Production of Highly Variable Hemagglutinin Antigens by *Lactococcus lactis* populations as a basis for an evolving vaccine

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Introduction

Humans have been in a biological war with viruses since the dawn of humanity. Evidence has been found of traces of human T cell leukemia virus type 1 (HTLV-1) in the human virome tracing back to more than 60,000 years ago. Only for a fraction of that period, we have been trying to produce vaccines for such viruses. Yet, viruses have evolved fast mutation rates in order to produce antigenically drifted viruses that avoid our protective immunity. The antigenic drift of HIV-1 and influenza A have made it a challenge to develop long-lasting treatments and vaccines for the viruses.

The identification of broadly-neutralizing antibodies (bNAbs) has sparked multiple efforts to design therapies tailored to the production of bNAbs. One popular effort is the sequential exposure to specific immunogens that yield the production of bNAbs, yet little success has been achieved. By modifying commensal bacteria for the production of HA antigens which longitudinally mutate, the ability of broadly-neutralizing immunity is increased by the exposure to most of the antigenically distinct strains of a virus.

Experimental Design

This study aims to determine the efficacy of endogenous production of highly variable hemagglutinin (HA) fragments by *Lactococcus lactis* as a means of eliciting a humoral immune

response against multiple strains of Influenza A. In the present framework, 2 plasmids are used for the orthogonal replication and transcription of the HA antigen. The first of the plasmids contain HA along with a signal peptide sequence for secretion and the T7 promoter. The second plasmid hosts the replication and transcription machinery. It contains the TP-DNAP, activation-induced cytidine deaminase (AID), error-prone T7 RNA polymerase (RNAP), and other primary replication transgenes. These plasmids are used to transform a *Lactococcus lactis* population in-vitro.

Two key metrics will be used as indicators that the aforementioned system has potential therapeutic applications. The first being the impact of the pro-mutagenesis molecular machinery on the bacterial host cell genome. The test will be split into three groups and a control. One containing only the TP-DNAP transgene, one containing both AID and the TP-DNAP transgenes, & one containing both error-prone T7 RNA polymerase and the TP-DNAP transgenes.. Population-wide sequencing of *Lactococcus lactis* will be done before and after the study to assess the effects of each group on the mutation rate of the host genome, whereby an ideal result would show little to no change in the mutation rate of the bacteria.

The second metric will be the mutation rate in the produced HA antigens. This will help validate if the endogenous production system can work at producing antigenically distant HA antigens. From each group, the plasmids will be sequenced on the first and last day as well as the proteins every 2 days. Ideally, the plasmids are producing antigenically distinct antigens at a mutation rate about 1.0×10^{-6} s.p.b.

Results and Interpretation

Protein Sequencing: In order to detect mutation rates in HA, proteins are subsequently sequenced after isolation by 2D Gel electrophoresis. To prepare the protein sample, the sample is first diluted into a Lysis solution to 0.1-1mg/mL. After the protein concentration is determined, 10-100ug of the protein sample is transferred to a new tube with an adjusted volume of 100µlwith the lysis solution. For reduction and alkylation, 50µlof the respective solutions are added to the sample and mixed and then incubated at 95ºC for 10 minutes.

In order for protein digestion into peptides, 500µlof enzyme reconstitution solution is first added to a Trypsin/Lys-C protease Mix. 50µlof this solution is subsequently added to our previous protein solution which is then incubated at 37ºC and shaking for 1-3 hours. 50µlof digestion stop solution is then added. The sample is then centrifuged 3 times for 2 minutes and the flowthrough is discarded. The solution is then washed with 300µlof a wash solution and then centrifuged for another 2 minutes.

The sample is eluted by adding 300µlof an elution solution followed by 2 minutes of centrifugation and then dried using a vacuum centrifuge. It is then put into 100µlof 0.1% formic acid and is subsequently analyzed by MS to determine the protein sequence.

Preparing Plasmid & Genomic DNA for sequencing: DNA is eluted in a 100 µl buffer and treated with 2 µl RNase (100 mg/ml) and incubated at room temperature for 1 h. RNase-treated DNA is then purified using the High Pure PCR Template Preparation Kit. 100 µl of binding buffer is added to RNase treated DNA and incubated at 70°C for 10 min. 50 µl of 2-Propanol is

added and transferred to a centrifuge spin column and spun at 8000 g for 1 min. The flow is discarded and the spin column is placed in a new collection tube. Another spin is performed at 8000 g for 1 min. The spin column is inserted into a 1.5 ml sterile microfuge tube 50 µl of pre-heated elution buffer is added to it and it is spun at 8000 g for 1 min to elute the purified DNA for next-generation sequencing.

Materials and Methods

Lactococcus lactis Preparation and Engineering: A stationary phase culture of the recipient of *Lactococcus lactis* (16-18 h) is inoculated (2% inoculum) into 10 ml of MRS broth and incubated in 5% CO, at 37°C without shaking. The cells are harvested from the broth in the early-log phase approximately 3 hours after incubation. This is done using a centrifuge at 4000-5000rpm for 5-10 minutes. The extracted cells are chilled on ice for approximately 5 minutes before being washed twice with an ice-cold washing buffer. The ice-cold cell suspension is mixed with a solution of plasmid DNA at a volume ratio of 50:1 µl inside a gene Pulser cuvette that has been chilled on ice for approximately 2 minutes. The solution is exposed to high voltage pulses at a peak field of 12.5Kv. The cells were then plated on selective MSR Agar containing Ampicillin.

Antigen Extraction: On plasmid A, a His6 tag is incorporated at the end of the HA protein sequence for identification after secretion. On successive days for measurement of the bacterial culture, antigen extraction is performed. Bacterial cells and media from a 10-cm dish are emptied into a 50mL centrifuge tube. 10mL of cell culture supernatant is isolated by centrifugation. 1-2 mL of PBS buffer is added to a Nickel Column clear insert. It is then centrifuged for 3 minutes

where the flow through is subsequently discarded. The 10mL cell culture supernatant is then added to the Nickel column insert. The column is centrifuged to allow for binding and to separate and discard flow through.

The Nickel column is then transferred into a new 50mL centrifuge tube where 6mL of Wash Buffer is added. The resulting solution is spun for 3 minutes and the column is transferred onto a new tube. To separate His6 tagged proteins from the column, 1mL of Elution Buffer is added. The tube is spun for 3 minutes and the resulting flow through contains the His6 tagged protein population.

2D Gel Electrophoresis: In order for the separation of slightly mutated proteins, 2D gel electrophoresis is used. To run the first dimension, Immobilized pH gradient (IPG) strips are used. Depending on the decided strip length and pH gradient, running time and voltage will vary. After the completion of isoelectric focusing (IEF), the strips equilibrate for 15 minutes with an equilibration buffer with DDT and then an additional 10 minutes with an equilibration buffer with iodoacetamide.

To prepare for the second dimension, the separation gel (10%) is prepared by mixing 4.1mL of water, 3.3 mL of Acrylamide, 2.5 mL of Tris-HCl, 100µlof 10% SDS, 10µlof TEMED, and 32µlof Ammonium persulfate (APS). This gel is then poured into the apparatus while leaving a couple of centimeters below the top to allow for the stacking gel. In order to remove bubbles and keep the gel from drying out, the top is layered with isopropanol. After 30 minutes, the stacking gel (4%) is prepared by mixing 6.1mL of water, 1.3 m: of Acrylamide, 2.5 mL of Tris-HCl, 100µlof SDS, 10µlof TEMED, and 100µlof APS. The stacking gel is then poured directly above

the separation gel.

IPG strips are placed on the now prepared SDS gel. Stains and desired samples are subsequently added for size-based separation. Proteins strips are subsequently eluted.

Genomic DNA Extraction: After experiment completion, the liquid culture media (200 µl) is centrifuged at 8000 g for 8 min in a sterile microfuge tube to pellet the cells. The cell pellet is suspended in an isotonic solution containing Tris, EDTA , glucose and RNase A. An alkaline solution containing sodium dodecyl sulfate (SDS) is then added to facilitate cell lysis and the complete denaturation of DNA. A potassium acetate solution is then used to neutralize the sample and separate the plasmid DNA from the gDNA. The solution is centrifuged forming a genomic DNA pellet while the plasmid DNA remains in solution.

Extracting plasmid DNA: Following the removal of the genomic DNA pellet, %97-%100 ethanol is used to precipitate the plasmid DNA. It is centrifuged again to produce a plasmid DNA pellet.

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