaction. Once samples are thawed, set up reactions according to the above protocol while omitting the restriction enzymes until beginning the appropriate reactions.

PureLink™ Plasmid Miniprep Kit

Note: Inoculate 5-mL LB-ampicillin (60µg/mL) broth overnight at 37 ℃ before starting. All centrifugation steps used are at 13,000 x g. Before starting, prepare buffers according to instructions and place labeled spin columns inside 2-mL wash tubes.

1. Transfer 1.5-mL of the overnight culture to a clean microcentrifuge tube. Centrifuge for 30 seconds, then discard the supernatant. Repeat twice in the same tube to pellet cells from a total of 4.5-mL overnight culture.
2. Add 250 µL Resuspension Buffer (R3) to the pelleted cells, cap, and mix until cells are completely homogenized.
3. Add 250 µL Lysis Buffer (L7). Invert tubes 6-8 times gently, or until completely homogenized. Do not vortex. Incubate at room temperature for 5 minutes (Approximately 22-25 ℃).
4. Add 350 µL Precipitation Buffer (N4). Immediately cap and mix by inverting the tubes 6-8 times, or until completely homogenized. Do not vortex. Centrifuge for 10 minutes.
5. Being careful to avoid disturbing pelleted and floating cell debris, transfer the supernatant from step 4 into the spin column. Centrifuge for 1 minute. Discard the flow-through and return column to the wash tube.
6. Add 500 µL Wash Buffer (W10) to the column. Incubate at room temperature for 1 minute. Centrifuge for 1 minute. Discard flow-through and return column to wash tube.
7. Add 700 µL Wash Buffer (W9) to the column. Centrifuge for 1 minute. Discard flow-through, and centrifuge for an additional 1 minute.
8. Transfer spin column to a clean 1.5-mL recovery tube, and discard wash tube with remaining flow-through.
9. Add 75 µL TE Buffer (TE) to the center of the column. Incubate for 1 minute at room temperature. Centrifuge for 2 minutes. Discard column and store DNA at -20 ℃ until use.

PureLink™ Quick Gel DNA purification

Note: Before starting, prepare buffers according to the instructions. Set a water bath to 50 ℃. All centrifugation steps used are at 13,000 x g. It is preferable to run this protocol immediately after gel electrophoresis, but excisions can be stored at -20 ℃ if necessary. All gels used were ≤ 1% agarose.

1. Excise target gel band using a gel razor blade and/or scalpel. Record weight in milligrams, and place excision into a clean microcentrifuge tube.
2. Multiply weight by 3 to determine volume in µL for step 3.
3. Add Gel Solubilization Buffer (L3) calculated on step 2. Incubate at 50 ℃ for 10 minutes, inverting 3-4 times every 3 minutes.
4. Incubate an additional 5 minutes at 50 ℃.
5. Load ≤ 850 µL of the dissolved gel buffer into a column placed inside a wash tube. Centrifuge for 1 minute. Discard flow-through, return column to wash tube. Repeat until all dissolved gel buffer has been used.
6. Add 500 µL Wash Buffer (W1) to the column. Centrifuge for 1 minute. Discard flow-through, return column to wash tube. Centrifuge an additional 2 minutes.
7. Transfer spin column to recovery tube, and discard wash tube with remaining flow-through.
8. Add 50 µL Elution Buffer (E1) to the column. Incubate at room temperature for 1 minute. Centrifuge for 1 minute to elute DNA. Discard column. Store at -20 ℃ until use.

Alkaline Phosphatase, Calf Intestinal Phosphatase (CIP)

Note: The standard protocol calls for a 20 µL reaction. For my research 60 µL reactions were performed. Thermocycler was set to 37 ℃ before starting.

1. Add 49 µL restriction digestion product to a clean PCR tube.
2. Add 6 µL CutSmart Buffer (10x).
3. Add 4.5 µL molecular-grade water.
4. Add 0.5 µL CIP
5. Lightly mix by flicking. Quick Spin.
6. Incubate at 37 ℃ for 30 minutes to dephosphorylate. Purify DNA using DNA Clean & Concentrator™-5 kit.

DNA Clean & Concentrator™-5

All centrifugation steps should be performed at 13,000 x g.

1. Add 120 µL DNA Binding Buffer to the 60 µL from step 6 of CIP dephosphorylation. Vortex briefly, and quick spin.
2. Transfer mixture to a Zymo-Spin™ column in a collection tube.
3. Centrifuge for 30 seconds. Discard flow-through.
4. Add 200 µL DNA Wash Buffer to the column. Centrifuge for 30 seconds. Repeat once.
5. Transfer column to a clean microcentrifuge tube. Discard collection tube and flow-through.
6. Add 20 µL DNA Elution Buffer to the center of the column. Incubate at room temperature for 1 minute. Centrifuge for 30 seconds to elute DNA. Discard column, and store at -20 ℃ until use.

***E. coli* Standard and Colony PCR - Thermocycler Settings**

	Step	Temperature (℃)	Duration
	Initial Denaturation	95	See below**
40 Cycles	Denaturation	95	30 sec
	Primer Annealing	54	30 sec
	Elongation	72	1 min/kb
	Final Elongation	72	5 min
	Hold	4	Infinite

**For standard PCR, initial denaturation of 3 min. For colony PCR, initial denaturation was 10 min.

VITA

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