Identification of Lactate Export Systems in *Escherichia coli* through Genetic Screens and Substrate Similarity Search

by

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A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved April 2022 by the
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ARIZONA STATE UNIVERSITY

December 2022

ABSTRACT

The purpose behind this research was to identify unknown transport proteins involved in lactate export. Lactate bioproduction is an environmentally beneficial alternative to petroleum-based plastic production as it produces less toxic waste byproduct and can rely on microbial degradation of otherwise wasted biomass. Coupled with appropriate product refinement, industrial microbial producers can be genetically engineered to generate quantities of bioplastic approaching 400 million metric tons each year. However, this process is not entirely suitable for large investment, as the fermentative bottlenecks, including product export and homeostasis control, limit production metrics. Previous studies have based their efforts on enhancing cellular machinery, but there remain uncharacterized membrane proteins involved in product export yet to be determined. It has been seen that deletion of known lactate transporters in *Escherichia coli* resulted in a decrease in lactate production, unlike the expected inhibition of export. This indicates that there exist membrane proteins with the ability to export lactate which may have another similar substrate it primarily transports.

To identify these proteins, I constructed a genomic library of all genes in an engineered lactate producing *E. coli* strain, with known transporter genes deleted, and systematically screened for potential lactate transporter proteins. Plasmids and their isolated proteins were compared utilizing anaerobic plating to identify genes through sanger sequencing. With this method, I identified two proteins, *yiaN* and *ybhL-ybhM,* which did not show any significant improvement in lactate production when tested. Attempts were made to improve library diversity, resulting in isopropyl-β-D-1-

thiogalactopyranoside induction as a likely factor for increased expression of potential fermentation-associated proteins. A genomic library from *Lactobacillus plantarum* was constructed and screened for transport proteins which could improve lactate production. Results showed that isolated plasmids contained no notable inserts, indicating that the initial transformation limited diversity. Lastly, I compared the results from genomic screening with overexpression of target transporter genes by computational substrate similarity search. Induced expression of *ttdT*, *citT* and *dcuA* together significantly increased lactate export and thus production metrics as well as cell growth. These positive results indicate an effective means of determining substrate promiscuity in membrane proteins with similar organic acid transport capacity.

ACKNOWLEDGMENTS

I would like to acknowledge the many researchers and peers which helped in my learning process and scientific pursuit. Firstly, Dr. Xuan Wang was a great mentor and lab director who encouraged my independent experiments while providing me the means to succeed. I would also like to thank Rodrigo Martinez and Moses Onyeabor for the guidance, support, and training during my undergraduate and graduate semesters. I would like to thank Aidan Schneider's contribution to computational research in this thesis. And thank you to Desiree and Mica for being amazing friends and helping with projects during long days.

Lastly, thank you to my committee members, Dr. David Nielsen and Dr. Arul Varman for taking the time to review my research and offer wisdom. This thesis is the culmination of many months of work and much combined feedback, and I am glad to have the opportunity for multiple lab directors to provide input.

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INTRODUCTION

Lactic Acid Bioproduction and Use

Worldwide, there is a heavy reliance on fossil fuels and petroleum-based plastic production. Based on global consumption rates, and the associated by-products with the refinement of petroleum, estimates indicate that the over-production of petroleum-based plastics will consume known oil reservoirs by 2050, cost over $10 billion in energy production, and emit over 78 million metric tons of greenhouse gasses[1; 2]. In addition to this, most plastic is not recycled, resulting in costly disposal or chemical treatment of waste[3]. It is important that we look to reduce our carbon footprint and plastic production. While there have been more advances and time dedicated to plastic recycling, biodegradable materials have become a staple of sustainability[4]. Compostable straws, bags, cups, and clothing are all important revolutions which improve the capacity of societies to mass-produce materials, but with a reduced impact on environmental contaminants[4; 5]. With utilization of microbes, the possibility for producing environmentally friendly plastic alternatives is achievable[6]. The methods involved in bioproduction have been explored in many organisms and through various metabolic pathways covering the production of many organic acids, polymers, and amino acids[7; 8; 9].

Overview of Lactic Acid Fermentation

Lactic acid is an important material for the production of biodegradable plastics, such as poly-lactic acid (PLA) and the production of medical supplies and drugs[4]. With the biodegradable aspect of PLA, it is more economically viable and environmentally beneficial to compost and naturally degrade these bioplastics[10]. As an alternative to fossil

fuels, lactic acid is a biomaterial which can be produced by the fermentation of various microbial species including *E. coli* (strain TG114)[6; 11; 12]. This is possible through anaerobic growth; glycolysis of incoming sugars leads to the production of large quantities of pyruvate[13]. In order to replenish oxidative NAD^+, lactate dehydrogenases will convert pyruvate into lactate as a fermentation product and export it out of the cell[14]. This process is common in many bacterial and eukaryotic systems and can be seen in the production of yogurts from *Lactobacillus* species, and even in mammalian muscle fibers associated with the "burning" sensation in overworked muscles[15]. Although multiple organisms are used commonly for lactic acid bioproduction, *E. coli* holds many merits which make it a favorable model organism in the exploration of lactic acid metabolism. Namely, short duplication times, access to a variety of genetic modification techniques, a relatively well characterized genomic profile, and a capacity to utilize a variety of sugars or carbon sources[16].

Bioproduction Bottlenecks

Compared with current petroleum-based standards, production of bioplastics is not economically feasible to supplement current demands; it relies heavily on microbial producers which require large sugar supply, purification of lactic acid through acid treatment, and a continual removal or neutralization of fermentative by-products and acids[10; 17]. While previous research has been conducted to improve *E. coli* utilization of lignocellulose components and a wide array of sugars through directed evolution and mutagenesis, there is still difficulty in maintaining cost-effective bioproduction of acidic compounds[18; 19]. The methods involved in engineering metabolic pathways are diverse

and include targeting specific enzymes involved in the metabolism of sugars beginning with increasing sugar uptake mechanisms or improving the speed and efficiency of enzymes to process intermediates[20; 21; 22]. This can come in the form of mutagenesis, or the evolution of specific or general systems to find novel and possibly improved enzymatic activities[23; 24]. Alternatively, much work has been done in identifying homologous, or similarly structured, proteins in other organisms and expressing them in a different model organism[25]. This route could provide a vastly different effect on intracellular functioning, and typically requires adjustment of nucleic acid sequences or amino acids to ensure proper functionality. One major conflict with this experimental procedure is in the inherent nature of organisms to evolve naturally and efficiently on their own; most metabolic systems within the host microbe could have taken thousands of years to perfect their own metabolic processes and have well-balanced metabolites or redox balanced pathways[13]. Rather than targeting intracellular mechanisms, lactate transport is an overlooked mechanism in literature. *E. coli* must rely on key export proteins, which can reduce intracellular accumulation of the acidic products, but no extensive research has been performed to characterize all potential transporters capable of exporting lactic acid[15; 26]. Previous research has analyzed the primary function of and uptake capacity of known lactate transporters in *E. coli*, including *glcA* and *lldP*[26]. Testing with single deletions and double deletions confirmed the uptake capacity and of these proteins, but deletion of these transporters in TG114 only mildly reduced growth and production rates of lactic acid in our preliminary tests, indicating the presence of transport proteins which must possess some capacity to export lactic acid[6; 26]. This

implies that various export mechanisms exist within *E. coli* to primarily transport other substances, but have minor uses in the reduction of intracellular lactic acid. Exploration of these mechanisms is a major focus of this research, as it could lead to the discovery of novel transport mechanisms for further research on protein engineering or metabolism.

Lactic Acid Transporters

Transport proteins, or exporters, are essential membrane-bound channels or active pumps which both act to equalize concentrations of substances between the intracellular and extracellular membrane and move substances against their gradient[27; 28]. They work by creating small passageways in the cellular membrane and can simply allow small uncharged molecules to diffuse readily. More advanced components of membranes require active transport (usually secondary transport proteins) and the transport of larger, and sometimes charged, molecules. In lactic acid metabolism, cells encounter the issue of large increases in intracellular lactate buildup[28]. With a need to continuously metabolize glucose to provide energy and reducing equivalents, it is important that cells have efficient means of exporting the lactate to maintain chemical homeostasis (Figure 1). Currently, the means of lactate export is understood in a minor degree, but several systems in mammalian models (multiple carboxylate transporters) are well-known and have been explored. In *E. coli*, lactate permeases fill this roll, as they move lactate across the membrane with proton symport[12]. Focusing on the mechanism and essential genes involved in transport additionally increases understanding on product export which leans heavily on overall lactate production, offering the potential to enhance or increase current bioproduction metrics.

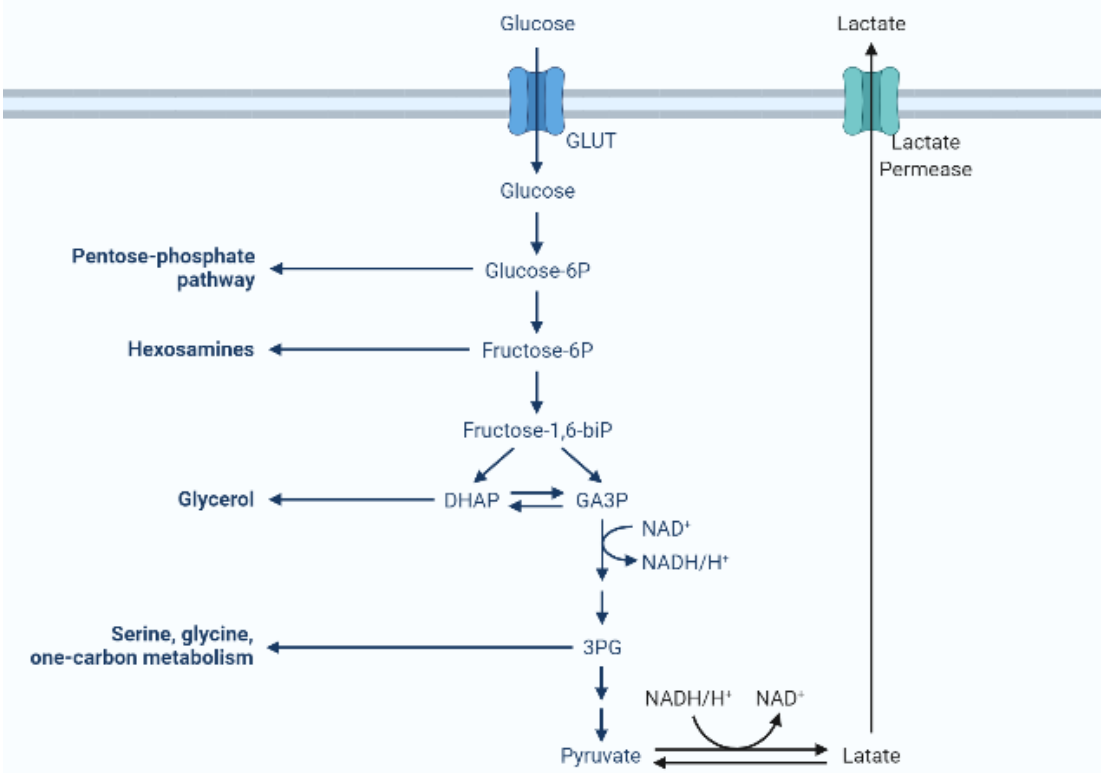

Figure 1: Schematic displaying the metabolic pathways utilized in lactic acid-producing *E. coli*. Currently displayed is the transport route deleted, with unknown homologous transporters yet undetermined.

Chemical Profiling of Transporters

Previous work from Aidan Schneider has been performed on identification on transport proteins, specifically on the chemical similarity of lactic acid with other organic acids produced by various organisms. Based on the carbohydrate structure, charge localization, and bonding patterns, we can generate an estimate of similarity between other charged molecules which behave like lactic acid and have respective transporters.

Several fingerprinting algorithms were compared to calculate similarity between multiple substrates, which were then processed and ranked to generate a list of chemicals with similar structures and bonds to lactate. By reviewing the current list of similar transporters and targeting relevant products or membrane proteins found in *E. coli*, there are more alternatives for genetic screening. Some preliminary tests have shown that the deletion of *citT* and *yhjE* further decreased lactate production in JX141 especially at early fermentation phase (Figure 2). While limiting the options available to a reasonable quantity of testable genes, this method of analyzing chemical structures can overlook chirality and reduce the specificity of chemical profiles.

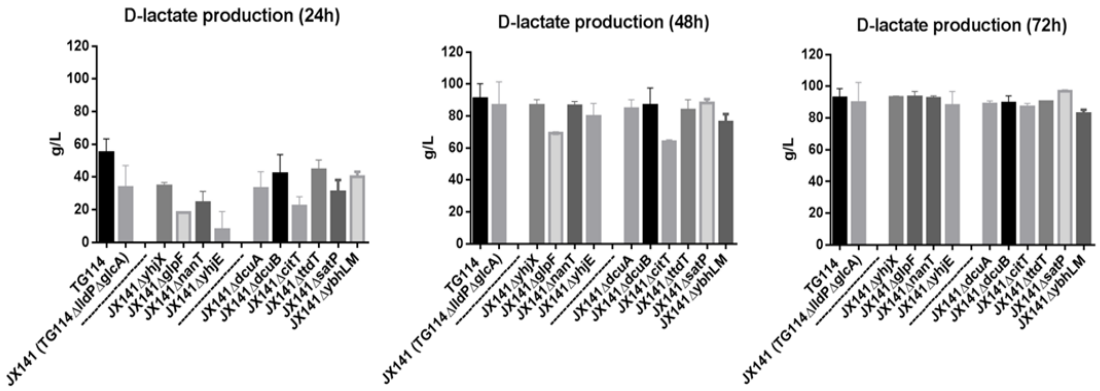

Figure 2: Baseline results and initial work in targeted deletions of JX141 (work from Aidan Schneider and Edward Nolan). Most significant differences observed in lactate production of deletions in citrate and succinate transporters including *citT* and the putative transporter *yhjE*.

Experimental Testing and Description of Results

The focus of this research is to identify unknown proteins responsible for lactate export by utilizing genetic screening[29; 30]. The potential this information offers is expansive; we can gain insight into microbial transporters which allows for the systematic analysis and testing of various potential transport mechanisms. This can then be translated into research on improving current transport procedures in various microbial producers, or even into potential applications in human muscle tissue lactate production or cancer biology[31]. To accomplish this, a genomic library (Figure 3) was constructed from the current strain (*E. coli* JX141) with deletions in all known lactate transporters (*lldP* and *glcA*), which was then expressed in the same deletion strain[30]. This method has been tested and shown in previous studies to effectively screen for unknown homologous genes associated with furfural resistance in fermenting *E. coli* strains[32]. Full growth inhibition is not observed, indicating the possibility of minorly effectual promiscuous transport proteins capable of completing lactate fermentation and exporting the final product. Observations of improved growth rates or a rescue of the deletion phenotype due to overexpression of homologous transporters will allow for further analysis of potential transporter proteins.

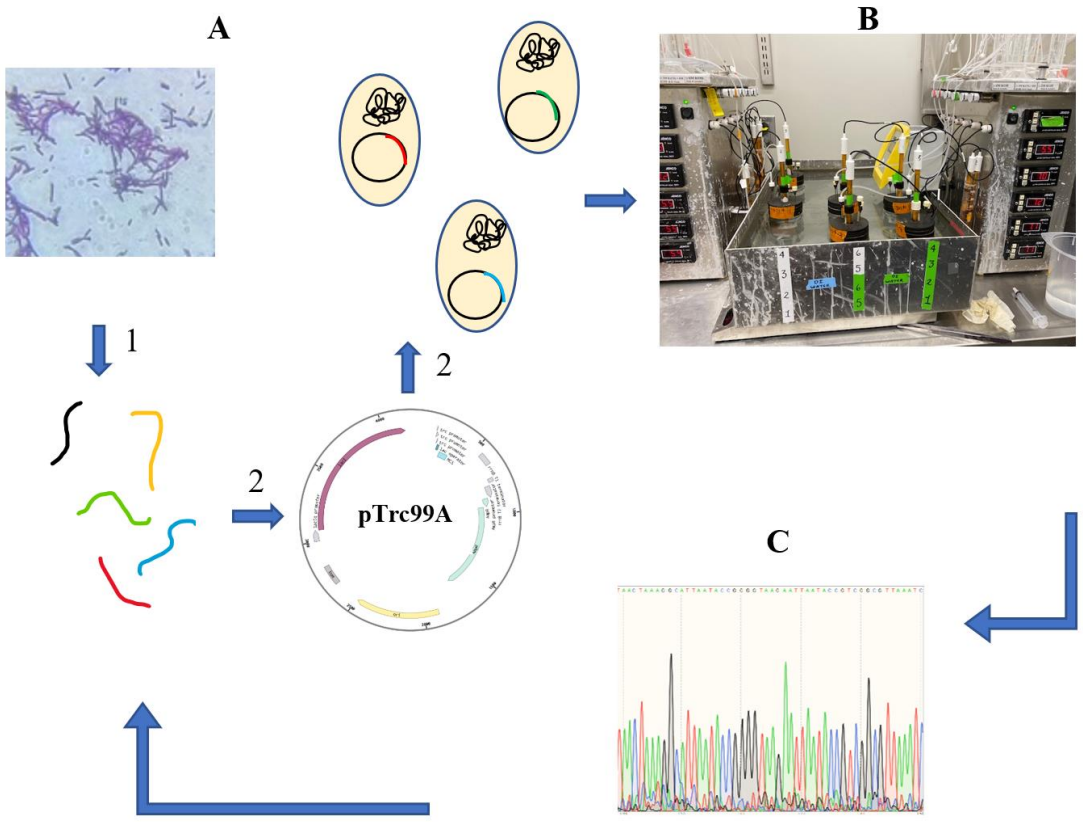

Figure 3: Diagram of the protocol for genetic screening. (A) Initial library construction beginning with (A-1) restriction digest of extracted genomic DNA from *E. coli* JX141, followed by (A-2) insertion of randomly generated fragments into pTrc99A vector, and ending with (A-3) transformation into JX141 deletion strain. (B) Enrichment batch fermentation setup with monitored pH, temperature, and minimal oxygen culture environments. (C) Sequencing analysis of top performer strains and gene fragments from isolated plasmids for identification of potential lactate transporters.

The results from the genomic library construction indicate that a complete library of JX141 was successfully generated. Extraction and concentration of genomic DNA

from the initial JX141 template was performed successfully and digested with Sau3A1 restriction enzyme. Sau3A1 is a 4bp recognition site restriction enzyme capable of recognizing GATC anywhere in the genomic DNA; as this pattern is common it produces many variable fragments, up to a size of about 5 kilobases (5kb), which can house entire operons[33]. Partially digested fragments were produced, as they contain relatively small sequences of DNA, and can produce more variability with the effect to screen every possible gene in *E. coli* JX141.

A description of strains and plasmids utilized and produced is found below (Table 1) in the materials and methods section. Initial testing of JX141 with known lactate transporter, *lldP*, was performed to confirm the function of lactate transporters and determine if overexpression of known transporters will improve growth. Results indicate that a noticeable improvement in growth and lactate production was observed in this control experiment as compared to the baseline empty vector (EV) transformants. Continuing with enrichment testing, the JX141 transformed with the genomic library (JX141-Lib1) underwent several rounds of fermentations and independent population transfers during each fermentation trial. Through this, it was expected to narrow down the population to only the most efficient growers within the library, which could be attributed to an improvement in fermentative growth or lactic acid production[15]. The first round of enrichment trials relied on anaerobically grown liquid cultures before transferring to the fermentation jar setup. These were allowed to grow for 24 hours before sampling measurement were recorded, and finally transferring a volume of the fermentation to a new jar with a starting OD of 0.05. Results displayed a common trend; a significant

improvement in growth and lactate production in the initial transfer, followed by less difference and even a reverse in relative growth as compared to the EV by the second and third transfers. The cause of this loss of improvement in growth could be related to a lack of initial screening pressure or loss of the functional genes in the library[34]. Cultures from all transfers were plated, individual colonies were isolated, and their plasmids were purified. Sanger sequencing displayed a combination of whole genes/operons and truncated proteins were isolated, confirming that the library was successfully prepared and transformed. Further enrichments were performed to approach the selection process by increasing IPTG concentration and increasing gene expression of the unknown membrane proteins within the library. These results were more successful in isolating transport proteins, and several target genes from both rounds of enrichments were back transformed into JX141. However, only slight improvements were displayed (as seen in the two plasmids containing *yiaN* and *ybhL-ybhM)*, unlike the previous noticeable increase in the *lldP* control experiments. An additional third round of enrichment was performed to improve the initial diversity and selection of the fermentation culture. This relied on plate-based growth, transferring whole populations to a fermentation jar, and avoiding any potential interference in selection through liquid seed cultures. Results indicated a similar pattern to the previous enrichment rounds, and additional plasmid isolation confirmed that variable genes were present, with no clear indication of similarity in functionality. Enrichment trials were then performed incorporating genomic DNA from phylogenetically similar *L. plantarum* to evaluate any transport proteins which could have homologous structures in *E. coli*. These results were less accurate, as

any potential interference in results may have been associated with the initial library transformation rejecting most of the large plasmid inserts.

In addition to enrichment trials, several malate transporters were overexpressed in JX141, according to data in chemical similarity and enhanced lactate production[35]. These genes were specifically targeted for overexpression as compared to the genome-wide selection process of the enrichments. Transporter genes were tested based on substrate similarity to lactate. Three genes were tested for homologous lactate transport capacity: *dcuA/citT/ttdT*. All were cloned into the same plasmid backbone and expressed simultaneously in JX141. Results from the targeted gene testing of chemically similar transporters showed an improvement in growth similar to the positive control, *lldP* overexpression. This poses the need for additional tests to confirm the growth and lactate production benefits of the individual transporters, and for a comprehensive review of more chemically similar organic acids including succinate, glycerate and tartrate and their respective transport proteins. Although prior single deletions have been performed in this strain, multiple deletions of chemically similar transporters have yet to be tested. Based on current successes with overexpression of potential lactate transporters, this would provide a unique comparison to the enrichment screen.

MATERIALS AND METHODS

Strains and Plasmids Utilized

All strains utilized in this research are summarized below (Table 1)[35; 36] The parental lactate producer (TG114) was genetically modified, utilizing a two-step method

of gene knockout and deletion, to produce the deletion strain JX141 which was the primary focus of this research[24]. Further transformations were performed with pTrc99A backbone and variable inserts including genomic libraries and individual genes.

Table 1: List of strains and plasmids used in this study

Strain/plasmid	Description	References
TG114	*E. coli B-Δ*(*focA–Z. mobilis pdc-adhB-pflB*) *adhE*::FRT Δ*ackA*::FRT Δ*frd, frdA*::*E.chrysanthemi celY lacY*::*K. oxytoca casAB pfl*::(*pdc*$^{+}$-*adhB*$^{+}$)-*recA*	36
JX141	TG114Δ*lldP*Δ*glcA*	Aidan Schneider's honors thesis
pTrc99A	pBR322_Ori, P*ampR/ampR,* P*trc*/*lacI*	Lab Collection
JX141-EV	JX141-pTrc99A	This Study
JX141-*lldP*	JX141-pTrc99A/lldP	This Study
JX141-Lib1	JX141 transformed with genomic library (Lib1)	This Study
JX141-TF2-4	JX141 transformed with isolated plasmid pTF2-4	This Study
JX141-TF3-10	JX141 transformed with isolated plasmid pTF3-10	This Study
JX141-L.P1-4	JX141 transformed with *Lactobacillus plantarum* genomic library	This Study
JX141-*dcuA/citT/ttdT*	JX141 transformed with pTrc99A-*dcuA*/*citT*/*ttdT*	35

Growth and Batch Fermentation Media

For most cell growth conditions, culturing was performed using LB medium, and conditions for transformations or plasmid purifications were performed with TB medium under aerobic growth conditions at 37℃[37]. AM1 mineral salts medium was used for fermentative trials with 10% glucose, 100 µg/ml ampicillin, and 10 µM IPTG for induction of genes with the *trc* inducible promoter present in pTrc99A[38]. Concentrations

of ampicillin were adjusted accordingly, allotting for testing under reduced antibiotic conditions at 50% ampicillin (50µg/ml). IPTG concentration was increased 10-fold to measure growth under increased expression profiles at 100 µM IPTG. Growth conditions relied on environmental temperatures of 37℃ with external supply of neutralizing 6M KOH to maintain pH 7.0 over the course of three to four days or until most cultures began the death phase[39].

Genomic Library Construction

Genomic library construction was performed through the partial digestion of strain JX141 with restriction enzyme Sau3AI [30] . Genomic DNA was extracted utilizing a Wizard DNA extraction kit and stored using sterile filtered water[40; 41; 42]. Approximately 5 µg DNA samples were prepared with 0.1% final concentration of SDS from a 6x NEB Loading dye and incubated with an appropriate buffer for 4 minutes at 37℃. The samples were immediately run on an agarose gel, and fragments with band sizes from 1kb to 4kb were extracted. This was repeated four times, purified DNA samples were pooled, and ethanol precipitation was performed[43]. Plasmid pTrc99A digestion was done with 1 µg of purified DNA digested with BamHI to generate compatible ends for ligation with Sau3AI digested genomic DNA. This fragment was dephosphorylated with alkaline phosphatase calf intestinal phosphatase (CIP) during the 1-hour digestion followed by column extraction and plasmid purification. The purified digested vector was ligated with the partially digested JX141 genomic DNA in a 5:1 ratio of insert to vector overnight at 16℃ with a final inactivation of the ligase.

Transformation Protocols

TOP10F', the deletion strain JX141 and the parental strain TG114 cells were prepared for chemical transformation with a heat shock protocol, and approximately 15 µl – 5 µl of the ligated plasmid were transformed into the deletion strain JX141 and the parental strain TG114[44]. 100 µl of freshly prepared competent cells were defrosted and mixed with plasmid DNA for 30 minutes before incubating at 42℃ for 45 seconds followed by 2 minutes at 4℃ (or on ice). Cells were immediately mixed with room temperature SOC medium supplemented with $MgCl_2$ to a final concentration of 10 mM[37]. Growth of individual colonies on LB plates was recorded and utilized in the calculation of cloning and transformation efficiency. The resulting growth was then uniformly collected, and the plasmids from TOP10F' transformants were purified to generate a complete genetic library of JX141.

Fermentation Procedure and Sampling

Batch fermentations required cells to be first streaked on AM1 minimal media plates, followed by growth under microaerobic conditions in liquid AM1 and MOPS buffer solution until optical density (OD 550 nm) 1 - 2 was reached[45]. Fermentation jars were prepared under sterile conditions and a volume of cell culture totaling to an initial OD of 0.05 was pelleted and resuspended in the AM1 jars. Cultures were monitored over three to four days and sampled at regular daily intervals, allowing for OD measurements, and storage of HPLC samples. 6 M KOH was utilized to maintain pH 7 of all cell cultures during the fermentation trial.

Enrichment fermentations began with the initial anaerobic growth of freshly transformed seed cultures in liquid medium until an approximate starting OD of 0.05 was

recorded, and seed cultures were transferred to 300 ml of AM1 media. Daily sampling and isolative transfers of the initial fermentation culture produced three subsequent generations of screened library mutants, which were measured for growth at OD 550 nm and 500 µl of the supernatant was collected for analysis utilizing HPLC[46].

Anaerobic Plating and Plasmid Isolation

Several trials of anaerobic plate growth-based experiments were performed on diluted samples and freshly transformed cells. From anaerobic growth plates, individual colonies were sequenced, genomic inserts were prepared with polymerase chain reaction (PCR) amplification and sequencing was performed for further analysis. Results were collected and organized to determine gene profiles and appropriate transmembrane genes were additionally characterized. Sequenced plasmids were individually transformed into the deletion strain JX141 and effects on fermentative growth were quantified with batch fermentations. Growth rates over the course of three to four days were recorded and HPLC samples for lactate export and glucose consumption were recorded.

HPLC Analysis

Fermentation samples measuring approximately 500µl were stored at 4℃ and 10µl of each sample was mixed with 884 µl of nanopure H_2O, 6 µl of 0.5 M H_2SO_4 and 100 µl of 1% methanol before mixing and running through the HPLC sampler. Individual peaks corresponding to glucose, lactate, and methanol (control baseline) were recorded and compared in graphing software alongside previously determined standards for concentrations (data not shown).

Computational Approach to Identify Putative Transporters with Substrates Similar to Lactate

Aidan Schneider used molecular signature fingerprints at depth 2 to compare carboxylic acids and aromatics, which we carefully selected with chemical substructure and property filtering. Each chemical structure was expressed using 20 diverse chemical fingerprinting methods over a range of depths (2, 3, 4 and default) with a combination of PyFingerprint and KNIME CDK nodes. Cosine, Tanimoto, Euclidean, dice, and Manhattan distance matrices between all transporter substrates and each set of target chemicals (aromatics and dicarboxylates separately) were created. The distance measure chosen plays a major role in evaluations of chemical similarity. For comparability across different fingerprinting methods, distance matrices were converted to rank matrices. For each set of target chemicals and distance measures, the rank matrices of all fingerprints were stacked into a tensor of dimension (number of candidate chemicals x number of target chemicals x number of fingerprints). To extract the fingerprint-specific components within the tensor we performed a CPD/PARAFAC tensor decomposition using the tensor tools python package. Rank was estimated by applying the Kneedle algorithm to the reconstruction error plot because reconstruction similarity was consistently high across ranks. We then projected each fingerprint onto a multidimensional space defined by the component fingerprint vectors. To group similar fingerprint methods together in a robust manner we used consensus and ensemble K-means clustering. We selected k value by consensus using the Monti algorithm with 90% resampling frequency and validated this using the Kneedle algorithm on SSW. Ensemble

clustering was repeated with random initializations until a consistent optimal partitioning was determined by a majority voting algorithm implemented in the OpenEnsembles python package. Rank matrices were first averaged within each cluster and then second across clusters. Then finally the rank matrices across all distance measures were averaged to yield a final consensus. This comprehensive approach aims to appreciate diverse representations for each substrate and rank chemical similarity by broad consensus. Since many transporters exhibit substrate promiscuity, the next step is to identify the transporters with substrates chemically similar to target molecules. The Transporter Classification Database (TCDB) is a website which contains up-to-date information about reported transporters and their characterized functions. The website was scraped with Beautiful Soup to retrieve the names of all known chemical substrates using the PubChem Identifier Exchange Service for each transporter. Ultimately, over 5,000 characterized transporter:substrate interactions were identified and used to generate putative transporters for target molecules. Then we used this information to identify both native and homologous transporters from *E. coli* with potential activities toward lactate. Homologous transporters in *E. coli* were identified based on protein sequence similarities. By combining cheminformatics and bioinformatics tools, putative transporters for lactate were eventually generated.

RESULTS

Genomic Library Construction

A genomic library was constructed with the deletion strain JX141 to test the overexpression of individual genes for their effect to confer an increase in growth comparable to the parental strain. To begin, the genomic DNA of JX141 was partially digested with Sau3A1 to create a random fragmentation of genes from 1 kb to 4 kb (Figure 4A), which were then successfully cloned into a plasmid backbone and transformed into JX141 (Figure 4B). Over 9000 distinct colonies were formed, with a cloning efficiency of 85%. All colonies were collected and pooled together to generate a complete coverage of all possible genes present in *E. coli*, estimated at about 5000 genes (Figure 4C) for complete coverage[47]. With effective screening methods, this library can be tested for any beneficial genes which confer an increase in growth to the deletion strain when grown under selective pressures[48; 49].

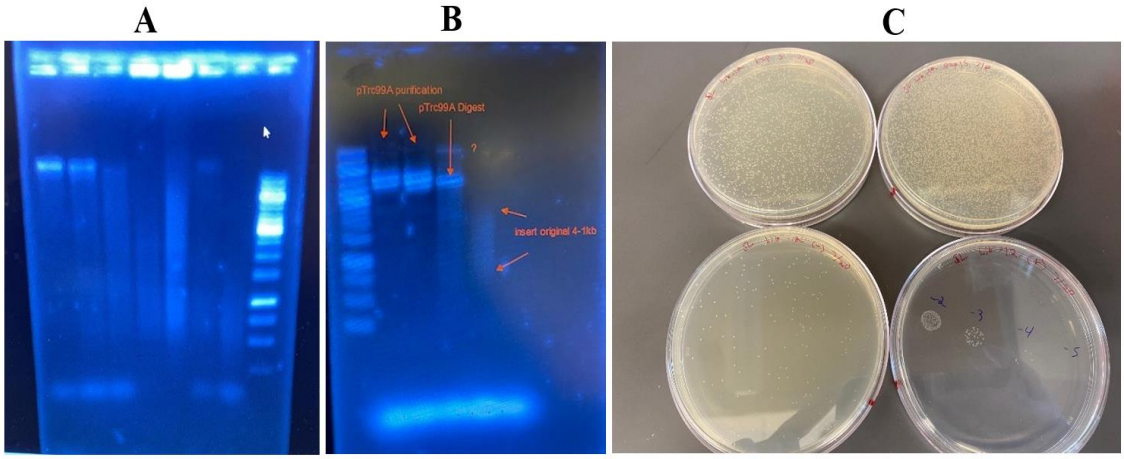

Figure 4: Generation of a complete genetic library was successfully performed and transformed into JX141. (A) Partial digestion of genomic DNA and identification of optimal parameters for incomplete digestions of 1 kb to 4 kb fragments at 37°C; *Lane 1:*

1 µg DNA with 2 minutes digestion time, *Lane 2*: 1 µg DNA with 3 minutes digestion time, *Lane 3:* 1 µg DNA with 4 minutes digestion time, *Lane 4:* 1 µg DNA with 5 minutes digestion time, *Lane 5:* 5 µg DNA + 0.1% SDS with 5 minutes digestion, *Lane 6:* 5 µg DNA + 1% SDS with 5 minutes digestion time, *Lane 7:* 5 µg DNA + 0.5% SDS with 8 minutes digestion time, *Lane 8:* Ladder. (B) Purification of plasmid backbone and gel extraction of partial digestion; *Lane 1:* Ladder, *Lane 2:* pTrc99A backbone undigested, *Lane 3:* pTrc99A backbone undigested, *Lane 4:* pTrc99A backbone + BamHI digestion, *Lane 5:* Extracted JX141 partial genomic DNA digestion. (C) Transformation results from plating indicates growth of over 9000 individual transformants with 5 µl (Top left plate) and 15 µl (Top right plate) from the plasmid library.

Library Enrichment and Plasmid Isolation

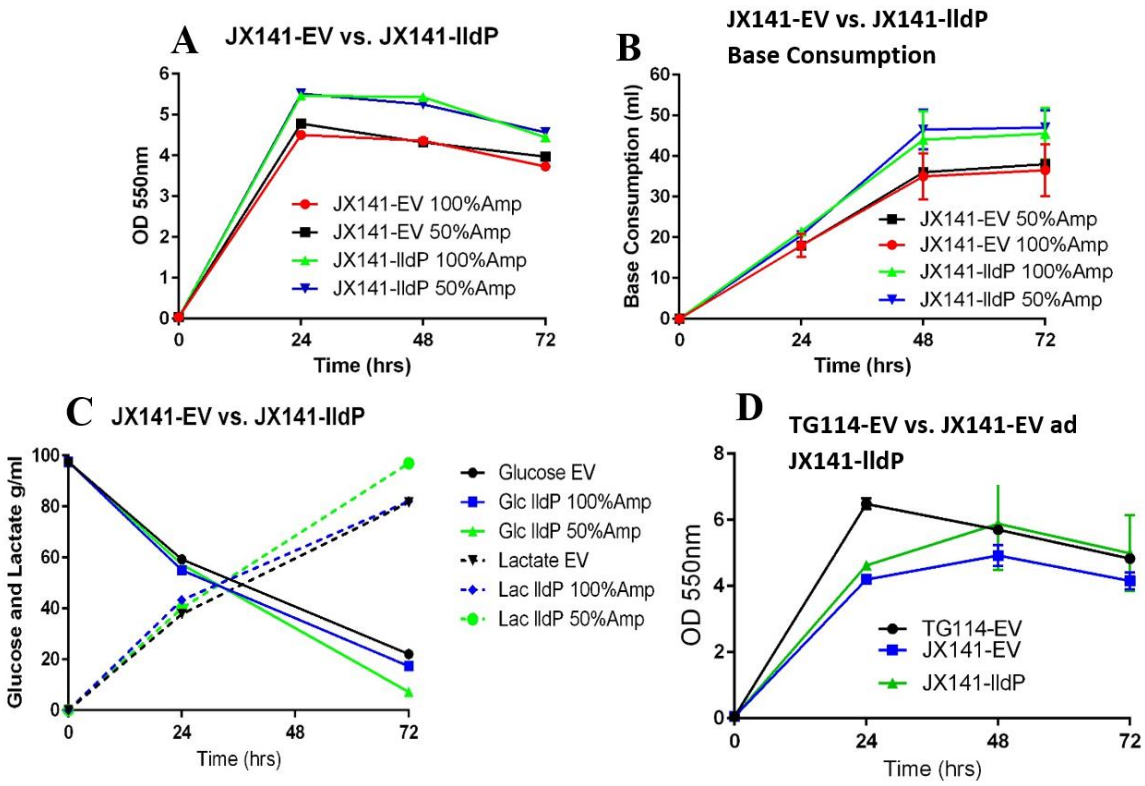

Figure 5: Fermentative growth and lactic acid production from JX141 transformed with previously deleted and known lactic acid transporter *lldP* gene. (A) OD growth of JX141-EV vs. JX141-*lldP* at 100 µg/ml (100%) and 50 µg/ml (50%) ampicillin concentrations. (B) Base consumption associated with lactic acid production in JX141-*lldP*. (C) Glucose and lactate consumption and production. (D) Comparison of growth in controls TG114-EV and JX141-EV against JX141-*lldP*.

Several baseline experiments were conducted supporting the data from JX141 and TG114 lactate production (data not shown), and the establishment of positive restorative growth conditions with *lldP* complement tests. Cells were transformed with the pTrc99A-*lldP* plasmid (producing JX141-*lldP*) and grown on minimal medium plates before

transferring to microaerobic seed cultures till the appropriate OD of 1-2 was reached. Once transferred to the fermentation setup, JX141-*lldP* indicated a significant improvement in growth over the empty vector (EV) control (Figure 5A and 5B), but still did not reach growth rates of the parental TG114 (Figure 5C). Relative improvement in growth averaged about 33% increase in OD in the JX141-*lldP* transformant by the first day, where most of the cultures have already begun stationary phase. At maximum OD, the *lldP* controls reached approximately 5.4 OD, whereas the baseline EV only reached to about 4.32 OD, indicating approximately 25.4% increase in growth. Additional tests involved varying the ampicillin concentrations in these preliminary experiments (Figure 5A and 5B), which essentially showed no difference between growth. HPLC samples over several days indicated a similar improvement in lactate produced and glucose consumed in the *lldP* controls. Approximately 60.8% more glucose was consumed in the *lldP* 50% controls with a slightly smaller increase in consumption by approximately 13.6% glucose as compared to the EV. Similarly, a greater quantity of lactate was produced in the *lldP* transformed cultures, showing an improvement of 22.8% (Figure 5D).

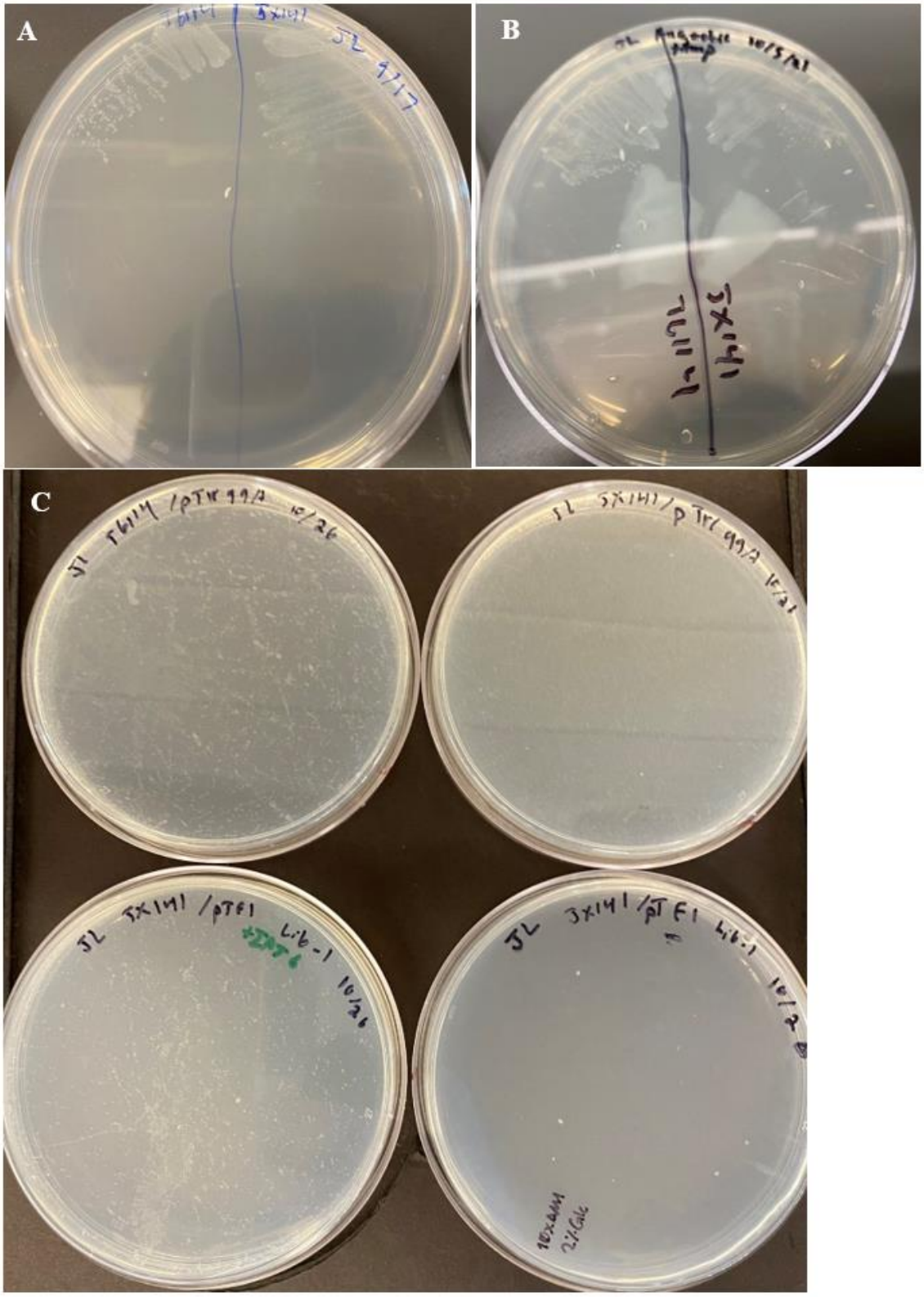
A
B
C

Figure 6: Plate-based Growth tests to determine colony morphology differences and evaluate metabolic advantages in cells. (A) Plate-based growth tests in oxygen-rich environments on minimal medium plates with TG114 and JX141. (B) Plate-based growth tests in anoxic environments. (C) Plate-based growth tests with transformed JX141 cells with the genomic library.

During the initial trials, the cultures were sampled for HPLC analysis and a -80℃ culture sample was stored for long-term preservation and eventual testing with anaerobic plating screens (Figure 6). Samples underwent a secondary screen on plates for a comparison of colony morphology on solid versus liquid media (Figure 6A). Anaerobic plates confirmed the benefit of the library transformants due to the similarity in colony size and density as compared to the positive control TG114. Additionally, the observation of a range of varying colony sizes can confirm the variable identity and benefits of the individual genes most prevalent in the library after transfers (Figure 6B). The presence of non-beneficial genes and empty plasmid vectors was detected through PCR and sequencing. Rather, this method did not yield optimal results for identifying growth-advantaged cells, as the difference in morphology was difficult to confidently determine. Additionally, the cell morphology of these strains under fermentation conditions should be explored more in the future, as this could change depending on the phase of the medium.

Round one enrichment fermentations were performed to compare improvement in growth in the deletion strain transformed with the library (JX141-Lib1) as compared to

the empty vector (EV). The isolated plasmid library was transformed into JX141, and immediately transferred to an anaerobic seed culture tube with minimal medium and allowed to grow until an approximate OD of 1-2 could be reached. These cultures were pelleted and resuspended in the fresh fermentation medium and observed for growth before transferring to the subsequent fermentation jars. In the first transfer of the initial round, there was an observed improvement in growth of approximately 72.2% in the library transformant JX141 culture as compared to the empty vector transformant JX141 (Figure 7A). While these initial enrichment results may have indicated the selection of potential beneficial genes, this trend did not replicate itself in subsequent transfers on day two and day three; instead, results from the second transfer showed an opposite trend to the first batch fermentations, where the empty vector outperformed the library transformant by approximately 24% after the second day (Figure 7B). This trend remained inconsistent during the third transfer, as the growth rates and maximum OD matched completely between the empty vector and the library (Figure 7C).

30 individual colonies were randomly isolated, their plasmids purified, and the inserted gene amplified utilizing colony PCR to identify the genes present and their approximate size (Table 2). Upon analysis with Sanger sequencing, results for potentially beneficial plasmids were organized and compared in the following Table 2 to distinguish transmembrane proteins and the predicted or known function of relevant genes. Approximately 34.7% of all genes from the multiple batch fermentation runs indicated they were transmembrane pumps, and only 50% were identified functional protein pumps for large organic molecules. This could indicate that the general screening methods

reviewed were not able to specifically isolate beneficial transporters [30]. Mostly, this technique of genetic screening could have isolated genes involved in extraneous metabolic functions which may still confer an initial improvement to growth, but do not directly alter lactate fermentation or export [15]. Several of these key suspect transporter homologues were re-introduced into JX141, and additional batch fermentations were performed to confirm the improvement in growth of the subset of transmembrane genes. Among these, two beneficial plasmids, and the associated genes *yiaN* and *ybhL-ybhM*, showed a slight improvement in growth with a measured increase in OD of approximately 10% and 13% respectively (Figure 7D).

Lastly, HPLC analysis of the round 1 enrichment provided data on lactate production and glucose consumption profiles. As expected, instances of high growth corresponded to large sugar uptake/utilization peaks, and large shifts in base usage were indicative of high lactic acid production and fermentative growth. In total, results show a slight improvement in glucose consumption during the initial transfer of about 10.9% µg/ml, while lactate production was significantly higher at around 69.3% (Figure 7E and 7F). Trends for the second and third transfer follow the OD measurements more closely and reveal closely ordered data values in the fermentations which, although much greater than the previous transfer, do not indicate any improvement in the library transformants over the EV (Figure 7E and 7F).

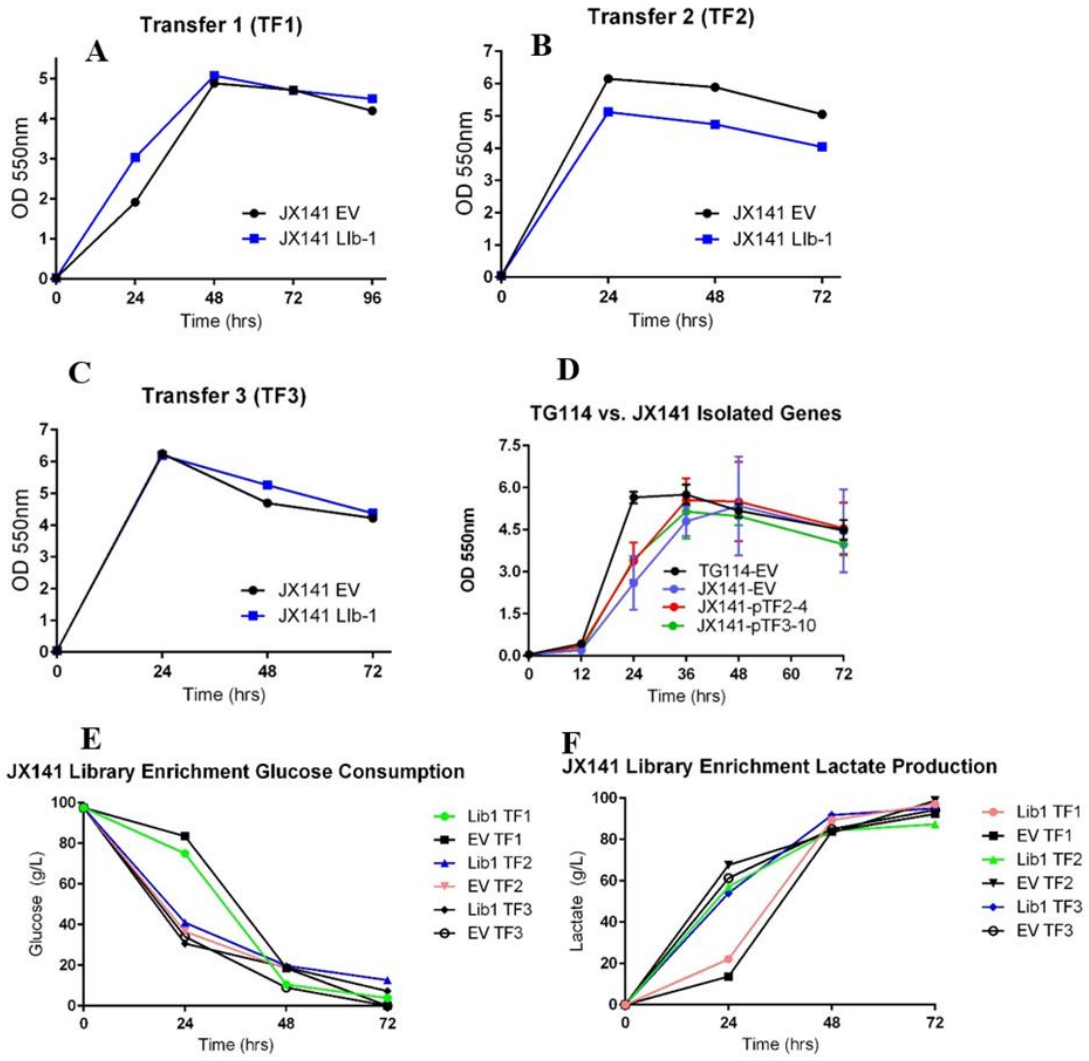

Figure 7: OD and HPLC data from round 1 enrichment tests. (A) OD measurements from library transformed JX141 during the initial transfer, followed by (B) a second sub-population transfer and (C) a third transfer. (D) Isolated plasmids from previous transfers were back-transformed and tested in Fermenting JX141 to determine individual gene overexpression. (E) Glucose and (F) Lactate HPLC data from enrichment testing.

A second round of enrichment was performed, beginning with increasing the expression of membrane-bound proteins through IPTG induction at 10 µM concentration. It was assumed that any slight positive effect conferred by the library proteins would show increased effect under additional expression, and this trend was consistent in the subsequent enrichment trials. As indicated in the OD readings (Figure 8A), a positive increase of approximately 21.4% OD difference is observed in the initial transfer as compared to the EV, while a significant improvement is seen in subsequent transfer days, reaching an OD of almost 6.5, similar to the TG114 phenotype. HPLC analysis was performed to review the glucose and lactate concentrations in the fermentation culture, revealing a considerable improvement in glucose consumption during the growth phase in both transfers as compared to the EV control (Figure 8B). The maximum difference in growth rate is observed during the first 24 hours, reaching approximately 815% greater consumption in the library transformants. This trend was repeated in lactate production in the second transfer but was not easily seen in the initial transfer when compared to the EV. Production in the second transfer reached approximately 328% more lactate produced, while the initial transfer displayed a much larger improvement in lactate production reaching almost 567% (Figure 8C).

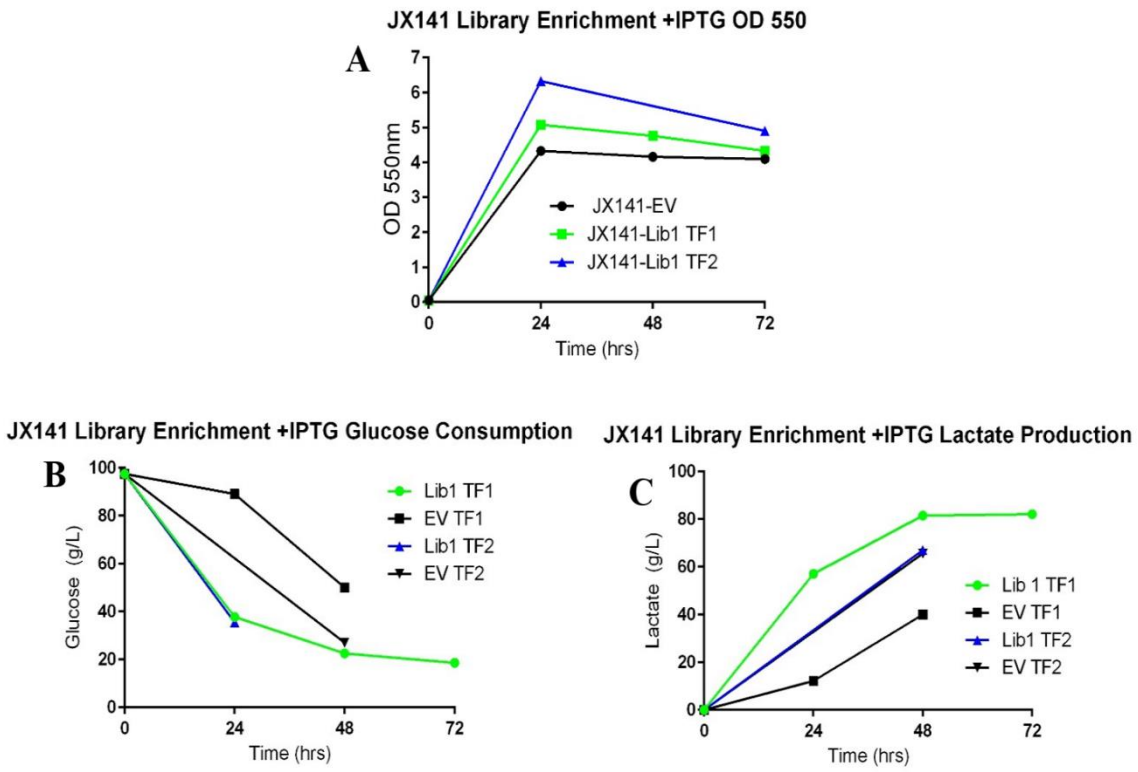

Figure 8: OD and HPLC analysis from IPTG-induced enrichment testing. (A) Glucose consumption profiles of JX141-Lib and JX141-EV. (B) Lactic acid production in JX141-Lib and JX141-EV.

Table 2: Isolated plasmids after enrichments

Plasmid	Description/Inserted Genes	Membrane Protein	Function	References
pTrc99A	P_{trc}, *ampR, lacI*	N/A	Ampicillin resistance, IPTG inducible promoter	Lab Collection
pTF1-1	pTrc99A-*mvpA, ypfJ*	No	Unknown	This Study
pTF1-2	pTrc99A-*ybjD, ybjX*	No	Toxin secretion	This Study
pTF1-6	pTrc99A-*murE, murF*	No	Peptidoglycan ligase and synthesis	This Study
pTF1-8	pTrc99A-*rrfB*	No	5s ribosomal RNA	This Study

pTF1-9	pTrc99A-*kefB*	Yes	K+ Transporter	This Study
pTF2-2	pTrc99A-*yhhN*	Yes	Ca+ Transporter	This Study
pTF2-3	pTrc99A-*tatE, lipA, ybeF, lysR*	Yes	Twin arginine transport subunit and associated transcription regulation	This Study
pTF2-4	pTrc99A-*yghW, hybO, hybA*	No	Unknown potential hydrogenase	This Study
pTF2-6	pTrc99A-*rpoB*	No	RNA polymerase subunit	This Study
pTF2-8	pTrc99A-*ycjO, gltP*	Yes	ATP transporter, Glutamate/Aspartate transporter	This Study
pTF2-10	pTrc99A-ECW_m3031, *lmpK*, ECW_m3032	Yes	Potential Type IV secretion system subunit	This Study
pTF3-5	pTrc99A-*infB*	No	Translation initiation factor	This Study
pTF3-6	pTrc99A-ECW_m1491, ECW_m1492	No	Unknown	This Study
pTF2-4-IPTG	pTrc99A-*yiaN*	Yes	L-dehydroascorbate importer	This Study
pTF2-7-IPTG	pTrc99A-*yejE*	Yes	Subunit of ABC transporter	This Study
pTF2-14-pIPTG	pTrc99A-*metE*	No	Homocysteine transmethylase	This Study
pTF2-16-IPTG	pTrc99A-*rpmL, rplT, pheM, pheS*	No	Ribosomal RNA subunit, phenylalanine ligase	This Study
pTF3-2-IPTG	pTrc99A-*bglA*	No	6-phospho-β-glucosidase	This Study
TF3-7-IPTG	pTrc99A-*ybiY, ybiW*	No	pyruvate-formate lyase activating protein	This Study
pTF3-8-IPTG	pTrc99A-*ubiH, pepP, visC*	No	Ubiquinone synthesis	This Study
TF3-10-IPTG	pTrc99A-*ybhL, ybhM*	Yes	Ca+ transporter	This Study
pTF3-12-IPTG	pTrc99A-*lacZ*	No	β-galactosidase	This Study

Third round enrichment trials attempted to address the potential diversity of the transformed population. This method of post-transformation recovery involved plate-based aerobic growth, which could potentially avoid any intrinsic stressors or selection methods which prioritized genes with minimal influence on lactic acid fermentation. Plates were grown with freshly transformed JX141-Lib1, and all colonies were collected and resuspended in the fermentation jars. Results from the OD measurements showed an increase in OD of the previously enriched library of approximately 34.9% and 42.8% in the second transfer and first transfer respectively, while the plate-based culture technique library (Lib3) showed a smaller difference when compared to the EV (Figure 9A). Comparison of glucose consumption in the library transformants confirmed results, as the initial transfers and previously enriched library both displayed the largest consumption in glucose, matching consistently with the OD recordings (Figure 9B). A significant glucose consumption of approximately 60 g/L was seen in the Lib3 initial transfer by day one, which shows an increase of about 25.1% over the Lib1 transfer. Lactate production showed similar results in lactate production in the two top performing transfers, with equivalent production metrics at the second day of approximately 58 g/L, which is 50% higher than the slower fermenting cultures at this time point (Figure 9C). Regardless, the growth of the remaining transfers usually reaches similar production values by the third day.

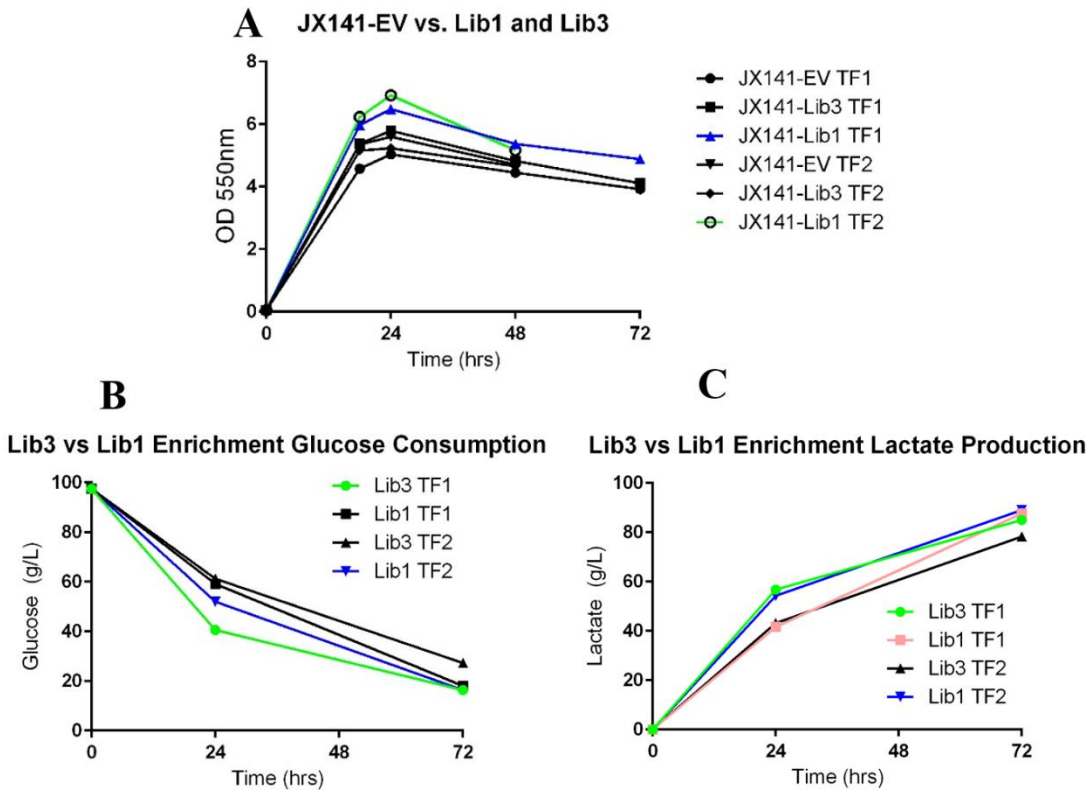

Figure 9: Third round library transformations and enrichment comparing different seed culture techniques, Lib3, against previously enriched library transformants. (A) OD comparison between Lib1, previously enriched library, and Lib3. (B) Glucose and (C) Lactic acid HPLC profiles from enrichment tests.

Screening with an alternative genome from *L. plantarum* was performed to evaluate the homologous proteins in phylogenetically similar lactic acid producing bacteria. Four individual plasmids containing separate regions of the *L. plantarum* genome (sublibraries) were prepared and transformed into JX141 strains yielding JX141-L.P1, JX141-L.P2, JX141-L.P3, and JX141-L.P4. Newly constructed strains were prepared and fermented similarly to the Round 2 enrichment trials, and OD

measurements were taken. Results indicated the L.P2 sublibrary performed consistently higher than the baseline EV, as both transfers displayed an OD improvement of 10.4% to 25.0% between the initial transfer and secondary transfer respectively. Further, the initial transfer of the L.P1 sublibrary displayed a slight increase in OD over the EV yet showed a reverse in this pattern as the secondary transfer (L.P1 TF2) was approximately 6.25% OD less than the EV baseline (Figure 10). Isolation and screening paints a different picture, as sequenced genes (data not shown) did not reveal any significant gene inserts in the plasmid backbone.

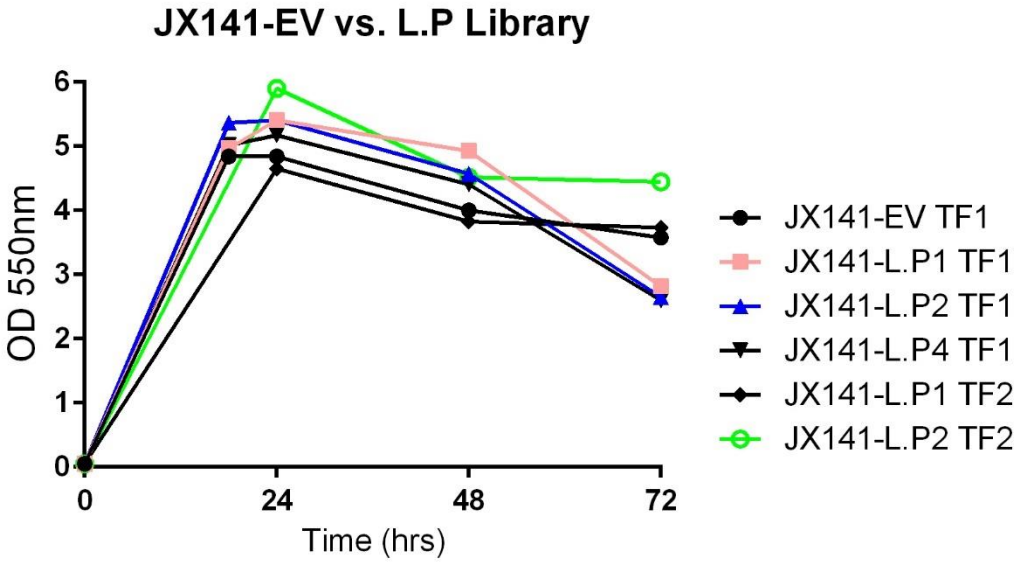

Figure 10: Genomic screen in *Lactobacillus plantarum* genomic library. Display of OD measurements over the course of several days and two individual transfers with JX141-EV control and four separate sub-libraries, L.P1-4 (L.P3 data not shown).

Chemical and genetic profiling was performed to identify potential transporters with lactate export activity in *E. coli* JX141 (Table 3), of which, several malate transporters were identified as potential targets using out computational algorithm. I overexpressed them in JX141 using a plasmid vector with IPTG-inducible expression system. One genetic construct was prepared, containing *dcuA, citT, and ttdT* on the same pTrc99A backbone, transformed into JX141, and fermented alongside EV controls in 10 µM and 100 µM IPTG. Results from the OD experimental values indicated that the 10 µM concentration transformants outperformed the EV control reaching a maximum OD of about 5.42, while the 100 µM trials displayed a slightly lower, yet significant improvement over the EV of about 11.6% (Figure 11A). HPLC analysis was performed to observe glucose consumption which showed consistent increase in consumption of about 52.6% in the top performing 10 µM concentration by day one as compared to the 100 µM trial which displayed an increase of 15.8% in the first day. Lactate production in these samples were recorded and displayed; by the first day, the 10 µM concentration produced about 66.7% more lactate over the EV and the 100 µM experiment produced roughly 30.3% more than the EV. Both samples produced around 78 g/L of lactate by day three, which significantly improved the production standard from the EV by 22.0% (Figure 11B).This preliminary result indicates that DcuA, CitT and TtdT (either all three or only some of them) have lactate export activities.

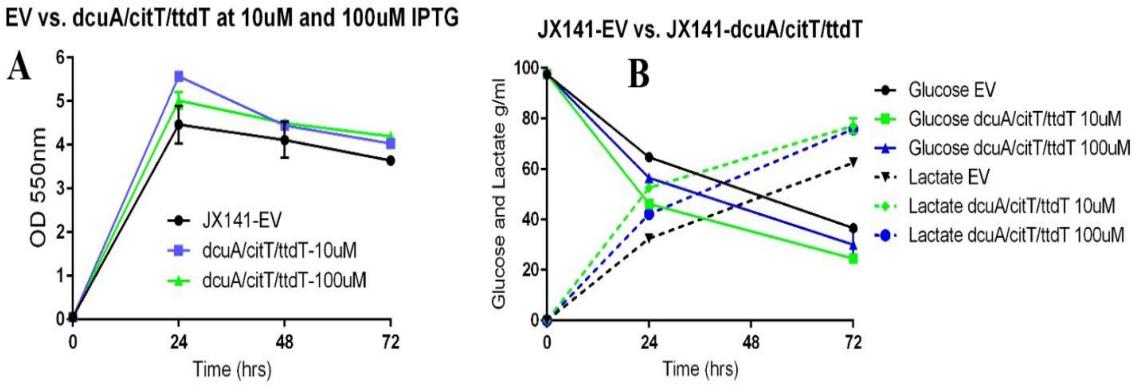

Figure 11: Fermentation tests with JX141 transformed with plasmid 3g (*dcuA/citT/ttdT*) containing previously identified malate transporters suspected of lactic acid transport capacity. (A) OD measurements from JX141-EV, baseline comparison, and JX141-*dcuA/citT/ttdT* at 10 µM IPTG and 100 µM IPTG concentrations including (B) glucose and lactic acid HPLC analysis.

Table 3: Substrate Similarity and Potential Transporters

Substrate	Putative Transporters	Alternative Genes
D-Lactate/L-Lactate	*lldP/glcA*	*glpF/aqpZ, nanX/nanT/prop, yhjX, yaaU, araE, ydjE, actP, yidK, panF*
Glycerate	*garP*	*yohK*
Malate	*dcuA ttdT, citT*	*dctA, ybhL, yfbS, yfdV*
Tartrate	*ttdT*	*yfaV, gudP, garP, lgoT, dgoT, exuT, citT*
Butyrate	No direct homologs	*actP, yidK, panF, atoE*
Succinate	*dcuAB, satP*	(See malate transporters), *yidE, ybjL, dcuC, yjjB,*
Valerate	*atoE*	
Glycerol	*glpF, aqpZ*	*galP, araE, xylE*
α-Ketoglutarte	*kgtP*	*ybhL, citT, ttdT*
Glycolate	*yohK*	*lldP, glcA, actP*

DISCUSSION

The research was conducted to identify unknown lactate transporters in *E.coli.* With the potential for increasing lactate production metrics, this research explores a new approach for characterizing export capacity of specific and unidentified genes in the entire genome[11; 15]. While prior research in our lab has been conducted to knockout specific genes *lldP* and *glcA* among other potential homologs, most of these methods have only been minorly effective; rather than eliminating export, some export mechanisms are still present as evidenced by the remaining lactate production and growth[26]. This research was significant because it forms a general basis of the process and potential in genetic screening and generates new hypotheses to perform future work including protein analysis and residue modification for the characterization of previously unknown proteins[10; 32; 39]. Genomic libraries have been utilized to generate information about whole systems of microbial organisms. Their use in sequencing and protein identification proves to be replicable for a number of metabolic pathways or bioproduction processes[8; 23; 32; 50]. In particular, this research highlights that genomic libraries, although inconsistent in results, have the potential to yield individual functions from an entire genomic profile[6; 47]. Further, this research is applicable to any number of genetically modified organisms with the caveat being that simple and reproducible methods are needed to effectively generate screening or selection pressures. Certain restriction enzyme digestions may not be applicable to other genomes, and effective engineering of plasmid libraries for optimal protein expression is a necessity[34].

Table 4: Summarized media and growth conditions for enrichments

Strain	Description	Growth, Seed Cultures, and Medium Used
Round 1	Initial screening method, with freshly transformed cells prepared in an anaerobic seed culture with minimal media	JX141 transformed with genomic library (Lib1), AM1/10%Glucose/Ampicillin 100 µg/ml
Round 2	Screening with increased membrane-bound protein expression	JX141 transformed with Lib1, AM1/10%Glucose/Ampicillin 100 µg/ml/IPTG 10 µM
Round 3	Removal of anaerobic seed culture, for improved initial diversity	JX141 transformed with Lib1, AM1/10%Glucose/Ampicillin 100µg/ml/IPTG 10 µM – full plate transfer to 300 ml jars
L. plantarum library	Screening with IPTG and *L. plantarum* library	JX141 transformed with L.P1-4, AM1/10%Glucose/Ampicillin 100 µg/ml IPTG 10 µM

The major contribution of this research presents the genomic library construction of a lactate producing strain of *E. coli*, JX141, and systematic testing to identify and isolate potential lactate transporter homologues. Three main aims were displayed in this research including being able to generate a genomic library of *E. coli* JX141, successfully performing enrichment fermentation trials while isolating potential gene candidates and comparing the effectiveness of whole genetic screens with substrate similarity computational analysis. The initial phase required proper creation and characterization of the genomic library; all genomic regions need to be accessible and inserted into the plasmid backbone for later screening to be as diverse as possible. Following this, enrichment protocols were developed and adjusted to improve selection pressures while

maintaining diversity and protein expression. This allowed for the isolation and identification of target plasmid sequences from the genomic library, which could then be individually transformed/expressed. Lastly, results from the enrichments need to be evaluated for efficacy, and so several malate transporters were identified according to chemical similarity and overexpressed in fermenting cells.

Results from the genomic library construction showed that a complete coverage was accomplished with fragments approximating 1 kb-4 kb in size. This method was successful in *E. coli* DNA and could likely be repeated for a number of bioproduction strains; this would allow for similar enrichment screening trials which may include diverse functions including sugar uptake and utilization routes[17; 51].

Initial positive control experiments were conducted with the complement of the deleted *lldP* in JX141 while comparing growth to EV transformants. While growth was significantly improved and lactate production reached a much higher value by the first 24 hours, there is still a difference in growth between the JX141-*lldP* control and the parental TG114. This was expected as the deletion strain still possesses other deletions (Δ*glcA*) which may affect the overall metabolic performance. Further tests may need to be performed to fully evaluate the effect antibiotic concentration has on fermentation conditions, as no effect was seen at 50% ampicillin[48; 52].

Results from fermentations and screenings describe an interesting approach to genomic library enrichment. The round 1 trial provided a useful basis for analysis of expression profiles and isolation timing. While an improvement was observed in growth in the library transformant over the EV, this trend did not continue in subpopulation

transfers on following days. This could lead to the assumption that selection pressures were not able to properly enrich for beneficial lactate transporters or other fermentation-associated genes. Rather, there was only the perceived initial improvement in growth, and any further reliance on those gene products were not necessary or helpful for fermentative growth. On the other hand, the potential for interference of lactate production selection could be masked with the antibiotic or IPTG concentration, indicating that selection pressures may be reduced in order to properly screen for beneficial genes associated with lactate fermentation. Additional screens may also be applied incorporating additional lactate (supplied in the growth media), or the adjustment of selective pressures including increased antibiotic concentrations or increased osmotic pressure through modifying sugar concentrations.

Following round 2 enrichment trials, significantly more target genes were characterized, and HPLC analysis revealed the improvement of growth to be more comparable to TG114. This poses another potential issue, as gene overexpression or IPTG-induced protein expression could remove selection pressures[25; 38]. The identified candidate genes *yiaN* and *ybhL-ybhM* have been characterized and share a similar role in pre-translocation molecule localization and binding. It is possible that the proteins play a role in lactic acid movement to the membrane and enhance the ability of pre-existing pumps to guide and export lactate, but do not largely play a role in lactate export otherwise.

Round 3 enrichments were performed to investigate a plate-based library transformation technique, and whether this method of avoiding initial seed cultures could

improve genetic diversity[14; 20]. Upon review of the fermentation, it was seen that the previously enriched library continued to outperform the EV and the plate-based library transformants. This could indicate that the method did not effectively allow for a diverse plasmid profile, and likely selected for fast growers with non-essential genes or junk DNA. Sequencing was performed on several plasmids (data not shown), which confirmed the prediction.

Plate-based colony morphology observations proved to be less effective in identifying potential growth benefits imparted by the library in anaerobically grown cultures. Although there were noticeable differences in colony size when comparing the wildtype TG114 with JX141, individual differences in the library transformants were too minimal to be repeatable, indicating the possibility that colony size is more dependent on initial growth conditions, and not improved lactate producers[20].

The additional testing performed with *L. plantarum* indicated some potential for identification of non-native transport homologs in *E. coli,* including the possibility of the expression of different lactate transporters with the ability to transport different chiral forms of the lactate[53]. However, the results were inconclusive as analysis of isolated plasmids revealed that no significant plasmid inserts could be detected, and that any difference in growth could be due to the initial plate-based seed growth conditions[53; 54].

Computational search for substrate similarity was found in several organic acids and can be seen summarized above (Table 3). Several algorithms were compared and chemical profiles analyzing acidic groups, charged regions, bonds present, and bond lengths were performed (data not shown). Of these, three genes (*dcuA, citT, and ttdT*)

were transformed and tested for fermentative growth in JX141. A combination of the three genes imparted a noticeable improvement in lactate production, comparable to the *lldP* controls. This would imply that one or all the genes likely possess some homologous transport capacity, and this is improved in the overexpressed plasmid construct. Analysis of protein sequences reveals a relative conserved pattern in several regions between the known lactate transporters and trio of genes (Figure 12). In a more targeted approach, protein analysis will be performed using the aligned sequence information, which can lead to future tests with engineered or mutated alleles to inhibit pump activity. Following this, deletion of the homologous transporters and transformation of the plasmid into the wildtype, TG114, will be performed to analyze if full growth inhibition can be accomplished, or if improved production metrics can be reached.

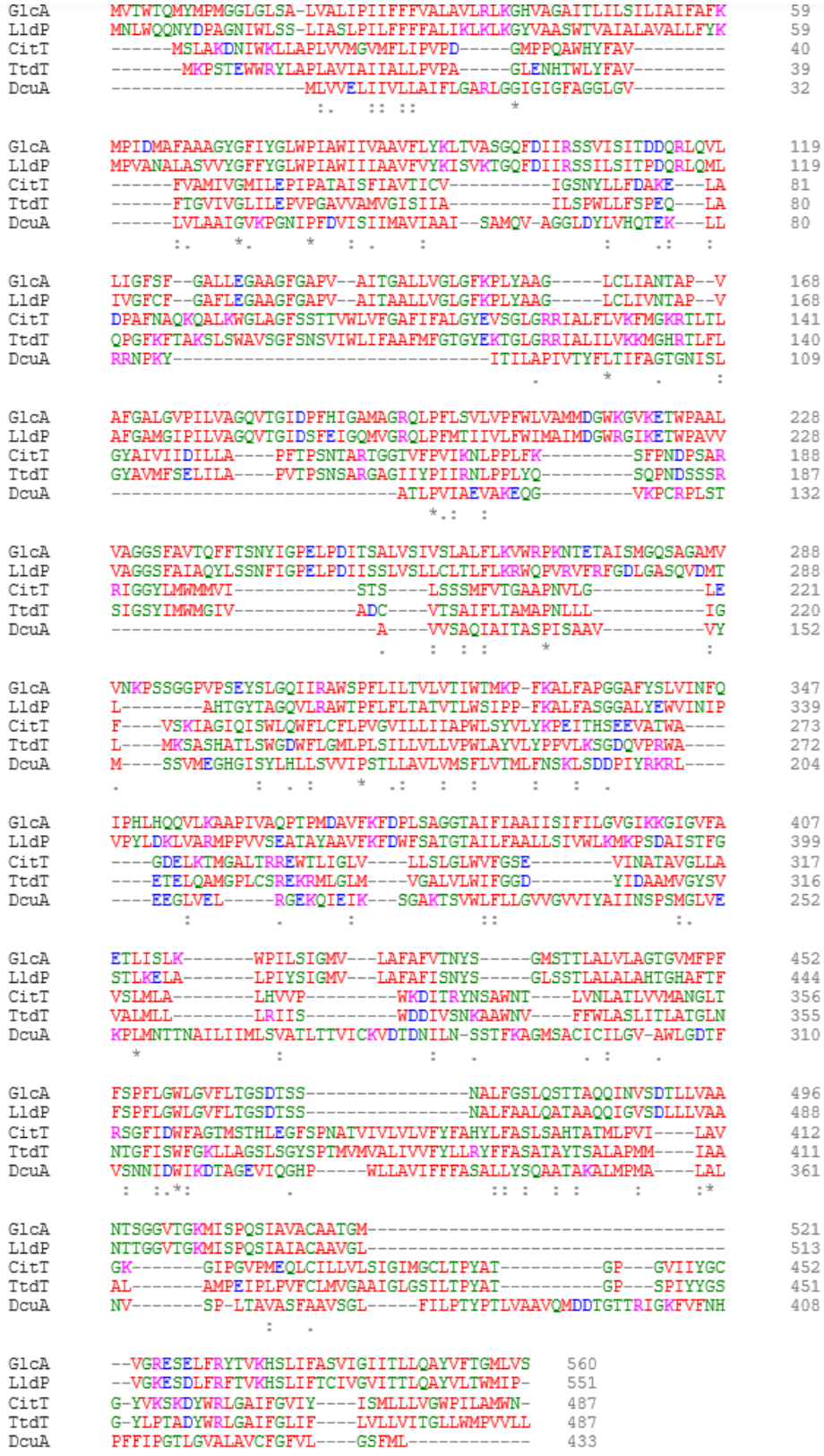
GlcA MVTWTQMYMPMGGLGLSA-LVALIPIIFFFVALAVLRLKGHVAGAITLILSILIAIFAFK 59
LldP MNLWQQNYDPAGNIWLSS-LIASLPILFFFFALIKLKLKGYVAASWTVAIALAVALLFYK 59
CitT ------MSLAKDNIWKLLAPLVVMGVMFLIPVPD-----GMPPQAWHYFAV---------- 40
TtdT -------MKPSTEWWRYLAPLAVIAIIALLPVPA-----GLENHTWLYFAV---------- 39
DcuA ------------------MLVVELIIVLLAIFLGARLGGIGIGFAGGLGV---------- 32
GlcA MPIDMAFAAAGYGFIYGLWPIAWIIVAAVFLYKLTVASGQFDIIRSSVISITDDQRLQVL 119
LldP MPVANALASVVYGFFYGLWPIAWIIIAAVFVYKISVKTGQFDIIRSSILSITPDQRLQML 119
CitT ------FVAMIVGMILEPIPATAISFIAVTICV----------IGSNYLLFDAKE---LA 81
TtdT ------FTGVIVGLILEPVPGAVVAMVGISIIA----------ILSPWLLFSPEQ---LA 80
DcuA ------LVLAAIGVKPGNIPFDVISIIMAVIAAI--SAMQV-AGGLDYLVHQTEK---LL 80
GlcA LIGFSF--GALLEGAAGFGAPV--AITGALLVGLGFKPLYAAG-----LCLIANTAP--V 168
LldP IVGFCF--GAFLEGAAGFGAPV--AITAALLVGLGFKPLYAAG-----LCLIVNTAP--V 168
CitT DPAFNAQKQALKWGLAGFSSTTVWLVFGAFIFALGYEVSGLGRRIALFLVKFMGKRTLTL 141
TtdT QPGFKFTAKSLSWAVSGFSNSVIWLIFAAFMFGTGYEKTGLGRRIALILVKKMGHRTLFL 140
DcuA RRNPKY----------ITILAPIVTYFLTIFAGTGNISL 109
GlcA AFGALGVPILVAGQVTGIDPFHIGAMAGRQLPFLSVLVPFWLVAMMDGWKGVKETWPAAL 228
LldP AFGAMGIPILVAGQVTGIDSFEIGQMVGRQLPFMTIIVLFWIMAIMDGWRGIKETWPAVV 228
CitT GYAIVIIDILLA----PFTPSNTARTGGTVFPVIKNLPPLFK---------SFPNDPSAR 188
TtdT GYAVMFSELILA----PVTPSNSARGAGIIYPIIRNLPPLYQ---------SQPNDSSSR 187
DcuA ----------ATLPVIAEVAKEQG---------VKPCRPLST 132
GlcA VAGGSFAVTQFFTSNYIGPELPDITSALVSIVSLALFLKVWRPKNTETAISMGQSAGAMV 288
LldP VAGGSFAIAQYLSSNFIGPELPDIISSLVSLLCLTLFLKRWQPVRVFRFGDLGASQVDMT 288
CitT RIGGYLMWMMVI----------STS----LSSSMFVTGAAPNVLG----------LE 221
TtdT SIGSYIMWMGIV----------ADC----VTSAIFLTAMAPNLLL----------IG 220
DcuA ----------A----VVSAQIAITASPISAAV----------VY 152
GlcA VNKPSSGGPVPSEYSLGQIIRAWSPFLILTVLVTIWTMKP-FKALFAPGGAFYSLVINFQ 347
LldP L--------AHTGYTAGQVLRAWTPFLFLTATVTLWSIPP-FKALFASGGALYEWVINIP 339
CitT F----VSKIAGIQISWLQWFLCFLPVGVILLIIAPWLSYVLYKPEITHSEEVATWA---- 273
TtdT L----MKSASHATLSWGDWFLGMLPLSILLVLLVPWLAYVLYPPVLKSGDQVPRWA---- 272
DcuA M----SSVMEGHGISYLHLLSVVIPSTLLAVLVMSFLVTMLFNSKLSDDPIYRKRL---- 204
GlcA IPHLHQQVLKAAPIVAQPTPMDAVFKFDPLSAGGTAIFIAAIISIFILGVGIKKGIGVFA 407
LldP VPYLDKLVARMPPVVSEATAYAAVFKFDWFSATGTAILFAALLSIVWLKMKPSDAISTFG 399
CitT ----GDELKTMGALTRREWTLIGLV----LLSLGLWVFGSE--------VINATAVGLLA 317
TtdT ----ETELQAMGPLCSREKRMLGLM----VGALVLWIFGGD--------YIDAAMVGYSV 316
DcuA ----EEGLVEL-----RGEKQIEIK---SGAKTSVWLFLLGVVGVVIYAIINSPSMGLVE 252
GlcA ETLISLK-------WPILSIGMV---LAFAFVINYS-----GMSTTLALVLAGTGVMFPF 452
LldP STLKELA-------LPIYSIGMV---LAFAFISNYS-----GLSSTLALALAHTGHAFTF 444
CitT VSLMLA---------LHVVP---------WKDITRYNSAWNT----LVNLATLVVMANGLT 356
TtdT VALMLL---------LRIIS---------WDDIVSNKAAWNV----FFWLASLITLATGLN 355
DcuA KPLMNTTNAILIIMLSVATLTTVICKVDTDNILN-SSTFKAGMSACICILGV-AWLGDTF 310
GlcA FSPFLGWLGVFLTGSDTSS----------NALFGSLQSTTAQQINVSDTLLVAA 496
LldP FSPFLGWLGVFLTGSDTSS----------NALFAALQATAAQQIGVSDLLLVAA 488
CitT RSGFIDWFAGTMSTHLEGFSPNATVIVLVLVFYFAHYLFASLSAHTATMLPVI----LAV 412
TtdT NTGFISWFGKLLAGSLSGYSPTMVMVALIVVFYLLRYFFASATAYTSALAPMM----IAA 411
DcuA VSNNIDWIKDTAGEVIQGHP-----WLLAVIFFFASALLYSQAATAKALMPMA----LAL 361
GlcA NTSGGVTGKMISPQSIAVACAATGM---------- 521
LldP NTTGGVTGKMISPQSIAIACAAVGL---------- 513
CitT GK-------GIPGVPMEQLCILLVLSIGIMGCLTPYAT----------GP---GVIIYGC 452
TtdT AL-------AMPEIPLPVFCLMVGAAIGLGSILTPYAT----------GP---SPIYYGS 451
DcuA NV-------SP-LTAVASFAAVSGL-----FILPTYPTLVAAVQMDDTGTTRIGKFVFNH 408
GlcA --VGRESELFRYTVKHSLIFASVIGIITLLQAYVFTGMLVS 560
LldP --VGKESDLFRFTVKHSLIFTCIVGVITTLQAYVLTWMIP- 551
CitT G-YVKSKDYWRLGAIFGVIY----ISMLLLVGWPILAMWN- 487
TtdT G-YLPTADYWRLGAIFGLIF----LVLLVITGLLWMPVVLL 487
DcuA PFFIPGTLGVALAVCFGFVL----GSFML---------- 433

Figure 12: Sequence similarity in putative transporters *citT, dcuA* and *ttdT* against known lactate transporters *lldP* and *glcA*. Multiple regions of overlap or residue similarity are observed in conserved regions of the lactate transporters against the three potential exporters.

This study does encounter some difficulty in highlighting specific gene targets; the method utilized highlighted a diverse set of genes, most of which do not contribute to lactate fermentation. Enzymes with repair or stress-tolerance functions could improve growth of fermentative *E. coli* in each batch fermentation. Moreover, the fundamental screening process may likely be selecting for randomly beneficial genes, as no clear patterns or similarities were identified in the sequenced plasmids. This confirms the importance of strict and specific screening mechanisms, and further increases the reproducibility or application of this genetic screening method in other models.

There exists the opportunity for targeted lactic acid transporters not only in microbial strains for the improvement of fermentative metabolism, but also routes into how lactate is transported in mammals for the purpose of cancer biology research identified in the Warburg Effect[31; 48]. Primarily, this research will be applied to review the potential of other transport mechanisms in *E. coli*, with an emphasis on sugar uptake routes and the generation of mutants for overall improvement of metabolism. This could likely involve methods of error-prone PCR for the comparative analysis of mutant transporters and their effects on fermentative metabolism.

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