Characterizing three *Pseudomonas* milk isolates and interrogating specific similarities to cystic fibrosis clinical isolate, *P. lundensis* AU1044

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Bachelors of Science
Honors Microbiology
at the University of Michigan — Ann Arbor
April 2020

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ABSTRACT

Pseudomonas lundensis are recognized as psychrotrophic bacteria that contribute to cold food spoilage (21). The only existing sequenced genomes of *P. lundensis* were isolated from spoiled meat until clinical strains of *P. lundensis* were recovered from the lungs of cystic fibrosis patients in 2018 (21), however the role of *P. lundensis* in respiratory disease and the overall lung microbiome remains elusive. Novel *Pseudomonas* species' dual presence in milk and in disease make it an important area to explore given the discrepancy between the bacteria's role (pathogenic and non-pathogenic) in each environment. This study aims to characterize three *Pseudomonas* strains (101, 103, 105) isolated from milk and determine their similarity to *Pseudomonas lundensis* AU1044, a strain isolated from a cystic fibrosis patient, in order to establish the feasibility that milk could be an environmental source of *P. lundensis* in the lungs of humans with respiratory disease. Growth morphology, Gram staining, 16S rRNA polymerase chain reaction (PCR), and *Pseudomonas* specific PCR were utilized to get rough estimates of isolate identities. Isolate identities were strengthened and confirmed by genetic analysis using the combination of 16S rRNA, *motA*, and *rpoB* genes as reference in NCBI's Basic Local Alignment Search Tool (BLAST). Hypotheses concerning isolates' environmental behavior were formed by looking at genes and proteins within three metabolic systems: respiration, type III secretion, and quorum sensing/biofilm formation. Experimentation showed isolate 103 to be distinct from isolates 101 and 105 in terms of growth morphology, growth temperature preference, and crude identification conclusions made from *Pseudomonas* specific PCR. Deeper genetic analysis confirmed the phenotype findings and further separated isolate 103 from isolates 101 and 105. Isolates 101 and 105 were identified as the same closely-related *P. lundensis* strain to *P. lundensis* AU1044 while isolate 103 was not. In dendrogram analysis, isolates 101 and 105 showed stronger evidence of being closely related *P. lundensis* strains to *P. lundensis* AU1044 than isolate 103. Multi-genic analysis suggested that the milk isolates and clinical isolates behave similarly; they prefer aerobic environments, utilize biofilm, and have a limited (or misunderstood) type III secretion system. The majority of *Pseudomonas* milk isolates exhibited strong similarity to clinical isolate, *P. lundensis* AU1044, which supports milk as an environmental source for *P. lundensis* present in respiratory disease. Isolate 103's distinction from the other milk isolates provides avenues for future directions where similarity to other closely-related *Pseudomonas* species could be studied for further clinical application.

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I. BACKGROUND

A. *Pseudomonas*

The *Pseudomonas* genus contains significant groups of bacteria. While they all share rod-shape, polar flagellum, and stain Gram-negative, *Pseudomonas* is recognized for its wide species diversity (1, 14, 22). Members of the *Pseudomonas spp.* are ubiquitous and found in almost every type of aerobic environment. They can reside in natural habitats such as soil, fresh water, and salt water, and they can be found in naturally occurring human body microbiomes, specifically ones in the oral cavity and lungs (14, 19). Notable species within the genus are *P. aeruginosa*, *P. fluorescens*, and *P. syringae*.

B. Food Spoilage

Food spoilage, particularly in dairy products, is often due to *Pseudomonas spp*. Refrigeration of raw milk throughout the dairy manufacturing chain prior to heat treatment selects for the growth of certain psychrotolerant (or psychrotrophic) bacteria which tend to belong to the *Pseudomonas* spp. (3). In a study sampling milk across seasons, 53% of all 103 isolates fell into species identification of either *P. fragi* or *P. lundensis* for their production of heat resistant proteases (13). Their proliferation begins with metabolizing non-protein nitrogen elements (2). Secreted heat resistant proteases and lipases that survive pasteurization and ultra-high temperature treatment are responsible for exerting the main structural defects to milk that cause spoilage (2, 3). Amino acids and fatty acids that are released as by-products give off typical food waste odors and signal rancidity. If allowed to continue, extracellular biofilm production will ensue along with visible, pigmented bacterial growth (2). While *Pseudomonas* microorganisms contribute in large part to decreased shelf life and sub-par quality of food, they are also implicated in some pathogenic infections in humans (14).

C. *P. lundensis*

Pseudomonas lundensis, part of the *P. fluorescens*-species complex, are popularly recognized as psychrotrophic bacteria that contribute to cold food spoilage (21). *P. lundensis*, like other *Pseudomonas*, is a Gram-negative bacillus that moves via polar flagellum. It's psychrotolerant characteristics allow it to grow best at temperatures 0-33℃ (15). The only existing sequenced genomes of *P. lundensis* were isolated from spoiled meat until clinical strains of *P. lundensis* were recovered from the lungs of cystic fibrosis patients in 2018 (21), however the role of *P. lundensis* in respiratory disease and the overall lung microbiome remains elusive. The 4.81 MB genome of *P. lundensis* isolate, strain AU1044, is available for the comparative analysis this study requires (21).

D. Genetic Markers

Each of these genes will be used to characterize the *Pseudomonas* milk isolates in terms of taxonomic relationship, broad identity, *Pseudomonas* genus comparison, and *P. lundensis* AU1044 comparison.

16S ribosomal RNA, or rRNA, is a component of the 30S small subunit of a ribosome in prokaryotic translation that binds the Shine-Delgarno sequence on incoming mRNA. This gene is highly conserved across bacterial and archaeal organisms for its major role in prokaryotic translation. It is often used to reconstruct phylogenies to understand and establish taxonomic relationships. 16S rRNA will be used in 16S rRNA amplification to establish isolates' broad identities and will inform UPGMA dendrogram analysis.

I2, a region unique to the *P. fluorescens*-species complex, encodes for a superantigen that contributes to autoimmune and enteric diseases in humans (27). I2 was originally discovered through Crohn's Disease selective peptide screening (27). The presence of I2 indicates a positive result for the P. *fluorescens* species within the *P. fluorescens*-species complex, thus *P. lundensis* should be negative for I2.

Pseudomonas ExoU is a type III secretory toxin and virulence factor that contains phospholipase activity which induces reduction of cell membrane integrity and cell death (20). It is expressed in 90% of clinical *P. aeruginosa* strains that are causative agents of disease, such as acute lung injury and/or sepsis (20, 18). There is evidence of ExoU homologs in 17 other *Pseudomonas* species and 8 either Gram-negative bacteria that are members of different genera which supports

similar avenues to cytotoxic function (18). Our laboratory has created PCR primers specific to e*xoU* in *P. lundensis,* which do not amplify e*xoU* in *P. aeruginosa*.

E. Metabolic systems

Bacterial genetics, specifically ones belonging to metabolic systems, provide a lot of information on how bacteria gain control of their environment. The respiratory system, type 3 secretion system, and quorum sensing in biofilm formation system were identified as essential metabolic systems to be characterized in this study to determine if genes in each respective system suggest benefit in environmental, clinical, both, or neither.

Respiration is essential to bacterial function and provides information into what sort of environment bacteria can live in. Genes governing aerobic and anaerobic respiration determine where the milk isolates and clinical isolate can proliferate and survive. *narX, narL,* and *narG* all exert central function in nitrate reduction, a common method for anaerobic bacterial respiration (7). *arcA* and *arcB* are oxygen sensors responsible for the switch between aerobic and anaerobic respiration depending on the environmental conditions (8). Their functions are well-described in Figures 22 and illustrated in Figure 23 (7, 8).

Looking at whether the milk isolates and clinical isolate contain genes for a type III secretion system will be telling of potential virulence. Comprised of protein complexes that cross two membranes (similar to a flagellar assembly complex) in Gram-negative bacteria, a type III secretion system allows for contact-dependent injection of toxic effector proteins that mainly targets eukaryotic cells (4). *hrcC*, *hrcJ*, *hrcR*, *hrcS*, *hrcU*, and *hrcV* are all highly conserved structural regions of the type III secretion system apparatus (4). Their functions are well-described in Figures 25 and illustrated in Figure 26 (4).

Quorum sensing is a term used to describe coordinated shifts in bacterial behavior and serves as a general framework to look into biofilm formation. Biofilms, something of a hallmark to *Pseudomonas* species, are predominant means for colonization as well as pathogenic capability that can be signaled to activate through quorum sensing. *lasR, rhlA,* and *rhlR* are well-identified components of quorum sensing in *Pseudomonas aeruginosa* (11, 16). Their specific functions are discussed in Figure 28 and illustrated by Figure 29 (10, 11, 12, 16). *mucA, algD,* and *ndvB* are the remaining genes in the system: *mucA* and *algD* are genes that are directly related to biofilm formation and *ndvB* has been implicated in conferring antibiotic resistance in *Pseudomonas* species (10, 12, 16). Again, their functions are described in Figure 28 and illustrated by Figure 30 (10, 11, 12, 16).

Gene and protein homologies across these three metabolic systems will supplement the general understanding of the isolates' environmental niche(s) and potential means for virulence. With general knowledge about *P. lundensis*'s dual presence in cystic fibrosis and milk, one a disease and the other a consumable product, genetic differences are expected in the way the isolates exercise control over their environment across all three metabolic systems.

F. Study Motivation

Novel *Pseudomonas* species isolated from the lungs of cystic fibrosis patients has thrusted *Pseudomonas* research even further into the clinical sphere, specifically P. *lundensis spp*. It's dual presence in milk and in disease make it an important area to explore given the discrepancy between the bacteria's role (pathogenic and non-pathogenic) in each environment. By identifying *Pseudomonas* milk isolates *in vitro* and comparing them back to clinical isolate *P. lundensis* AU1044, this work will seek to answer the question of whether milk could be a possible source of pathogenic *P. lundensis* colonization in diseased lungs.

II. OBJECTIVE STATEMENT

This study aims to characterize three *Pseudomonas* strains isolated from milk and determine their similarity to *Pseudomonas lundensis* AU1044, a strain isolated from a cystic fibrosis patient, in order to establish the feasibility that milk could be an environmental source of *P. lundensis* in the lungs of humans with respiratory disease.

III. APPROACH

Each isolate will be surveilled phenotypically and genotypically by a number of tests to determine its similarity to *P. lundensis* AU1044 and clinical consequence. Growth morphology, Gram staining, 16S rRNA polymerase chain reaction (PCR), and *Pseudomonas* specific PCR will be utilized

to get rough estimates of isolate identities. Isolate identities will be strengthened and confirmed by genetic analysis using the combination of 16S rRNA, *motA*, and *rpoB* genes as reference in NCBI's Basic Local Alignment Search Tool (BLAST). Isolate environmental behavior will be elucidated by looking at genes within three metabolic systems: respiration, type III secretion, and quorum sensing/ biofilm formation. By looking at gene homologies and subsequent protein homologies, hypotheses of the behavior of each unknown milk isolate can be made. This approach is laid out visually by Figure 1.

IV. MATERIALS AND METHODS

A. Bacterial strains

Unknown milk isolates were originally recovered from 1% pasteurized milk sourced from Meijer, Guernsey Farms, and Prairie Farms (25). Isolate numbers 101, 103, and 105 were chosen for this study because of the amount of pre-existing data on them.

B. Inoculation

For each unknown milk isolate, a broth culture and plate were prepared in duplicate for testing at room temperature (25℃) and 37℃. Broth cultures were prepared by inoculating 20 mL of Luria Broth with a loopful of cells from glycerol -20℃ freezer stocks. Plates were prepared by taking one loop of cells from the same glycerol stocks and streaking on Luria Agar plates. Both broth cultures and agar plates were incubated at the appropriate temperature for 48 hours. Broth cultures incubated with 125rpm agitation. Immediately following the 48 hour mark, descriptions and pictures were taken to catalog the growth and morphology of the broth and plates.

C. Pellet

1 mL of each broth culture was spun down at a bench top centrifuge at 13000 rpm for 10 minutes. Pellet formation was assessed.

D. Gram Staining

20uL of broth culture or 20uL of water mixed with streak of plate culture were dried onto a glass slide by sitting on a hot plate. The bacterial samples were heat-fixed by running through a flame three times. Next, the glass slide was flooded with crystal violet for 1 minute. After being washed with water for a few seconds, the slide was flooded with iodine for 1 minute. The slide was washed again with water before flooding with decolorizing agent for 15-20 seconds. A quick water rinse ensured removal of the decolorizer. The slide was flooded with counterstain (safranin) for 30 seconds to 1 minute. The slide was run under water until there was no color in the effluent. Results were observed under oil immersion using a compound light microscope with 100X objective (6). A pink stained organism indicated a Gram-negative bacteria and a purple stained organism indicated a Gram-positive bacteria.

E. 16S rRNA PCR

For each reaction, one Illustra Taq Ready To Go PCR bead was used. A total of 12 wells were needed per milk isolate: 3 replicate wells for each room temperature broth culture, 37℃ broth culture, room temperature colony growth, and 37℃ colony growth. Master mix was prepared in a 1.5mL Eppendorf tube which contained 20pmol of D88 forward primer (5'-GAG-AGT-TTG-ATY-MTG-GCT-CAG-3', 0.4uL per reaction), 20pmol of E94 reverse primer (5'-GAA-GGA-GGT-GWT-CCA-RCC-GCA-3', 0.4uL per reaction), and nuclease free water (24.2 uL per reaction). 25uL of master mix was added to each well containing a PCR bead. The mixtures were vortexed and spun to ensure full dissolution of the bead. Reaction wells were inoculated with either 1uL of broth or a single colony picked using a sterile toothpick and thoroughly mixed with a series of centrifugation and vortexing. Reactions were loaded into the thermocycler using program KLM which adhered to the following cycle parameters: initial melt at 94℃ for 2 minutes, 30 cycles of melt at 94℃ for 30 seconds, anneal at 50℃ for 45 seconds, elongation at 72℃ for 2 minutes, and then a final elongation at 72℃ for 5 minutes before 12℃ soak. The PCR products were run on a 1% agarose gel with a 1Kb ladder. The 1500 bp fragment was identified under ultraviolet (UV) light and cut out to be purified using Qiagen QIAquick Gel Extraction Kit. The purified DNA along with its primers were submitted to the University of Michigan Advanced Genomics Core for Sanger Sequencing.

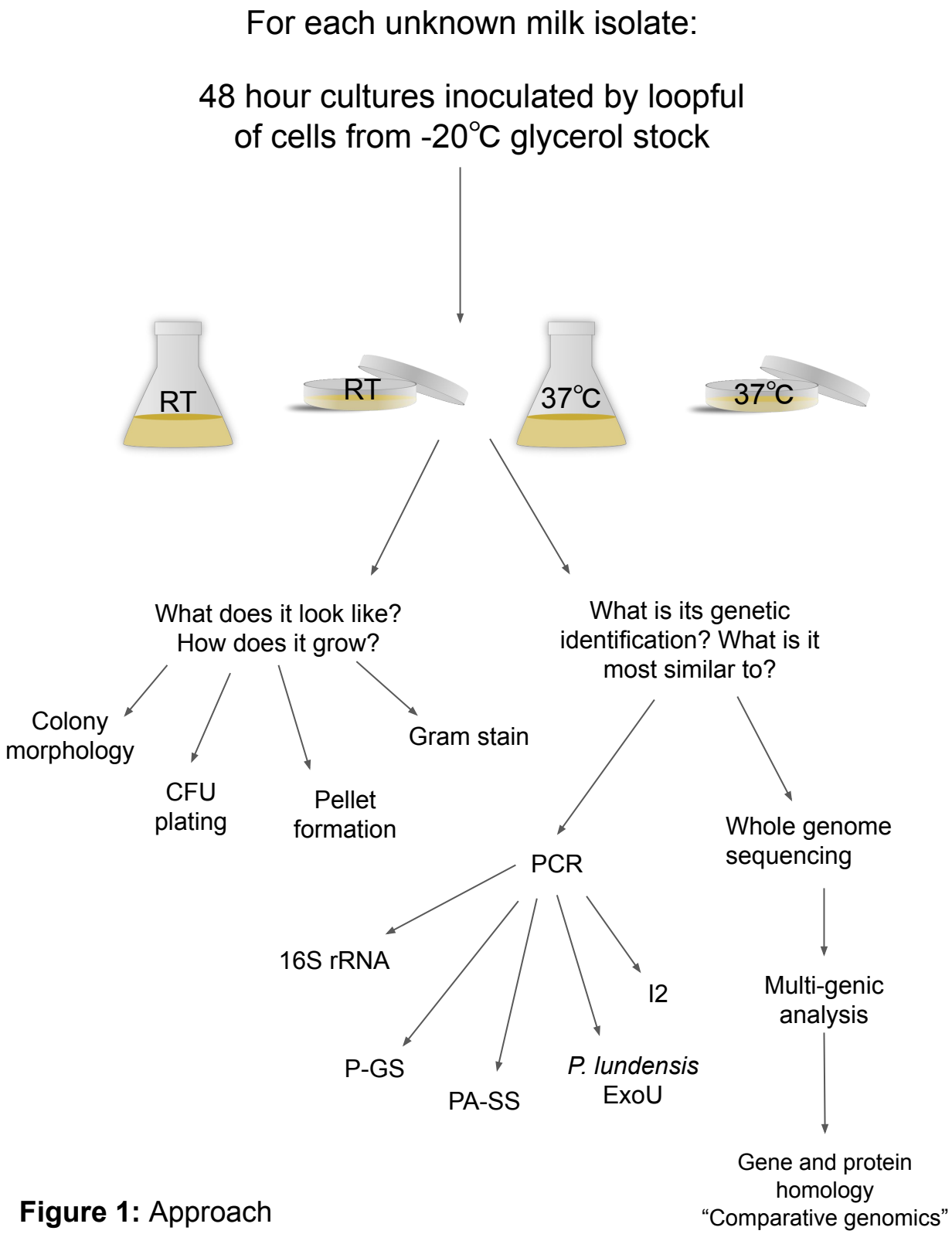

Figure 1: Approach

Sequences derived from the core were assessed using EZBioCloud for comparison to entire bacterial 16S rRNA database to determine rough taxon and strain identity. NCBI's Basic Local Alignment Search Tool (BLAST) was utilized to quantify the sequences' specific comparison to *P. lundensis* AU1044.

F. Quantification

Using a sterile round bottom 96-well plate, a 10 fold serial dilution (180uL dilutent:20uL culture) was performed on each broth culture up to 10E-8. These dilutions were plated on Luria Agar in duplicate and incubated at 37℃ for 24 hours. After 24 hours, bacterial concentration was determined by counting colony forming units (CFU) and taking an average count from the duplicate plating. End calculations yielded the number of viable bacteria per milliliter of culture.

G. *Pseudomonas* specific PCR

For each unknown isolate, CFU plates were referenced to determine which broth culture, room temperature or 37℃, had optimal growth. The growth temperature that showed the most growth was selected for Pseudomonas specific PCR. For each reaction, one Illustra Taq Ready To Go PCR bead was used. Total number of reaction wells differed per primer set but generally, 3 replicate wells were needed for the broth culture and 1 well was assigned per necessary control. Per primer set, master mixes were made in 1.5 mL Eppendorf tubes with 22uL of nuclease free water, 1uL or forward primer, and 1uL of reverse primer. The mixtures were vortexed and spun to ensure full dissolution of the bead. Reaction wells were inoculated with 1uL of broth or 1 uL of control bacteria DNA or water and thoroughly mixed with a series of centrifugation and vortexing. Reactions were loaded into the thermocycler using its respective program.

1. *Pseudomonas* Genus Specific PCR (P-GS) used controls *P. lundensis* AU1044 (positive control), *P. aeruginosa* PAO1 (positive control), *P. fluorescens* 8050 (positive control), *E. coli* K12 (negative control), and water (negative control) to confirm the isolates' identities as part of the *Pseudomonas* genus by targeting a subsection of the 16S gene that identifies the *Pseudomonas* genus away from other bacteria (24). The PCR program named "PGS" was run with P-GS forward primer (5'-GAC-GGG-TGA-GTA-ATG-CCT-A-3') and P-GS reverse primer (5'-CAC-TGG-TGT-TCC-TTC-CTA-TA-3') to yield the expected product size of 618 bp. The cycle parameters were as follows: initial melt at 95℃ for 10 minutes, 25 cycles of melt at 94℃ for 20 seconds, anneal at 54℃ for 20 seconds, elongation at 72℃ for 40 seconds, and then a final elongation at 72℃ for 1 minute before 12℃ soak. The PCR products were run on a 2% agarose gel with a 100bp ladder.

***2. Pseudomonas aeruginosa* Specific PCR (PA-SS)** used controls *P. aeruginosa* PAO1 (positive control), *P. fluorescens* 8050 DNA (negative control), *E. coli* K12 (negative control), and water (negative control) to confirm or deny the isolates' identities as *P. aeruginosa* by targeting a subsection of the 16S gene that identifies *P. aeruginosa* species away from other species in the *Pseudomonas* genus (24). The PCR program named "PASS" was run with PA-SS forward primer (5'-GGG-GGA-TCT-TCG-GAC-CTC-A-3') and PA-SS reverse primer (5'-TCC-TTA-GAG-TGC-CCA-CCC-G-3') to yield the expected product size of 956 bp. The cycle parameters were as follows: initial melt at 95℃ for 10 minutes, 25 cycles of melt at 94℃ for 20 seconds, anneal at 54℃ for 20 seconds, elongation at 72℃ for 40 seconds, and then a final elongation at 72℃ for 1 minute before 12℃ soak. The PCR products were run on a 2% agarose gel with a 100bp ladder.

3. I2 is a region that corresponds to a novel super antigen specific to the *P. fluorescens*-species complex (27). I2 Specific Primer (I2) used controls *P. fluorescens* 8050 (positive control), *P. aeruginosa* PAO1 (negative control), and water (negative control) to confirm of deny the isolates' identities as *P. fluorescens* AU8050. The PCR program named "I2" was run with I2 forward primer (5'-TCT-GCT-CAT-ACA-CGT-CAC-G-3') and I2 reverse primer (5'-CCG-TGG-GCA-TCC-AGT-CCG-3') to yield the expected product size of about 250 bp. The cycle parameters were as follows: initial melt at 95℃ for 5 minutes, 30 cycles of melt at 95℃ for 60 seconds, anneal at 65℃ for 60 seconds, elongation at 72℃ for 60 seconds, and then a final elongation at 72℃ for 5 minutes before 12℃ soak. The PCR products were run on a 2% agarose gel with a 100bp ladder.

4. ExoU codes for a phospholipase A that induces host cell damage and necrosis (20). It is present in both *P. aeruginosa* and *P. lundensis* (20), but the ExoU primer utilized in this protocol was specific to ExoU in *P. lundensis*. ExoU Primer (ExoU) used controls *P. lundensis* AU1044 (positive control), *P. aeruginosa* PAO1 (negative control), and water (negative control). The PCR program named "ExoU" was run with ExoU forward primer (5'-AGC-CGC-CCG-CCG-TTG-ACC-AG-3') and reverse primer (5'-GTG-ACC-GCG-CCG-CCC-TGC-TC-3') to yield the expected product size of 494 bp. The cycle parameters were as follows: initial melt at 95℃ for 10 minutes, 25 cycles of melt at 94.3℃ for 20 seconds, anneal at 64.6℃ for 20 seconds, elongation at 72℃ for 40 seconds, and then a final elongation at 72℃ for 1 minute before 12℃ soak. The PCR products were run on a 2% agarose gel with a 100bp ladder.

H. Multi-genic identification

Multi-genic identification began with searching for annotated genes for *motA*, *rpoB*, and 16S rRNA for each strain on NCBI. *P. aeruginosa* PAO1 was selected to be the base comparison for finding genes in each isolate using the "blastn" function on NCBI's Basic Local Alignment Search Tool (BLAST). Concatenated files of *motA*, *rpoB*, and 16S rRNA were created for each strain, then combined into a larger concatenated file of multi-genic sequence containing all strains. Using data from "blastn" searches, relative gene identities between all strains were determined and put into a similarity matrix. A distance matrix was generated from the similarity matrix to inform unweighted pair group method with arithmetic (UPGMA). Using the tool, DendroUPGMA, a dendrogram was generated from the distance matrix.

I. Metabolic systems: gene and protein homology

The procedure supporting gene and protein homology started by finding each gene in the given genomes from NCBI gene bank searches. Genes in isolates were identified by using NCBI's BLAST "blastn" function; from there, the aligned sequences were downloaded. Parameters for percent identity significance are given by a query coverage greater than 50% and an e-value less than 1E-25. Query origin was consistently *P. aeruginosa* PAO1 because it served as a suitable positive control for most of the genes.* Homology between genes were assessed in "blastn" by aligning two or more sequences. Genes were converted to proteins using "blastx." Once nucleotides were converted to amino acid sequences, protein homologies were compared aligned two or more sequences in "blastp."

* When it came to analyze type III secretion system genes, there had to be multiple positive controls because of the lack of inter/intraspecies system homogeneity and presence of system in *P. aeruginosa* PAO1, despite the deliberate choice to select genes with high conservation.

V. RESULTS

A. Colony Growth Morphology

After 48 hours, plated colony growth for milk isolates 101, 103, and 105 were very similar. For both room temperature and 37℃ incubation temperatures, isolates exhibited round colonies with glossy edges that were slightly yellow in color, though plates left at 37℃ tended to support colonies with slightly larger circumferences (Figure 2).

B. Broth Growth Morphology

After 48 hours, room temperature and 37℃ broth cultures were equally cloudy for all isolates and gave off a molding, food waste odor. The broth cultures at 37℃ were consistently more yellow, with the exception of isolate 103. Isolate 103 at 37℃ in broth was green with a slight film on top. The obvious change in color is the earliest form of difference that isolate 103 shows from isolates 101 and 105. Although contamination can never be ruled out entirely, tests further along in the experimental approach strongly suggest that 103 is distinct from 101 and 105 (Figure 3).

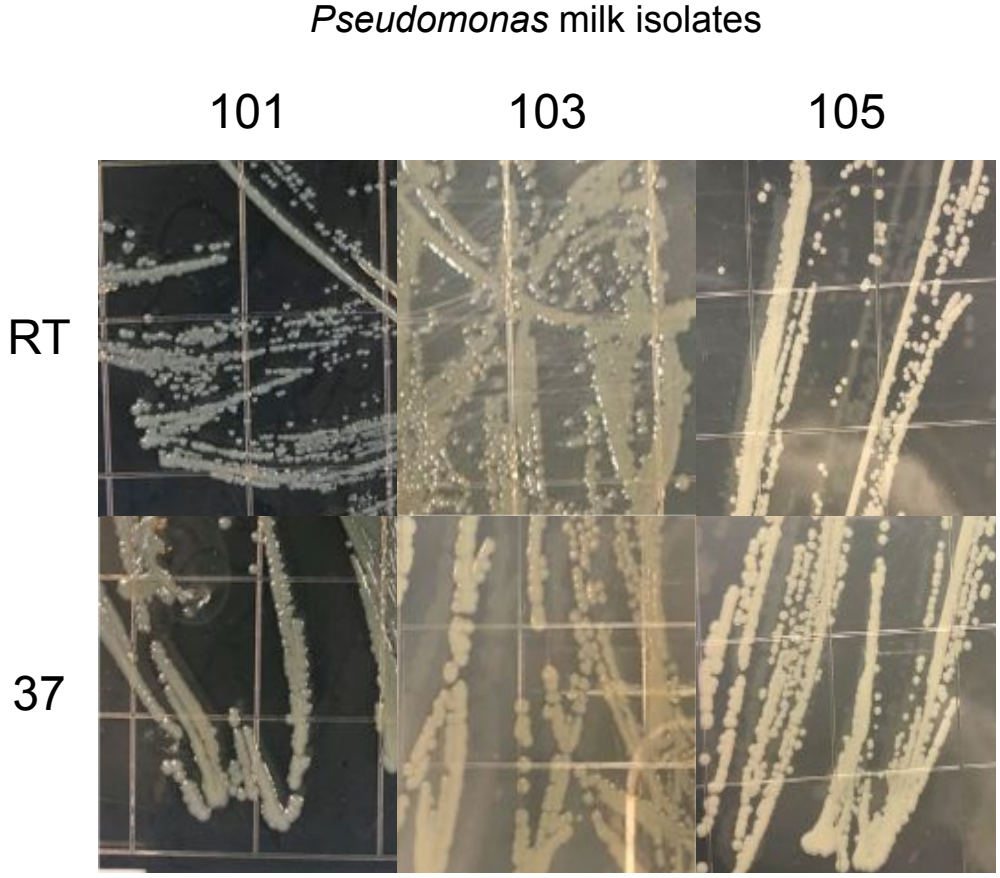

Figure 2: Colony growth morphology
Luria agar plates inoculated with one loopful of cells from -20℃ glycerol freezer stocks of *Pseudomonas* milk isolates were incubated for 48 hours at either room temperature (25℃) or 37℃.

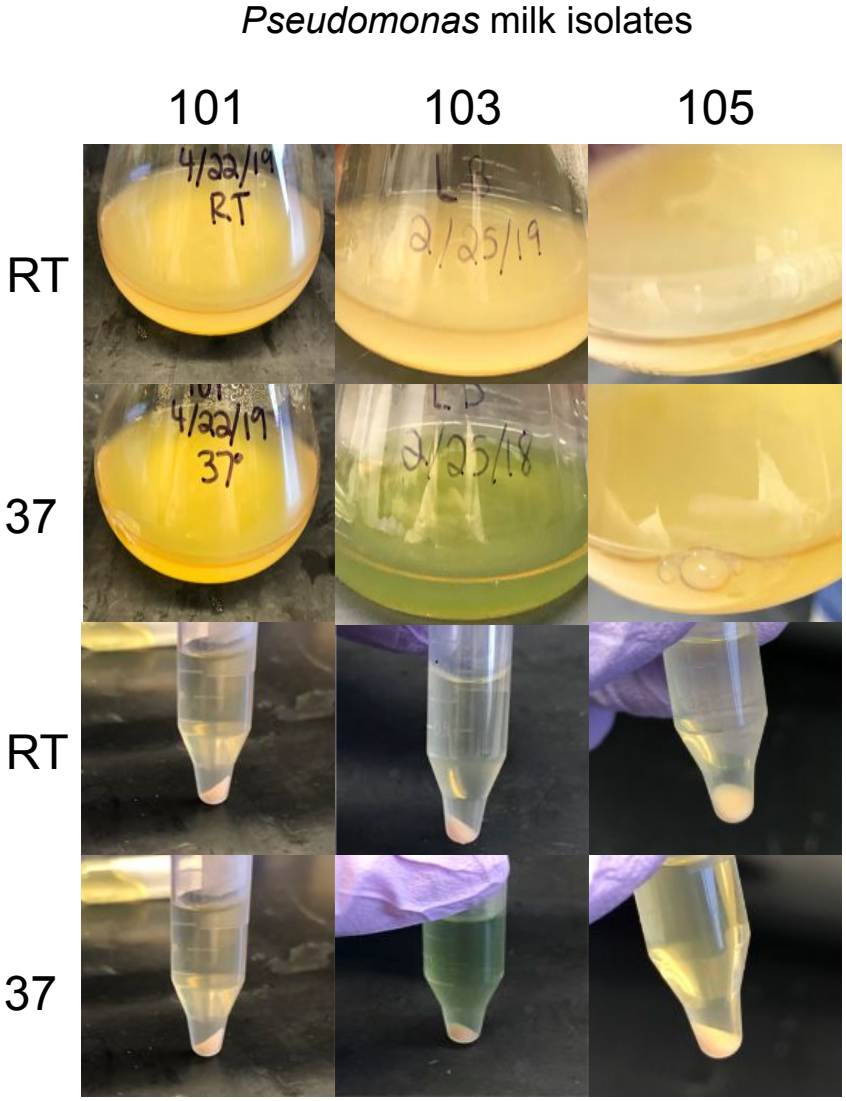

Figure 3: Broth growth morphology and pellet formation
Luria broth inoculated with one loopful of cells from -20℃ glycerol freezer stocks of *Pseudomonas* milk isolates were incubated for 48 hours at either room temperature (25℃) or 37℃.

C. Pellet Formation

All isolate broth cultures at room temperature and 37℃ yielded a hard, pinkish pellet after centrifugation for 10 minutes at 13x1000 rpm (Figure 3).

D. Gram Staining

Under oil immersion, all unknown milk isolate cultures on plates and in broth at room temperature and 37℃ were identified as Gram-negative bacilli. There was no evidence of contamination because the stained cells appeared homogenous (Figure 4).

E. 16S rRNA PCR

Since broth cultures showed visible bands following gel electrophoresis more reliably than toothpick selected colonies, DNA extraction and purification products from broth cultures were chosen for Sanger Sequencing. Against the whole database, *P. lundensis* DSM 6252 was the top taxon and strain hit for isolates 101 and 105. 103 provided conflicting data following search against the whole database. There were multiple top taxon and strain hits: *P. salmonii* CFBP 2022, *P. taetrolens* DSM 21104, and *P. corrugata* ATCC 29736. At this point, the results suggest two things. The first is points to lack of strength in the amplified sequence because of the inconsistent top taxon and strain hits. The second continues to support 103's distinction from 101 and 105 since *P. lundensis* DSM 6252 was not a single taxon or strain hit. In specific comparison to *P. lundensis* AU1044, isolates 101 and 105 showed high 16S rRNA gene homology with query coverages ranging from 94-99% and percent identities between 96.75-99.9%. Isolate 103's 16S rRNA gene homology to *P. lundensis* AU1044 was slightly lower and occupied a broader range with query coverage from 61-97% and percent identities between 93.29-97.66%. These results support that isolates 101 and 105 are more closely related to *P. lundensis* AU1044 than 103. The data from top taxon and hit strains further supports this takeaway by placing 101 and 105's 16S rRNA in alignment with *P. lundensis* DSM 6252, and 103's 16S rRNA with another *Pseudomonas* species entirely (Figure 5).

F. Quantification

A logarithmic scale was used to standardize the CFU/mL to account for different countable dilutions. Isolates 101 and 105 had higher CFU/mL counts at room temperature than at 37℃. Isolate 103 showed differences once again because it had a significantly higher CFU/mL count at 37℃ than room temperature. The results indicate that while 101 and 105 prefer to grow at room temperature, 103 prefers to grow at 37℃. This further differentiates isolate 103 from 101 and 105 (Figure 6).

G. Pseudomonas Specific PCR

1. *Pseudomonas* Genus Specific PCR (P-GS)

Pseudomonas genus specific PCR amplified a subsection of the 16S gene that identifies the *Pseudomonas* genus away from other bacteria (24). All isolates tested positive for the *Pseudomonas* genus which means that all isolates are a type of *Pseudomonas* (Figures 7-9).

2. *Pseudomonas aeruginosa* Species Specific PCR (PA-SS)

P. aeruginosa species specific PCR amplified a small part of the 16S gene that identifies *P. aeruginosa* species away from other species in the *Pseudomonas* genus (24). All isolates tested negative which indicates that none of the isolate identities align with P. *aeruginosa*. The original study that established these primers, "PCR-Based Assay for Differentiation of Pseudomonas aeruginosa from Other Pseudomonas Species Recovered from Cystic Fibrosis Patients" (24), tested 42 culture collection strains (including 14 *P. aeruginosa* strains and 28 strains representing 16 other closely related *Pseudomonas* species) and 43 strains that had been previously identified as belonging to 28 nonpseudomonal species also recovered from CF patient sputum (24). *P. lundensis* was not among the tested strains. Although it seems unrelated to the goal of this particular assay, 101 and 105 showed banding at ~1500 bp while 103 showed none. The weak banding indicates the presence of a homolog gene that is very close to the *P. aeruginosa* sequence selected for amplification. This could imply that isolates 101 and 105 contain a similar gene to that of *P. aeruginosa* and isolate 103 does not, which further separates 103 from the other isolates (Figures 10-12).

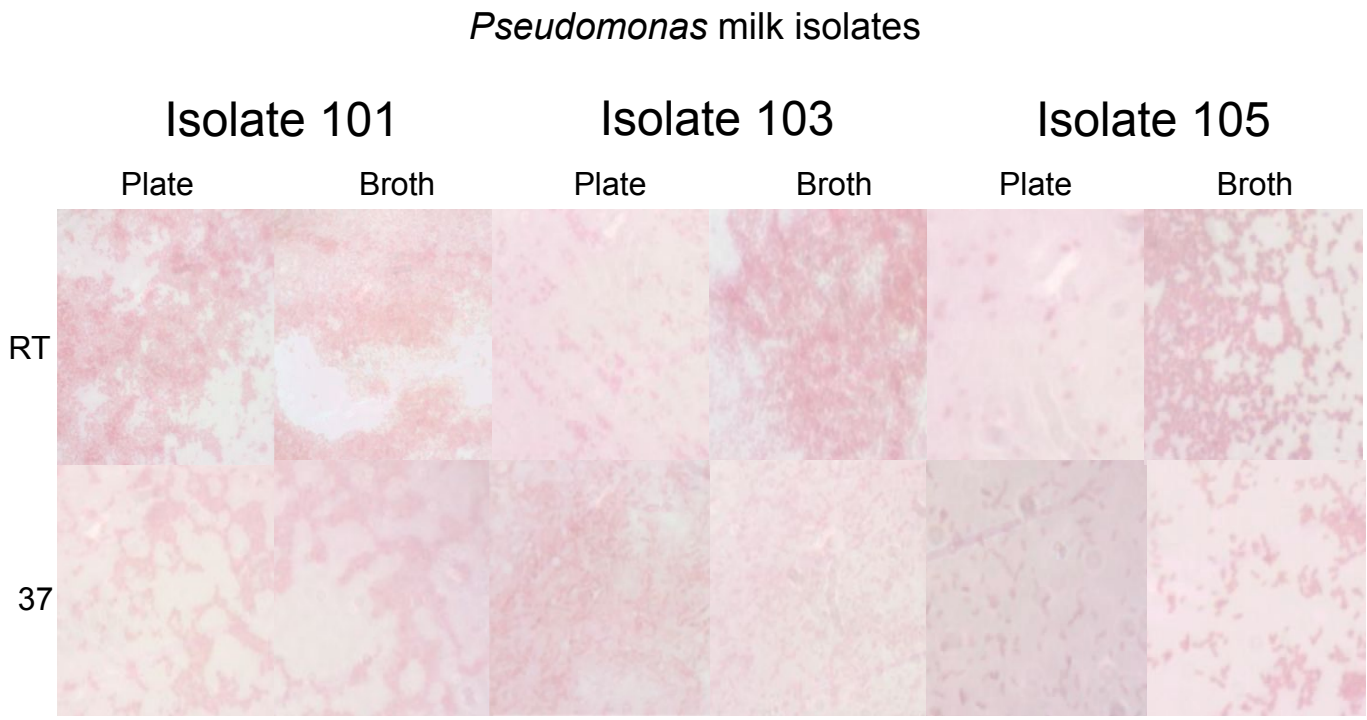

Figure 4: Gram stains

Luria agar plates and broth inoculated with one loopful of cells from -20℃ glycerol freezer stocks of *Pseudomonas* milk isolates were incubated for 48 hours at either room temperature (25℃) or 37℃. Gram stain was completed immediately following 48 hour incubation.

			Comparison to whole bacterial 16S database	Comparison to *P. lundensis* AU1044		
Isolate	Leuria Media	Growth Temp	Hit taxon and strain	Query Coverage	E value	Identity
101	Broth	RT	*P. lundensis* DSM 6252	98%	0	99.90%
	Broth	RT	*P. lundensis* DSM 6252	99%	0	98.27%
	Broth	RT	*P. lundensis* DSM 6252	99%	0	96.95%
	Broth	37	P. lundensis DSM 6252	98%	0	99.45%
	Broth	37	P. lundensis DSM 6252	98%	0	99.34%
	Broth	37	P. lundensis DSM 6252	98%	0	98.42%
103	Broth	RT	*P. salomonii* CFBP 2022 (T)	97%	0	97.66%
	Broth	RT	*P. taetrolens* DSM 21104 (T)	97%	0	98.97%
	Broth	RT	*P. corrugata* ATCC 29736 (T)	94%	2.00E-58	93.29%
	Broth	37	N/A	N/A	N/A	N/A
	Broth	37	*P. corrugata* ATCC 29736 (T)	94%	2.00E-58	93.29%
	Broth	37	*P. corrugata* ATCC 29736 (T)	61%	7.00E-44	94.59%
105	Broth	RT	*P. lundensis* DSM 6252	94%	0	98.30%
	Broth	RT	*P. lundensis* DSM 6252	95%	0	98.32%
	Broth	RT	*P. lundensis* DSM 6252	99%	0	97.87%
	Broth	37	*P. lundensis* DSM 6252 (T)	99%	0	97%
	Broth	37	*P. lundensis* DSM 6252 (T)	97%	0	96.75%
	Broth	37	N/A	N/A	N/A	N/A

Figure 5: 16S rRNA PCR, comparison to whole bacterial 16S rRNA database and only *P. lundensis* AU1044

Products from 16S rRNA gene amplification were submitted to the University of Michigan Advanced Genomics Core for Sanger Sequencing. Sequences derived from the core were assessed using EZBioCloud for comparison to the entire bacterial 16S rRNA database to determine rough taxon and strain identity. NCBI's Basic Local Alignment Search Tool (BLAST) was utilized to quantify the sequences' specific comparison to *P. lundensis* AU1044

Isolate	Growth Temperature	Countable Dilution	Average Count	Total CFU/ml	Log CFU/ml
101	RT	7	70	$7.0x10^{10}$	10.85
101	37°C	7	28.5	$2.85x10^{10}$	10.45
103	RT	5	42	$4.2x10^{8}$	8.62
103	37°C	9	57.5	$5.75x10^{12}$	12.76
105	RT	9	51	$5.1x10^{12}$	12.71
105	37°C	6	32	$3.2x10^{9}$	9.51

Figure 6: Quantification

10 fold serial dilutions (180uL dilutent:20uL culture) were performed on each broth culture up to 10E-8 on Luria Agar in duplicate and incubated at 37℃ for 24 hours. After 24 hours, bacterial concentration was determined by counting colony forming units (CFU) and taking an average count from the duplicate plating. End calculations yielded the number of viable bacteria per milliliter of culture.

Isolate 101 (RT Broth)
Pseudomonas Genus Specific, 618 bp product

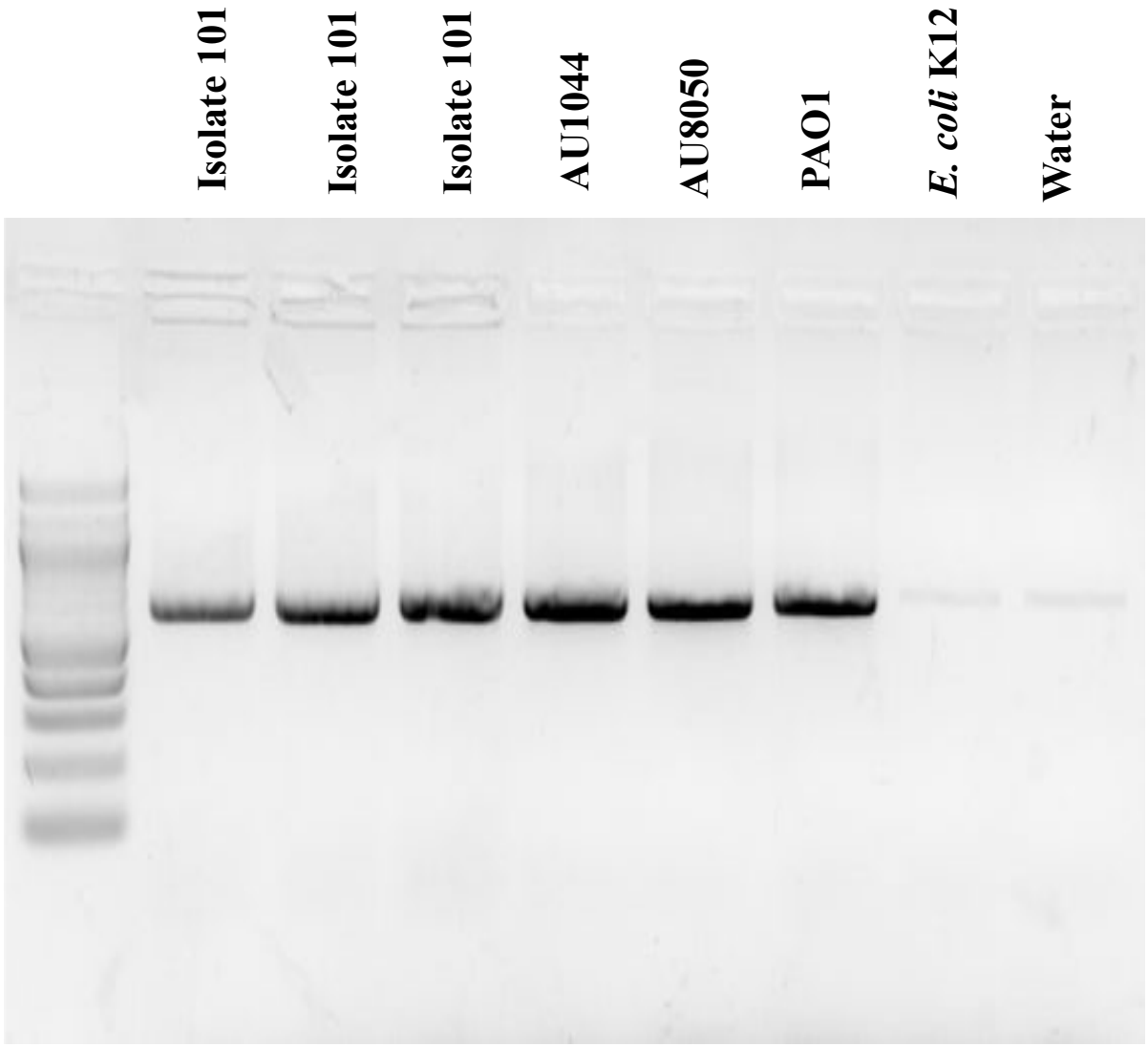

Figure 7: *Pseudomonas* genus specific PCR gel electrophoresis
PCR was carried out as outlined by the methods section.

AU1044 -- *P. lundensis* (Positive Control)
AU8050 -- *P. fluorescens* (Positive Control)
PAO1 -- *P. aeruginosa* (Positive Control)
E. coli K12 -- Negative Control
Water -- Negative Control

Isolate 103 (RT Broth)
Pseudomonas Genus Specific, 618 bp product

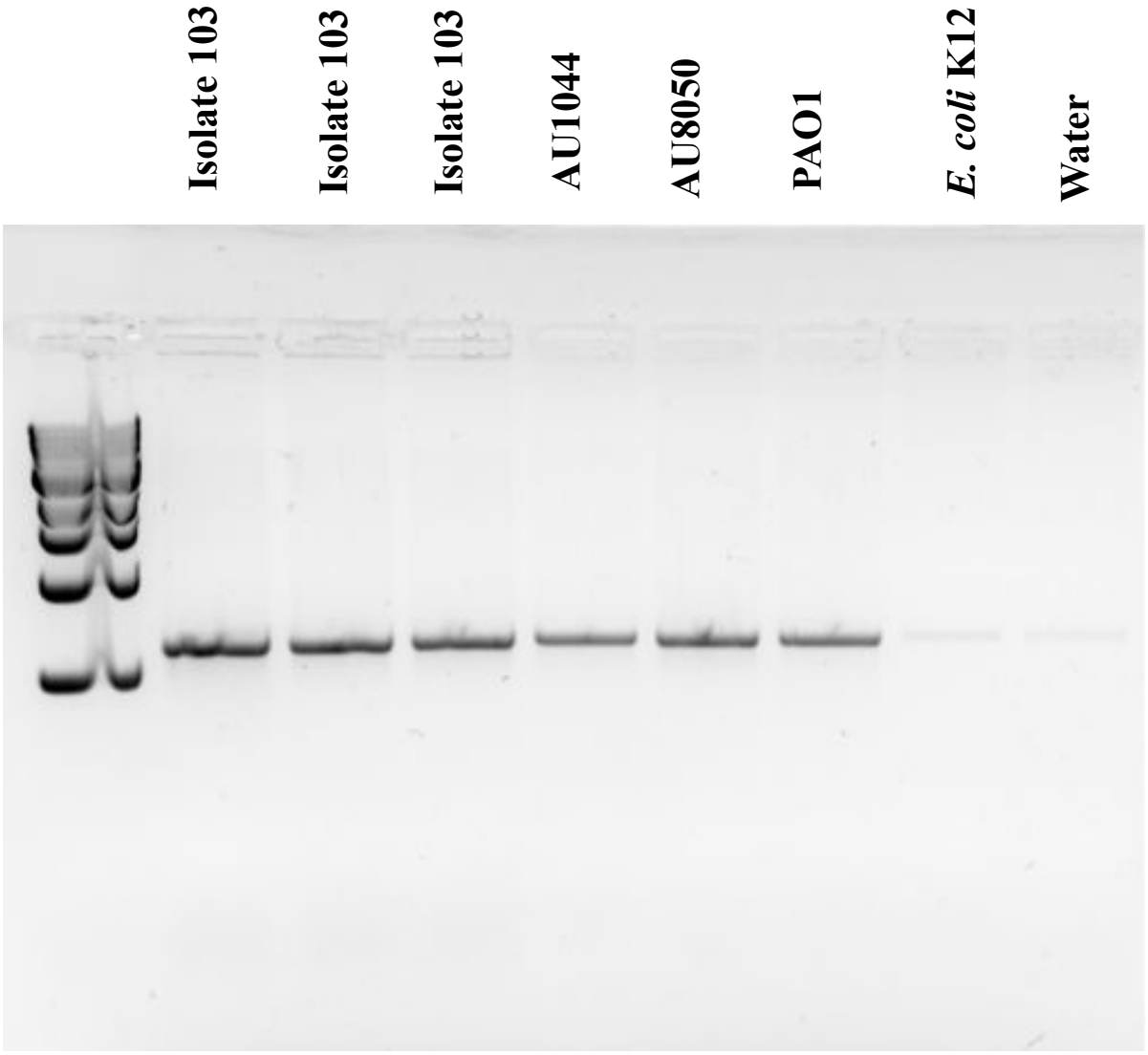

Figure 8: *Pseudomonas* genus specific PCR gel electrophoresis
PCR was carried out as outlined by the methods section.

AU1044 -- *P. lundensis* (Positive Control)
AU8050 -- *P. fluorescens* (Positive Control)
PAO1 -- *P. aeruginosa* (Positive Control)
E. coli K12 -- Negative Control
Water -- Negative Control

Isolate 105 (RT Broth)
Pseudomonas Genus Specific, 618 bp product

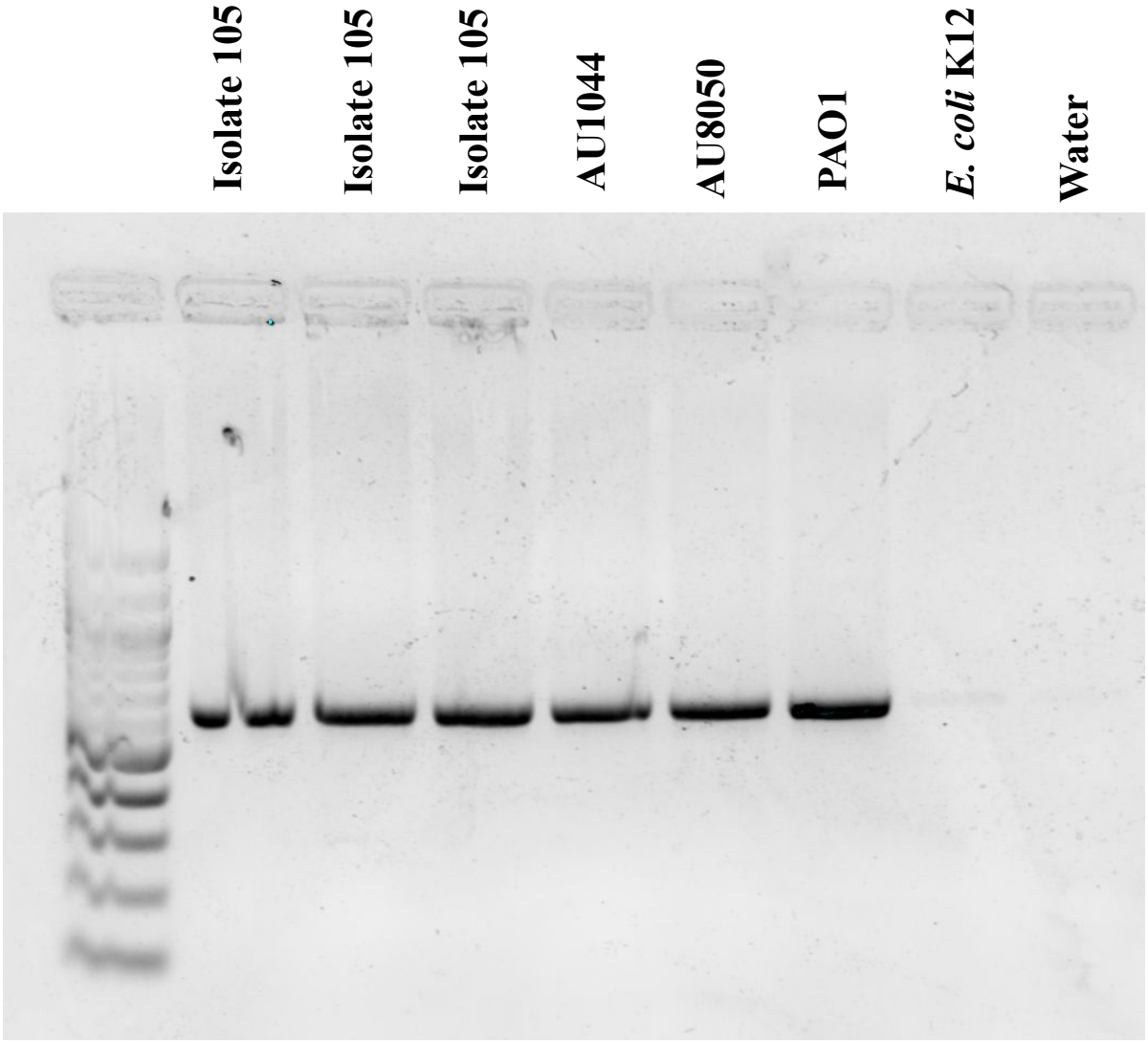

Figure 9: *Pseudomonas* genus specific PCR gel electrophoresis
PCR was carried out as outlined by the methods section.

AU1044 -- *P. lundensis* (Positive Control)
AU8050 -- *P. fluorescens* (Positive Control)
PAO1 -- *P. aeruginosa* (Positive Control)
E. coli K12 -- Negative Control
Water -- Negative Control

Isolate 101 (RT Broth)
P. aeruginosa Species Specific, 956 bp product

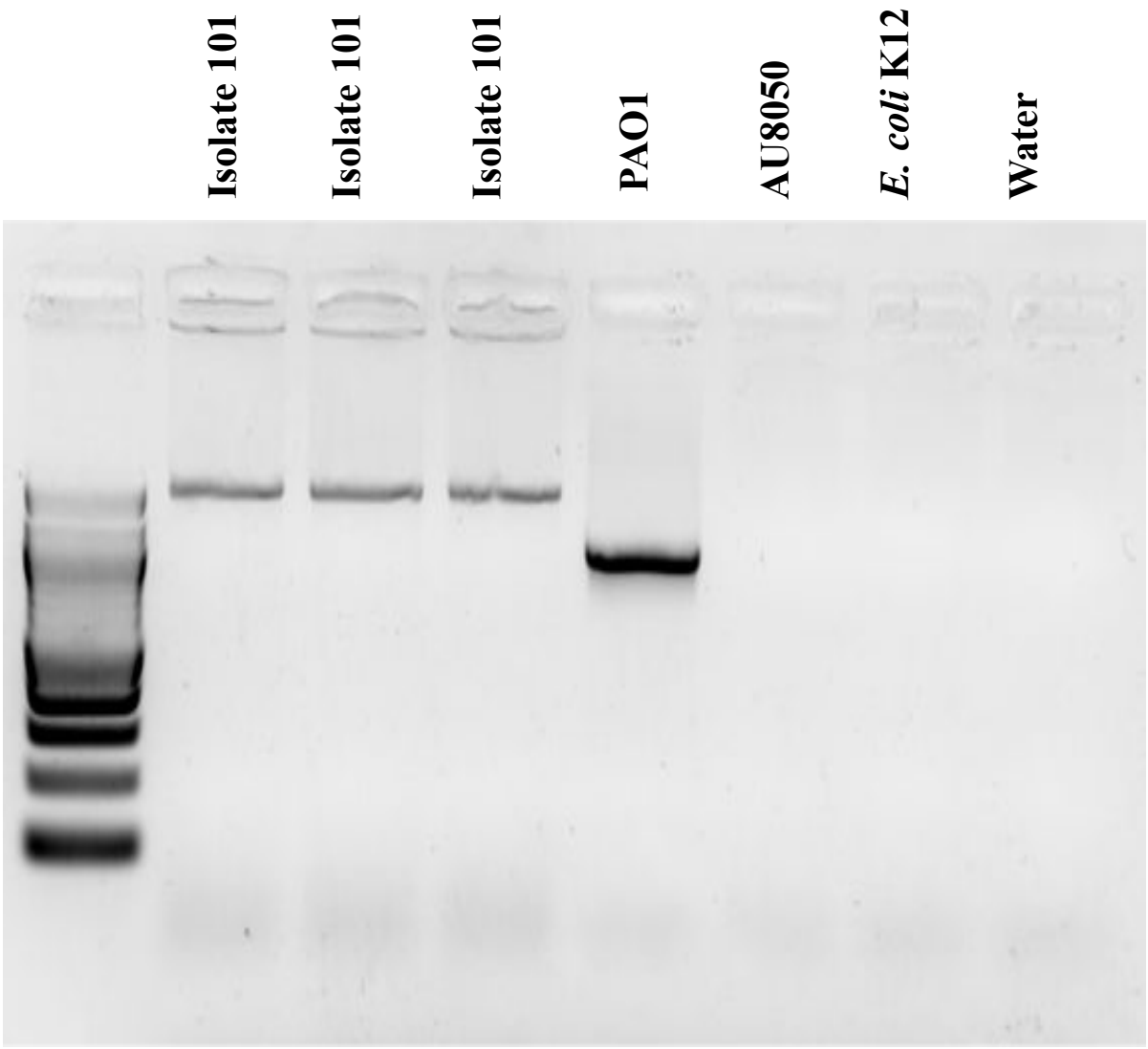

Figure 10: *P. aeruginosa* species specific PCR gel electrophoresis

PCR was carried out as outlined by the methods section.

PAO1 -- *P. aeruginosa* (Positive Control)
AU8050 -- *P. fluorescens* (Negative Control)
E. coli K12 -- Negative Control
Water -- Negative Control

Isolate 103 (RT Broth)
P. aeruginosa Species Specific, 956 bp product

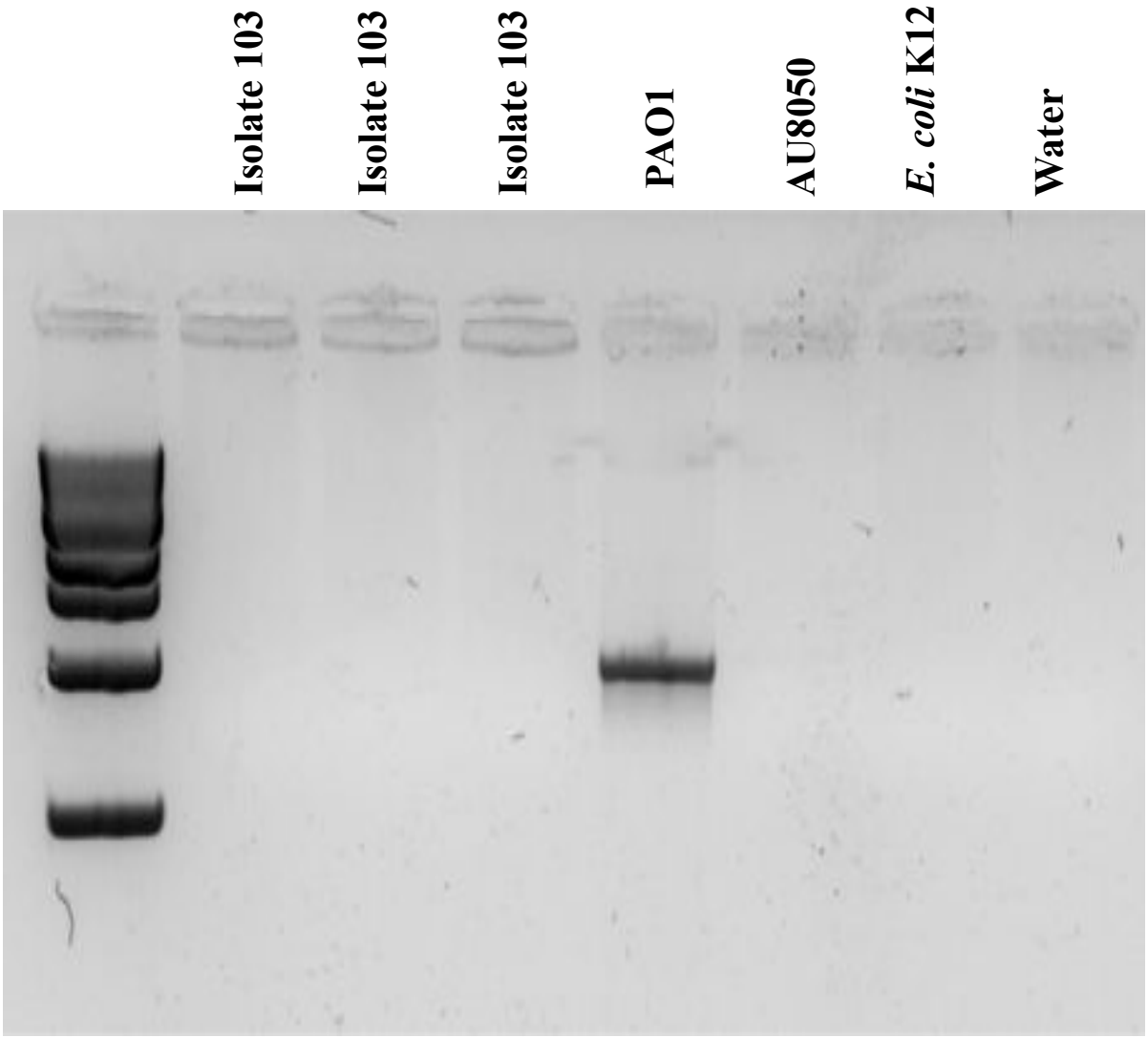

Figure 11: *P. aeruginosa* species specific PCR gel electrophoresis

PCR was carried out as outlined by the methods section.

PAO1 -- *P. aeruginosa* (Positive Control)
AU8050 -- *P. fluorescens* (Negative Control)
E. coli K12 -- Negative Control
Water -- Negative Control

Isolate 105 (RT Broth)
P. aeruginosa Species Specific, 956 bp product

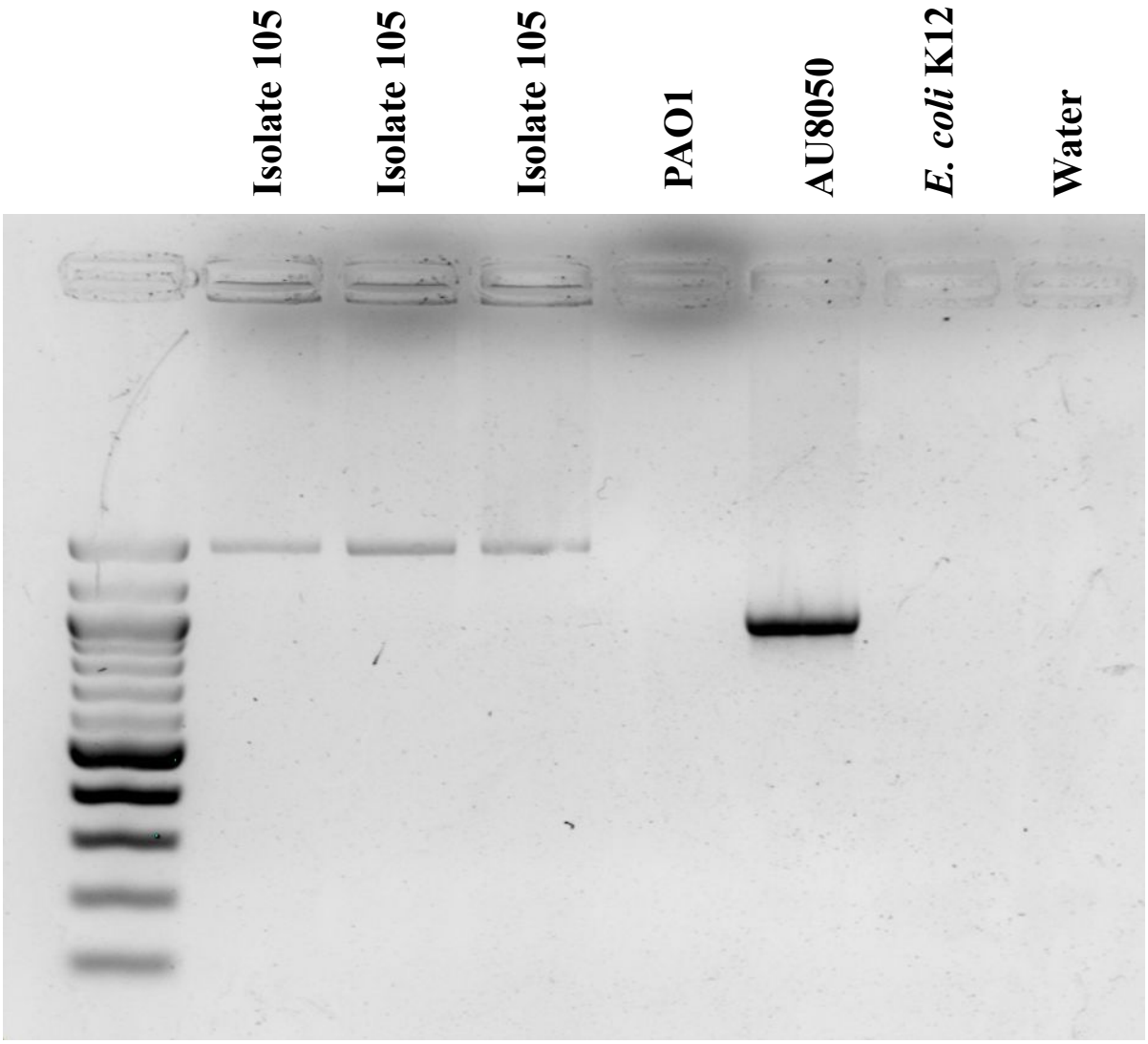

Figure 12: *P. aeruginosa* species specific PCR gel electrophoresis

PCR was carried out as outlined by the methods section.

PAO1 -- *P. aeruginosa* (Positive Control)
AU8050 -- *P. fluorescens* (Negative Control)
E. coli K12 -- Negative Control
Water -- Negative Control

3. I2 Specific PCR

I2 is a region that corresponds to a novel super antigen specific to the P. *fluorescens*-species complex (27). All isolates tested negative for I2 which means that none of the isolates belong to the P. *fluorescens*-species complex (Figures 13-15).

4. ExoU Specific PCR

ExoU codes for a phospholipase A that induces host cell damage and necrosis (20). While it is present in both *P. aeruginosa* and *P. lundensis* (20), our laboratory has created PCR primers specific to e*xoU* in *P. lundensis,* which do not amplify e*xoU* in *P. aeruginosa*. All isolates tested positive for ExoU which shows that they're extremely similar, if not identical to, *P. lundensis*. A faint band of smaller size shows up on each gel for *P. aeruginosa* PAO1 because its own *exoU* homolog was weakly amplified. (Figures 16-18).

Taken together, the results from all four *Pseudomonas* specific PCR protocols shows that the isolates are indeed part of the *Pseudomonas* genus, are not *P. aeruginosa* or *P. fluorescens*, and are extremely similar to *P. lundensis*. Presence of novel PA-SS banding exclusively shared between isolates 101 and 105 further differentiates isolate 103 (Figure 19).

H. Multi-genic Identification

Isolate identities were confirmed through 16S rRNA, *motA*, and *rpoB* gene homology (Figure 20); isolates 101 and 105 showed stronger evidence of being closely related *P. lundensis* strains to *P. lundensis* AU1044 than isolate 103. The dendrogram displays their relationship as a UPGMA tree (Figure 21).

I. Metabolic Systems

1. Respiration System

narX, narL, and *narG* were found in each isolate and *P. lundensis* AU1044, but did not meet the threshold for significant gene homology. NarL was the only protein out of the three previously mentioned to produce significant alignments, although they were only slightly above 50%. arcA and arcB were found in all isolates and *P. lundensis* AU1044. All showed similar gene and protein homology to *P. aeruginosa* PAO1, equal if not exceeding 80%. Across all subjects, there tended to be more significant alignments when it came to *arcB* and ArcB. FNR protein homology was greater than *fnr* gene homology for all isolates, milk and clinical. Taken together, the results tell us that the *P. lundensis* isolates may not be facultative anaerobes because of their weak *narL/X/G* gene and protein product homologies and strong *arcA/B/fnr* gene and protein product homologies (Figures 22-24).

2. Type III Secretion System

The type III secretion system homology data proved to be a bit confounding because of incongruous data for the *hrcC, hrcJ, hrcR, hrcS, hrcU,* and *hrcV* genes and their protein products. Their gene homology data was not significant enough to report but the protein sequences derived from the nucleotide sequences yielded significant alignments, which does not make sense and requires a follow up investigation (genes/proteins of interest are color-coded in Figure 27). This insufficiency encouraging looking into other well-identified genes in the type III secretion system: *escS, escV, yobB, yopR.* Like the originally selected genes, the workaround genes are part of the type III secretion system apparatus. The three milk isolates contained significant alignments for *escV and yopB*, but they were minimal. Searches in "tBLASTx" revealed proteins that are present and generally share homology, however there is not enough supporting data to suggest similar function. Function likely differs from the species investigated, as given by their distinctive environments (lungs and milk), but the proteins exist. To get a better idea of functionality, we'd have to find a well-studied type III secretion system specific to *P. lundensis*. Although in consideration of the data with what working knowledge we have, we have little reason to support the presence of a type III secretion system in the *P. lundensis* isolates (Figures 25-27).

Isolate 101 (RT Broth)
I2, 250 bp product

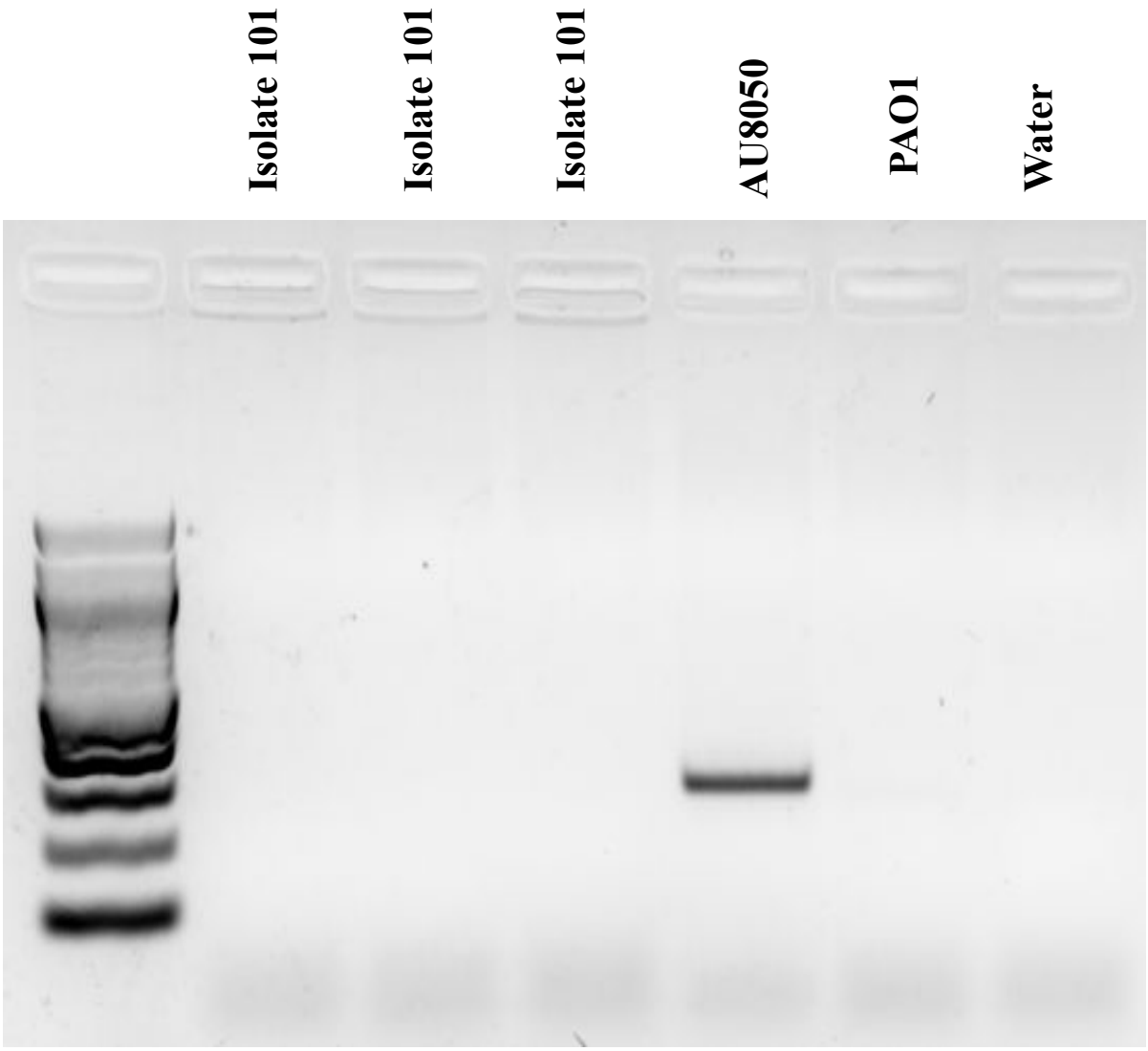

Figure 13: I2 specific PCR gel electrophoresis

PCR was carried out as outlined by the methods section.

AU8050 -- *P. fluorescens* (Positive Control)
PAO1 -- *P. aeruginosa* (Negative Control)
Water -- Negative Control

Isolate 103 (RT Broth)
I2, 250 bp product

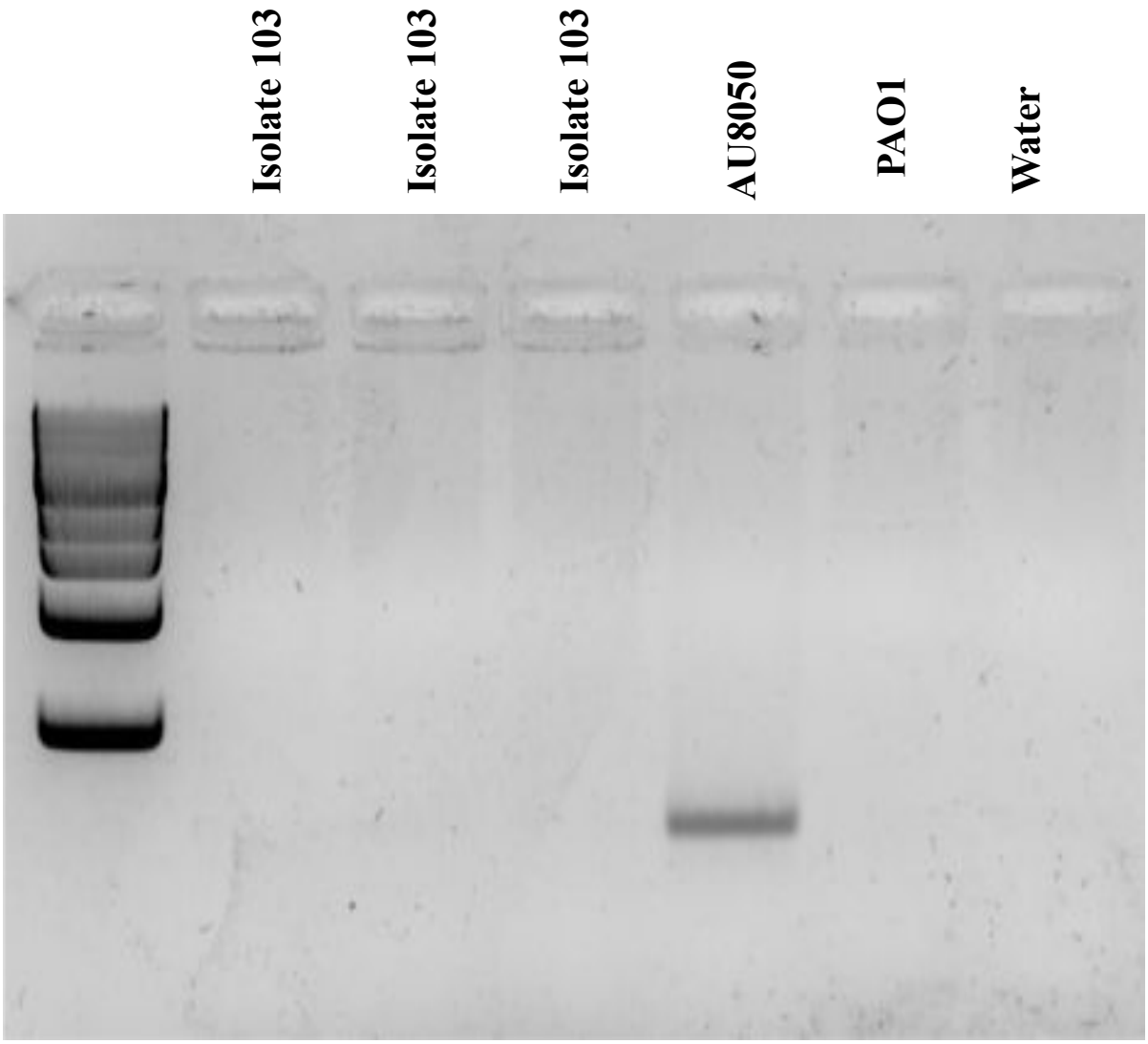

Figure 14: I2 specific PCR gel electrophoresis

PCR was carried out as outlined by the methods section.

AU8050 -- *P. fluorescens* (Positive Control)
PAO1 -- *P. aeruginosa* (Negative Control)
Water -- Negative Control

Isolate 105 (RT Broth)
I2, 250 bp product

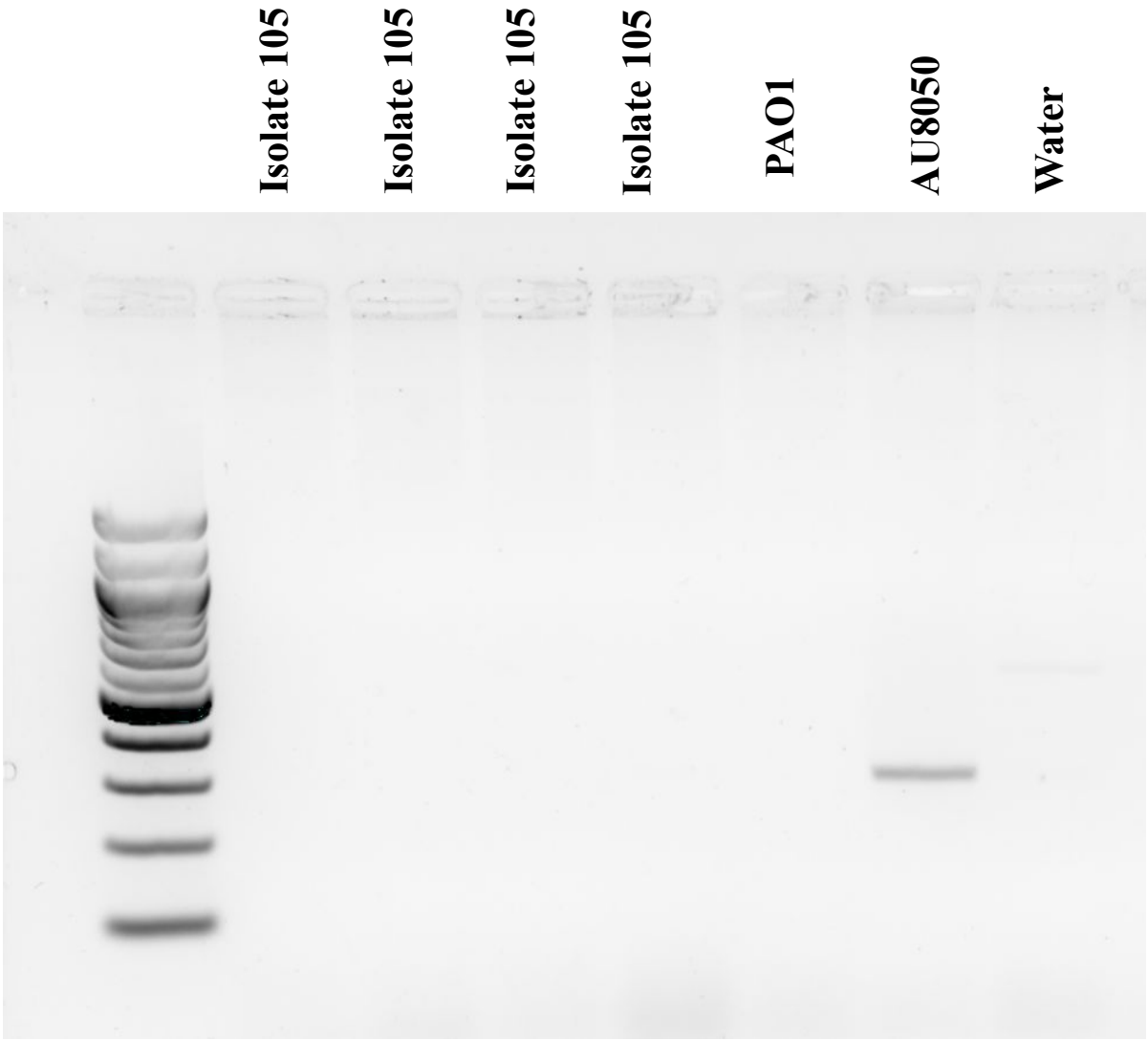

Figure 15: I2 specific PCR gel electrophoresis

PCR was carried out as outlined by the methods section.

AU8050 -- *P. fluorescens* (Positive Control)
PAO1 -- *P. aeruginosa* (Negative Control)
Water -- Negative Control

Isolate 101 (RT Broth)
P. lundensis ExoU, 494 bp product

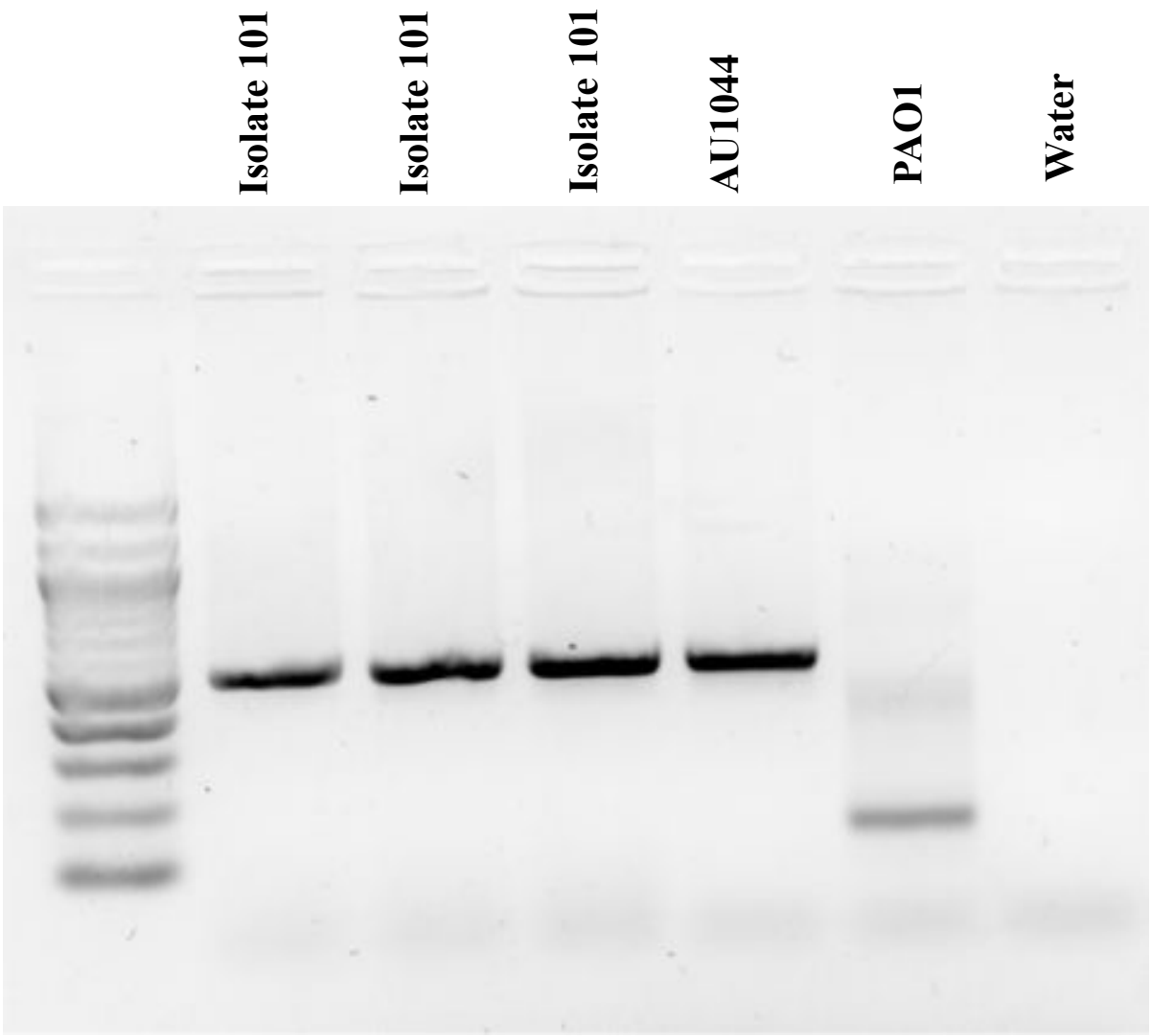

Figure 16: *P. lundensis* ExoU specific PCR gel electrophoresis

PCR was carried out as outlined by the methods section.

AU1044 -- *P. lundensis* (Positive Control)
PAO1 -- *P. aeruginosa* (Negative Control)
Water -- Negative Control

Isolate 103 (RT Broth)
P. lundensis ExoU, 494 bp product

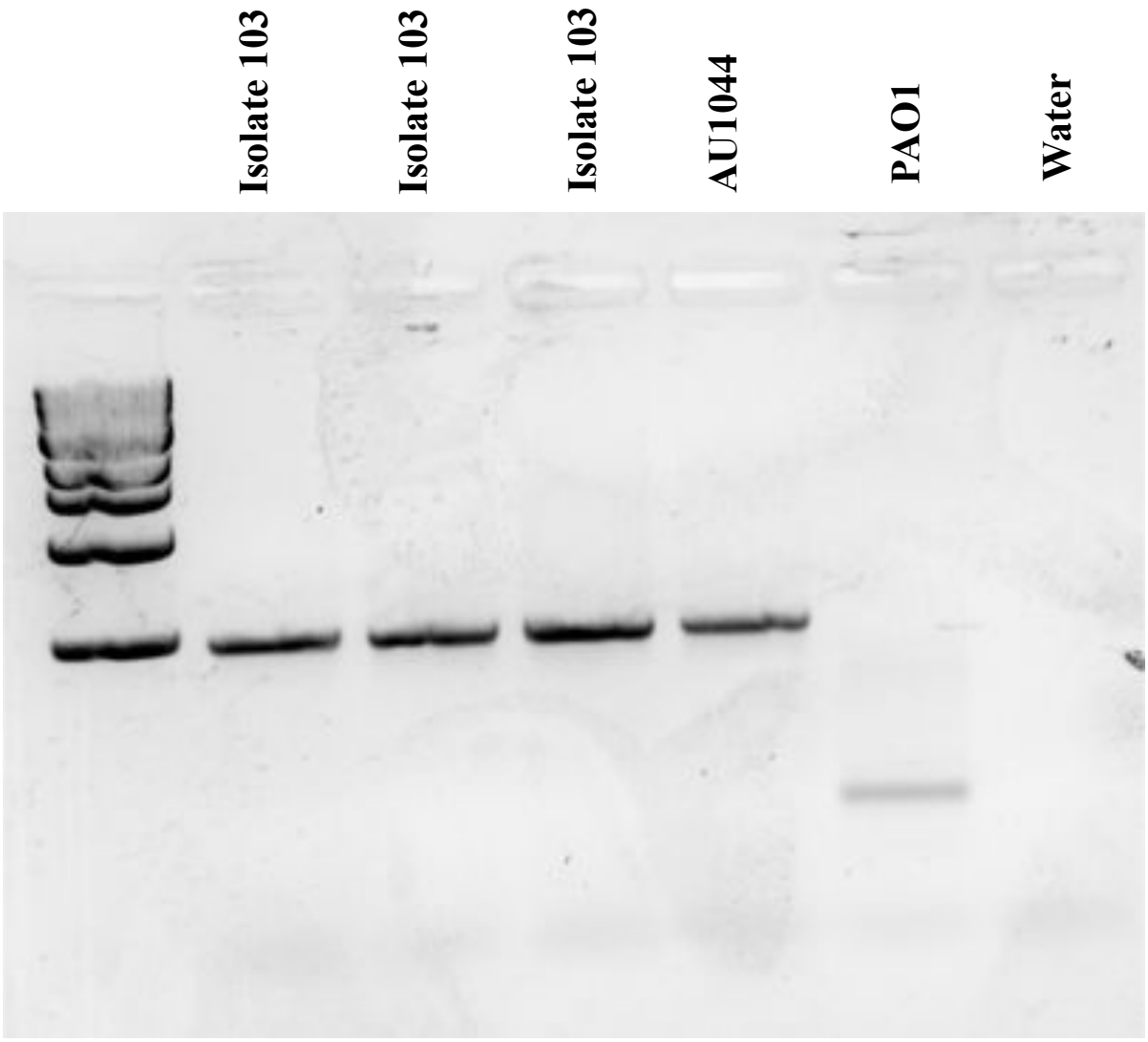

Figure 17: *P. lundensis* ExoU specific PCR gel electrophoresis

PCR was carried out as outlined by the methods section.

AU1044 -- *P. lundensis* (Positive Control)
PAO1 -- *P. aeruginosa* (Negative Control)
Water -- Negative Control

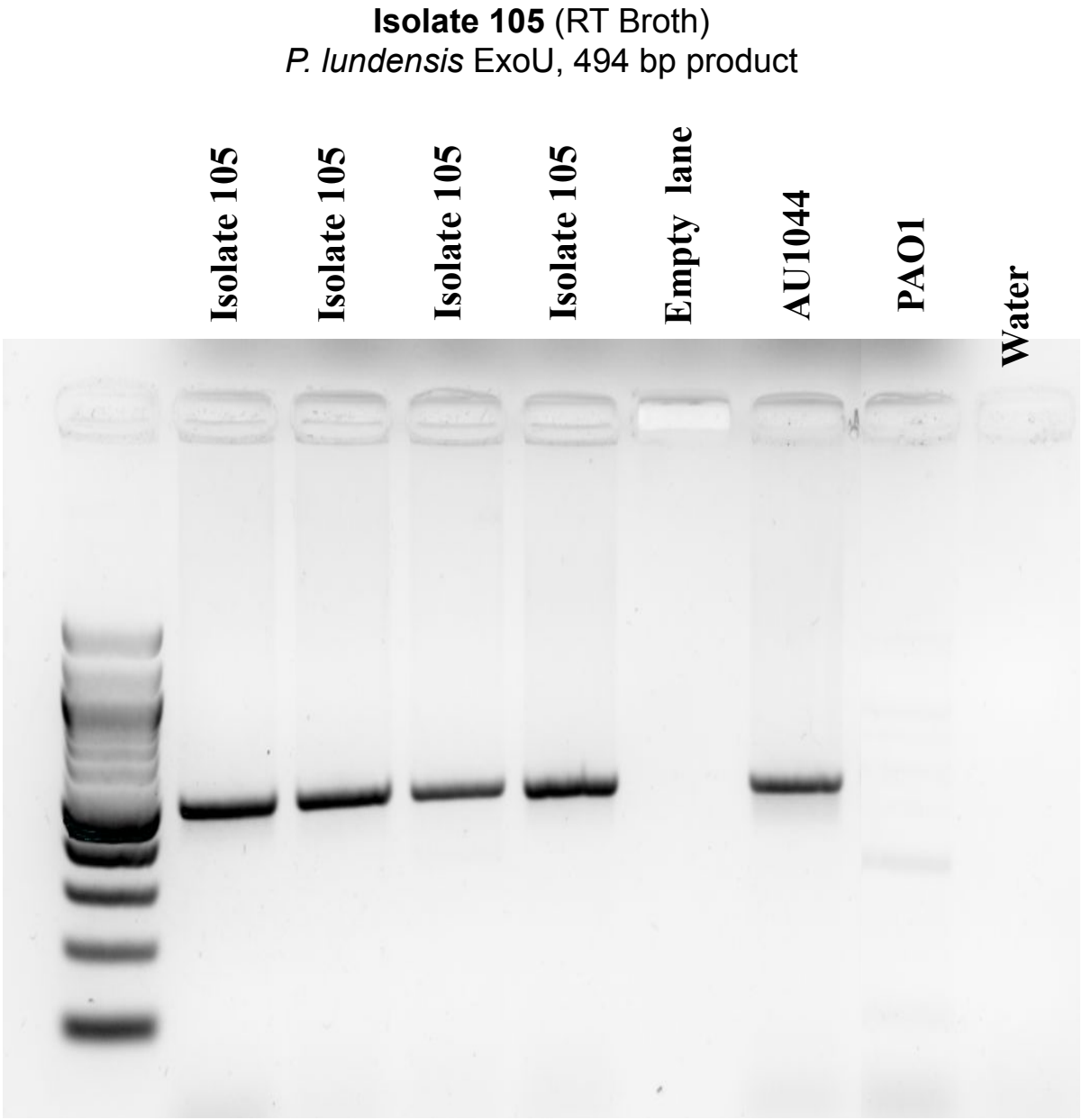

Figure 18: *P. lundensis* ExoU specific PCR gel electrophoresis

PCR was carried out as outlined by the methods section.

AU1044 -- *P. lundensis* (Positive Control)
PAO1 -- *P. aeruginosa* (Negative Control)
Water -- Negative Control

	P-GS	PA-SS	PA-SS novel band	I2	ExoU
101	+	-	+	-	+
	+	-	+	-	+
	+	-	+	-	+
103	+	-	-	-	+
	+	-	-	-	+
	+	-	-	-	+
105	+	-	+	-	+
	+	-	+	-	+
	+	-	+	-	+

+: bands present at corresponding positive control(s)
-: no bands present at corresponding positive control(s)

Figure 19: Summary of PCR Gel Electrophoresis Imaging

Species Name	101	103	105	K12	PAO1	L48	A506	SBW25	AU1044	KT2440	DSM10701	DC3000
Isolate 101	100	99.14	99.94	86.52	84.06	85.67	89.47	89.37	99.83	85.57	83.07	87.93
Isolate 103		100	99.14	85.81	83.84	85.44	89.13	88.98	98.97	85.32	82.78	87.53
Isolate 105			100	86.52	84.06	85.67	89.49	89.4	99.83	85.57	83.07	88
E. coli K12				100	85.99	86.19	86.8	86.58	86.67	86.39	86.73	86.11
P. aeruginosa PAO1					100	89.65	86.88	86.26	84.08	88.88	89.04	85.48
P. entomophila L48						100	88.76	88.44	85.71	94.71	84.43	87.47
P. flurescens A506							100	95.54	89.59	88.64	85.25	89.55
P. flurescens SBW25								100	89.49	88	84.92	89.01
P. lundensis AU1044									100	85.61	83.07	88.05
P. putida KT2440										100	86.76	87.41
P. stutzeri DSM10701											100	84.89
P. syringae DC3000												100

Figure 20: Distance matrix for UPGMA Dendrogram

Multi-genic identification began with searching for annotated genes for *motA*, *rpoB*, and 16S rRNA for each strain on NCBI. *P. aeruginosa* PAO1 was selected to be the base comparison for finding genes in each isolate using the “blastn” function on NCBI’s Basic Local Alignment Search Tool (BLAST). Concatenated files of *motA*, *rpoB*, and 16S rRNA were created for each strain, then combined into a larger concatenated file of multi-genic sequence containing all strains. Using data from “blastn” searches, relative gene identities between all strains were determined and put into a similarity matrix. A distance matrix was generated from the similarity matrix to inform unweighted pair group method with arithmetic (UPGMA).

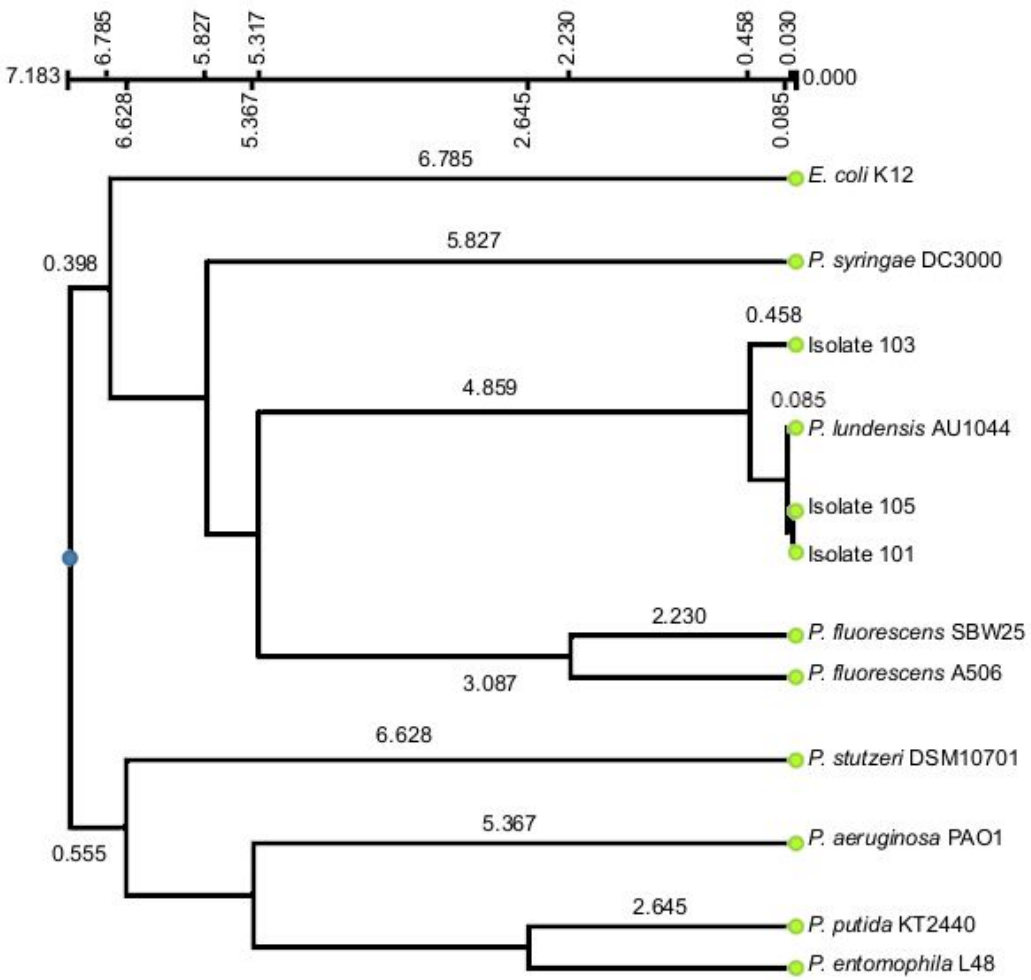

Figure 21: Dendrogram of *Pseudomonas spp.*, *E.coli* outgroup, and three isolates

Gene distance established using multigenic analysis using *rpoB, motA* and 16S rRNA. The concatenated nucleotide sequence files were analyzed via BLASTn search. Tree was generated from distance matrix (shown in Figure 20) using DendroUPGMA. Distance is shown as percent difference.

Description of Genes in Respiration System		
Gene	Product	Function
narL	nitrate/nitrite response regulator protein (NarL)	Activated in response to a signal from NarX; activates expression of narGHJI operon and other anaerobic respiratory genes
narX	nitrate/nitrite sensor protein (NarX)	Senses nitrate/nitrite in the environment and activates NarL by phosphorylation
narG	Respiratory nitrate reductase 1 alpha chain (NarG)	Reductase enzyme complex allows for nitrate to be used as an electron acceptor during anaerobic growth; alpha chain is site of reduction
arcA	Aerobic respiration control protein (ArcA)	Represses aerobic enzymes under anaerobic conditions; activated by ArcB
arcB	Aerobic respiration control sensor protein (ArcB)	Senses anaerobic conditions and activates ArcA by phosphorylation

Figure 22: Description of genes in respiration system

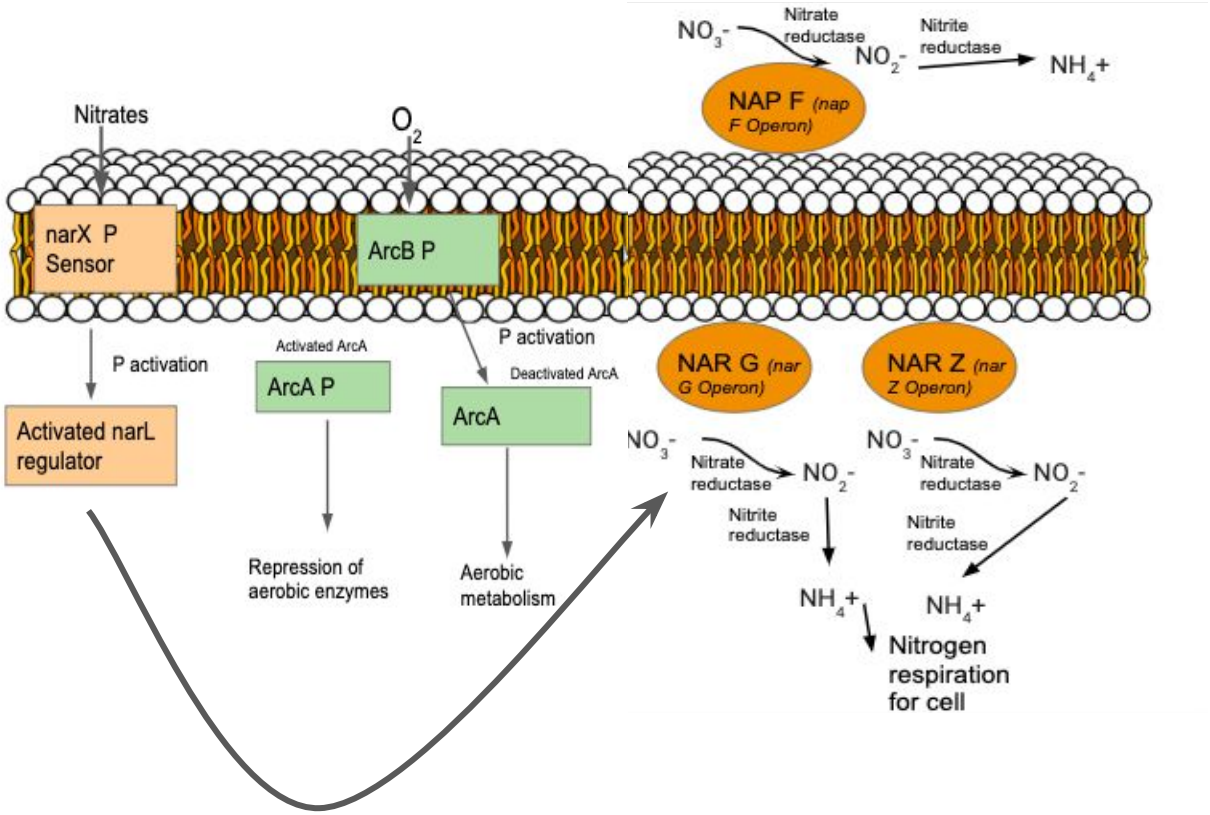

Figure 23: Diagram of respiration system showing respective roles of each selected gene/protein product

Query Origin	*P. lundensis* AU1044	Isolate 101	Isolate 103	Isolate 105
***P. aeruginosa* PAO1**				
arcA	80.4	80.58	80.11	80.57
arcB	82.06	82.1	81.48	82.1
fnr	80.06	80.06	78.71	80.06
narX	N/A	N/A	N/A	N/A
narL	N/A	N/A	N/A	N/A
narG	N/A	N/A	N/A	N/A
ArcA	81.58	81.58	81.01	84.80
ArcB	85.42	85.55	89.05	85.87
FNR	87.76	87.76	86.49	87.76
NarX	N/A	N/A	N/A	N/A
NarL	57.21	63.47	53.97	63.47
NarG	N/A	N/A	N/A	N/A

Figure 24: Gene and Protein Homology for Respiration System
This table shows the percent identity of genes as compared to a query “positive” using BLASTn searches and the protein homology (positives) in percentage, as compared to a query “positive” using tBLASTx searches. “N/A” cells indicated insufficient E-values ($>1^{-10}$).

Description of Genes in Type III Secretion System		
Gene	Product	Function
hrcC	T3SS secretin (YscC)	Building blocks for T3SS membrane structure
hrcJ	T3SS inner MS ring protein (YscJ)	Structural part of the T3SS ring structure extending into periplasm
hrcR	T3SS minor export apparatus protein (YscR)	Transmembrane proteins necessary for successful secretion
hrcS	T3SS minor export apparatus protein (YscS)	Transmembrane proteins necessary for successful secretion
hrcU	T3SS export apparatus switch protein (YscU)	Switches export apparatus focus from assembly and structure of hook to assembly of filament
hrcV	T3SS major export apparatus protein (YscV)	Cytosolic proteins necessary for successful secretion

Figure 25: Description of genes in type III secretory system

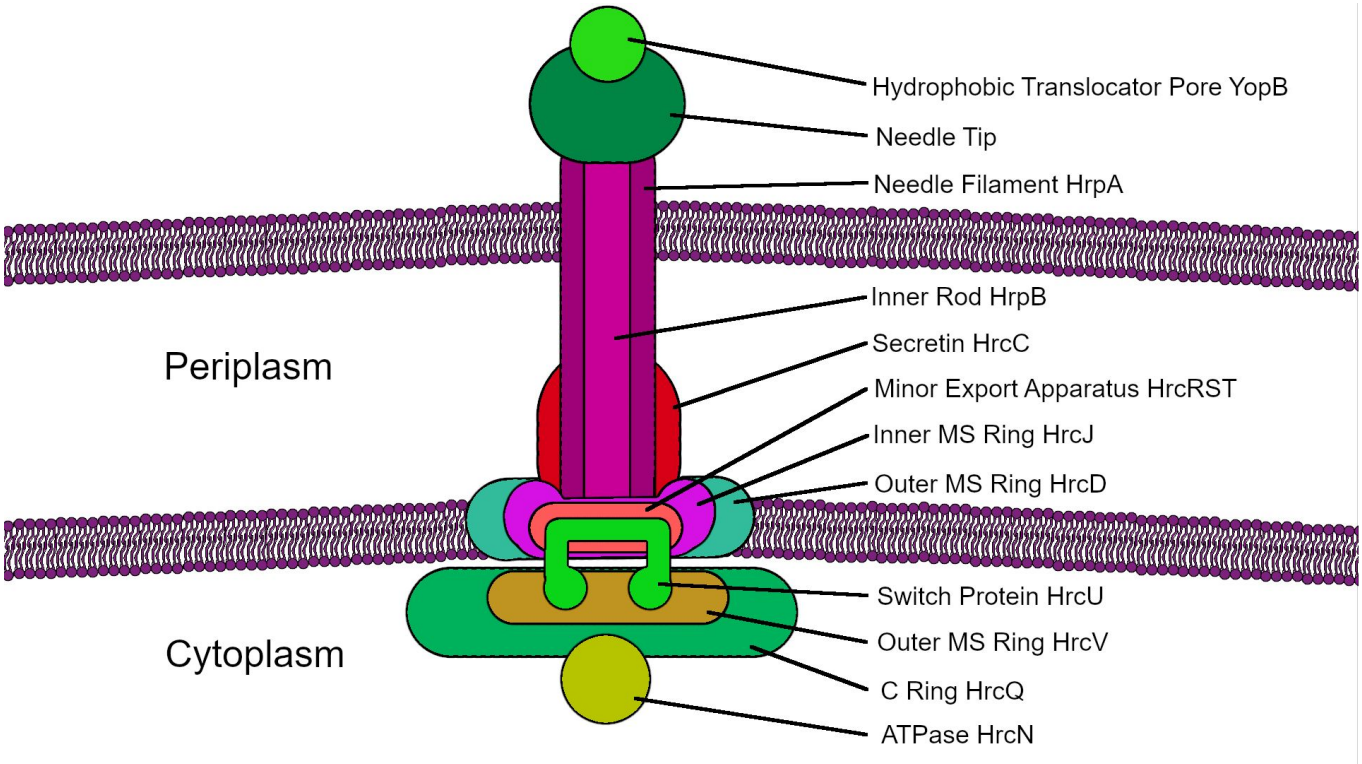

Figure 26: Diagram of type III secretory system showing respective roles of each selected gene/protein product

Query Origin	*P. lundensis* AU1044	Isolate 101	Isolate 103	Isolate 105
***P. syringae* DC3000**				
hrcC	N/A	N/A	N/A	N/A
hrcJ	68.91	68.91	68.39	68.91
hrcR	75.93	75.93	75.93	75.93
hrcS	N/A	N/A	N/A	N/A
hrcU	N/A	N/A	N/A	N/A
hrcV	N/A	N/A	N/A	N/A
YrcC	52	60	59	59
YrcJ	67	67	67	67
YrcR	69	65	69	65
YrcS	61	61	60	61
YrcU	61	61	60	61
YrcV	61	61	60	61
S. flexneri				
escS	N/A	N/A	N/A	N/A
escV	75.76	75.76	N/A	75.76
EscV	85	80	91	91
Y. enterocolitica 8081				
yopB	69.74	81.82	N/A	81.82
YopB	67	67	65	67
Y. pseudotuberculosis				
yopR	N/A	N/A	N/A	N/A
YopB	N/A	N/A	N/A	N/A

Figure 27: Gene and Protein Homology for T3SS System

This table shows the percent identity of genes as compared to a query "positive" using BLASTn searches and the protein homology (positives) in percentage, as compared to a query "positive" using tBLASTx searches. "N/A" cells indicated insufficient E-values ($>1^{-10}$). Only one showed sufficient homology to suggest shared function (EscV), while the remaining proteins that were present suggested only that they had related structure. Highlighted cells require follow up work due to inconsistent homology.

3. Quorum Sensing and Biofilm Formation

lasR and *rhlR* were absent from all isolates and *P. lundensis* AU1044. *rhlA* had limitations: it was only found in isolate 101 and 105. The results induce some skepticism because the gene homology consistently tended to be slightly greater than the protein homology. The same gene might show up with slight variation in different organisms, but the product, assuming the same protein, should be stronger proof of homology. Absence of *lasR* and *rhlR* combined with the presence of *rhlA* could mean that isolates 101 and 105 have a quorum sensing system without a regulator using PAI-2 and not PAI-1.

Although *mucA, algD,* and *ndvB* genes were found in all isolates and *P. lundensis* AU1044, MucA and AlgD were the only proteins to exist beyond that. *algD* had high gene similarity values between the unknown isolates and *P. aeruginosa* PAO1, but the *P. aeruginosa* PAO1 and *P. lundensis* AU1044 were less similar. The *mucA* and *ndvB* genes were present in all strains at relatively the same similarity (76%), but the NdvB protein was concluded missing with >1% query coverage. AlgD had 80% similarity to *P. aeruginosa* PAO1, but the MucA protein showed less homology at 63%. This supports basic genetic infrastructure for *Pseudomonas* biofilm formation, however absence of NdvB protein suggests lack of antibiotic resistance activity by the strains in the study (Figures 28-31).

VI. DISCUSSION AND CONCLUSION

The objective for this study was to characterize three *Pseudomonas* strains isolated from milk and determine their similarity to *P. lundensis* AU1044, a strain isolated from a cystic fibrosis patient, in order to establish the feasibility that milk could be an environmental source of *P. lundensis* in the lungs of humans with respiratory disease.

Phenotypically and genotypically, the results suggest that isolate 103 is distinct from isolates 101 and 105. This was observed as early as the 48 hour broth cultures. Regarding the 37°C growth, isolate 103's broth color was quite green, which deviated from the typical yellow hue of the other isolates. Gram staining ruled out the possibility of noteworthy contamination since all isolates were deemed Gram-negative bacilli. CFU plating showed that 101 and 105 shared the same temperature preference for optimal growth (RT) while 103 did not (37°C), further separating it. Colony PCR gave the first genomic data supporting isolate 103's distinction from 101 and 105. From 16S rRNA amplification, 101 and 105 were identified as a *P. lundensis* strain closely related to *P. lundensis* AU1044, *P. lundensis* DSM6265. 103 was identified as a few *Pseudomonas* members. While none of the top taxon or strain hits were *P. lundensis*, the heterogeneous data suggests that 103 is similar to a variety of *Pseudomonas* or perhaps the amplified sequence wasn't good enough to get the best comparison data. The several *Pseudomonas* hits could also be attributed to 103's original isolation, where undetectable phenotypic differences may have confounded establishing a single *Pseudomonas* identity.

In specific comparison to *P. lundensis* AU1044 16S rRNA, all of the isolate PCR products exhibited high homology. As a result of that, another identification measure was employed to construct a more comprehensive relationship framework around the unknown milk isolates and *P. lundensis* AU1044. From the multi-genic identification work using concatenated files of 16S rRNA, *motA*, and *rpoB*, isolate 103 proved to be distinct from the other milk isolates which keeps in line with the data thus far. The UPGMA-informed dendrogram shows isolate 101, isolate 105, and *P. lundensis* AU1044 sharing the same first common ancestor. Isolate 103 diverges slightly from the common ancestor that 101, 105, and *P. lundensis* AU1044 share. Taken together, these results bring 101 and 105 closer together in relation to P. *lundensis* AU1044 and further confirm 103 as different from the rest.

Gene and protein homologies were utilized to develop hypotheses about the range of metabolic niches that the *Pseudomonas* milk isolates can occupy through analyzing essential metabolic systems in respiration, type III secretion, and quorum sensing in biofilm formation. Isolate 103 showed slightly different gene and protein homology values across the three selected systems suggesting marginal differences in niche biology as compared to the other milk isolates. In the respiration scheme, weak narL, narG, and narX gene and protein homologies were observed in contrast to strong arcA, arcB, and fnr gene and protein homologies. Presence of a weak nitrate reduction system but strong oxygen sensing system suggests that the *P. lundensis* isolates are poor facultative anaerobes because they

Description of Genes in Quorum Sensing/Biofilm Formation System		
Gene	Product	Function
mucA	AlgU sigma factor regulatory protein	Activated *mucA* causes switch from mucoid to non-mucoid state resulting in repression of alginate biosynthetic genes
algD	GDP-mannose-6-dehydrogenase	Processes precursor for alginate; alginate is essential for *Pseudomonas* biofilm formation
lasR	Transcriptional activator protein (LasR)	Directs synthesis of autoinducer PAI-1
rhlR	Regulatory protein (RhlR)	Necessary for transcriptional activation of *rhlAB* encoding the rhamnosyltransferase
rhlA	3-(3-hydroxydecanoyloxy)decanoate synthase (RhlA)	Required for rhamnolipid surfactant production essential for directing synthesis of autoinducer PAI-2
ndvB	Produces NdvB which has been recently found to confer antibiotic resistance in *Pseudomonas* species	Required for synthesis of periplasmic glucans that prevent antibiotics from reaching their targets

Figure 28: Description of genes in quorum sensing and biofilm formation systems

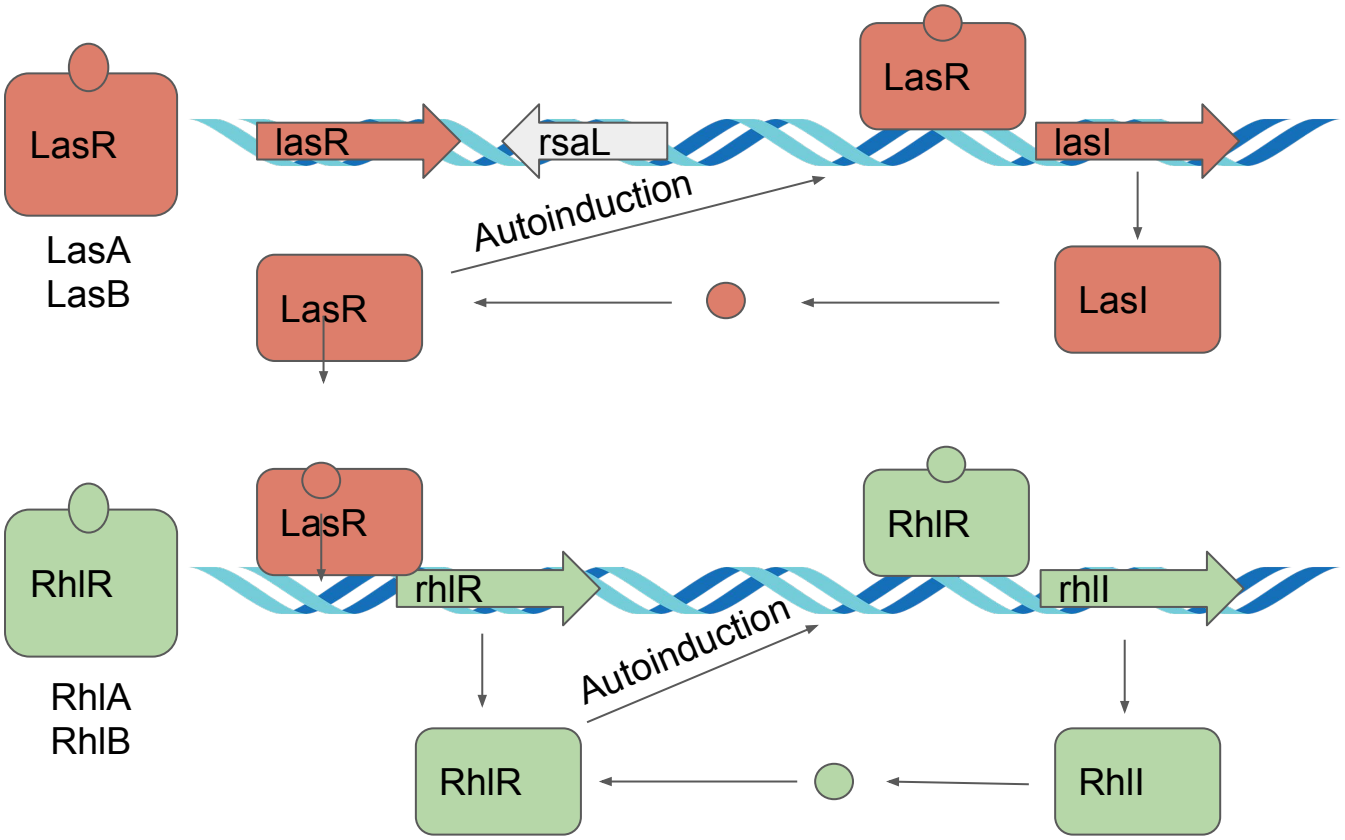

Figure 29: Diagram of selected virulence factors in quorum sensing system

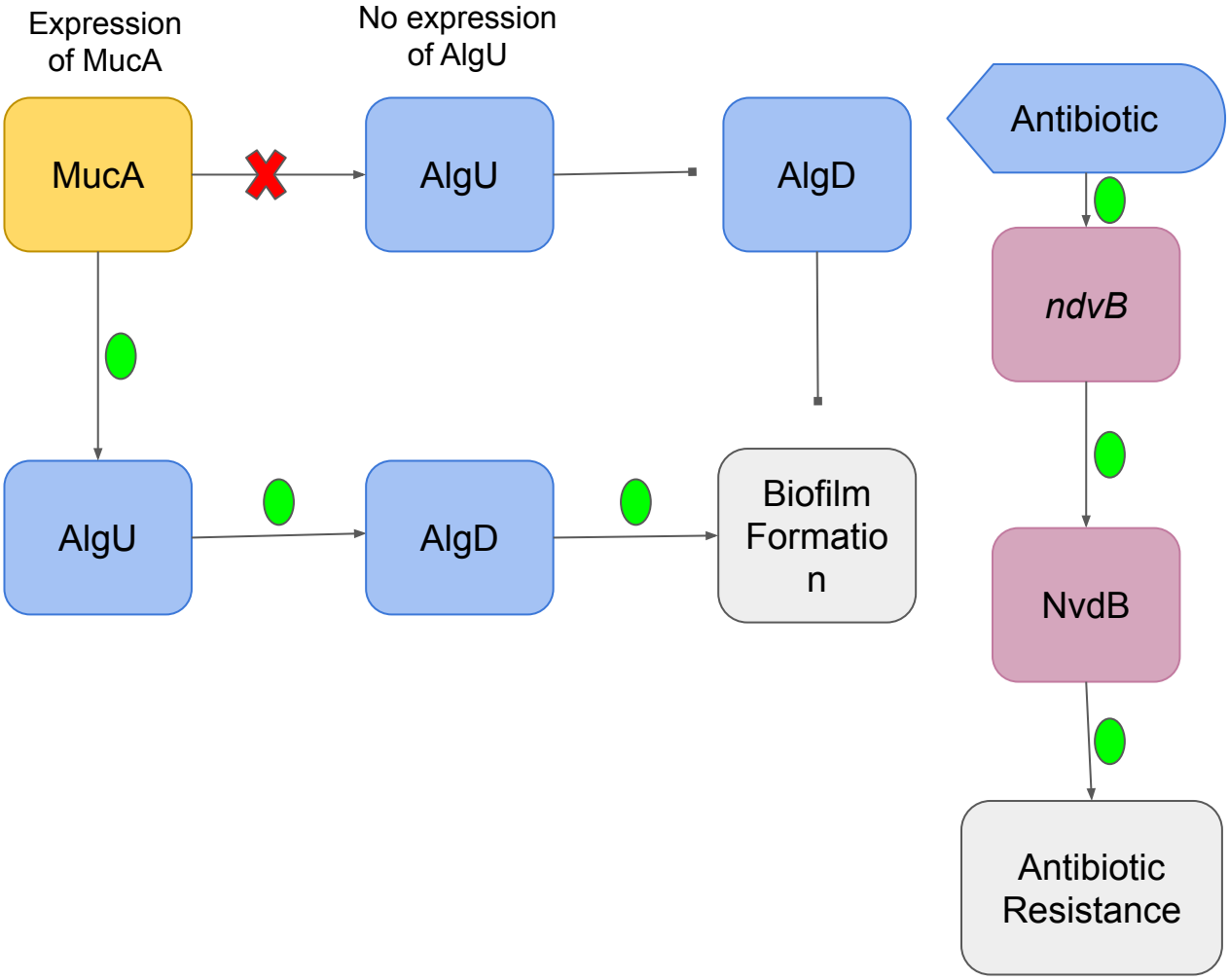

Figure 30: Diagram of biofilm formation system showing respective roles of each selected gene/protein product

Query Origin	*P. lundensis* AU1044	Isolate 101	Isolate 103	Isolate 105
***P. aeruginosa* PAO1**				
lasR	N/A	N/A	N/A	N/A
rhlR	N/A	N/A	N/A	N/A
rhlA	N/A	71.9	N/A	72.5
mucA	70.74	78.48	77.67	78.62
algD	76.77	99.4	98.63	99.57
ndvB	76.77	76.77	76.77	76.77
LasR	N/A	N/A	N/A	N/A
RhlR	N/A	N/A	N/A	N/A
RhlA	N/A	63.16	N/A	72.22
MucA	64.47	60.87	63.73	64.47
AlgD	78.72	81.48	81.18	81.18
NdvB	N/A	N/A	N/A	N/A

Figure 31: Gene and Protein Homology for Quorum Sensing/Biofilm Formation System

This table shows the percent identity of genes as compared to a query “positive” using BLASTn searches and the protein homology (positives) in percentage, as compared to a query “positive” using tBLASTx searches. “N/A” cells indicated insufficient E-values ($>1^{-10}$).

Gene/Organism	ID/ Location
***Pseudomonas lundensis* AU1044**	NZ_CP017687.1
rpoB	1673967-1679262
motA	2788051-2789157
16S rRNA	2393515-2395050
***Pseudomonas aeruginosa* PAO1**	NC_002516.2
arcA	5822380-5823636
arcB	5823716-5824726
fnr	1681071-1681805
narX	4345010-4346878
narL	4346875-4347534
narG	4338261-4342046
lasR	1558171-1558890
rhlR	3889925-3890650
rhlA	3892121-3893008
mucA	831826-832585
algD	3962628-3964331
ndvB	1260165-1263557
rpoB	c4780617-4776544
motA	c5559872-5559021
16S rRNA	722096-723631
***Pseudomonas syringae* DC3000**	NC_004578.1
hrcC	1528890-1530989
hrcJ	1526079-1527127
hrcR	1534238-1534840
hrcS	c1534230-1533964
hrcU	c1534230-1533964
hrcV	c1534230-1533964
rpoB	678489-682562
motA	2170575-2171315
16S rRNA	666727-668265

Gene/Organism	ID/ Location
Shigella flexneri	NZ_CP033511.1
escS	36303-36563
escV	29692-31752
***Yersinia enterocolitica* 8081**	NC_008791.1
yopB	c11798-10593
Yersinia pseudotuberculosis	NZ_CP032567.1
yopR	56811-57308
***Pseudomonas stutzeri* DSM10701**	NC_018177.1
rpoB	c3683992-3679922
motA	204626-205498
16S rRNA	679691-681226
***Pseudomonas putida* KT2440**	NC_002947.4
rpoB	537623-541696
motA	c5576148-5575297
16S rRNA	524934-526470
***Pseudomonas fluorescens* SBW25**	NC_012660.1
rpoB	6057880-6061953
motA	577958-578809
16S rRNA	119719-121255
***Pseudomonas fluorescens* A506**	NC_017911.1
rpoB	5307093-5311166
motA	574331-575182
16S rRNA	4478412-4479948
***Pseudomonas entomophila* L48**	NC_008027.1
rpoB	502974-507047
motA	c5262236-5261385
16S rRNA	115492-117028
***Escherichia coli* K12**	NC_000913.3
rpoB	4181245-4185273
motA	c1977139-1976252
16S rRNA	4166659-4168200

Figure 32: Gene ID and location numbers from BLAST

require greater oxygen availability to survive. In an aerobic environment, there is no need for nitrate-reducing capability. Type III secretion systems were genetically identified in all the *Pseudomonas* isolates, however their homology was poor. This finding encouraged the question of whether there's a different function of the protein products aside from what we know about the highly conserved elements. Without an existing model to represent type III secretion in *P. lundensis*, no hard and fast conclusions can be made about what the presence of a different type III secretion system might imply other than stating that there is no virulence benefit through these means. In quorum sensing analysis, absence of *lasR* and *rhlR* combined with the presence of *rhlA* could mean that isolates 101 and 105 have a quorum sensing system without a regulator using PAI-2 and not PAI-1. For biofilm formation, there appears to be basic genetic infrastructure for *Pseudomonas* biofilm formation, as given by mucA and algD, however absence of NdvB protein suggests lack of antibiotic resistance activity by the strains in the study.

The results from each system's gene and protein analysis suggest that the milk isolates and clinical isolate are not strong facultative anaerobes and likely do not contain a type III secretion system, but they can produce biofilm via a PAI-2 quorum sensing pathway without antibiotic resistance. These findings imply that their preferred environment is one with available oxygen, they grow effectively via biofilm formation, and their virulence capabilities are limited by the absence of a type III secretion system.

This study has demonstrated strong similarity between clinical isolate *P. lundensis* AU1044 and *Pseudomonas* milk isolates 101 and 105, which establishes the feasibility that milk could be an environmental source of *P. lundensis* in the lungs of humans with respiratory disease. Genetic identification analysis and the dendrogram are the strongest pieces of evidence that the clinical isolate and milk isolates (only 101 and 105) share environmental commonalities. Isolate 103, although similar to *P. lundensis* AU1044 according to the dendrogram, is not the same. A future direction for this study could be expansion on milk isolate 103. Additional genomic analysis against *P. fragi*, the next closest *Pseudomonas* species to *P. lundensis*, could help solidly identify isolate 103 and accurately characterize it among the other isolates. Other studies concerned with oral and respiratory colonization routes could also be referenced to develop a working model for *P. lundensis* lung colonization and its permanence. Depending on the outcome of that work, aggressive examination of cystic fibrosis causality from specific bacterial colonization could shed light on potential routes for preventative treatment action.

VII. ACKNOWLEDGEMENTS

The completion of this thesis would not have been possible without the support of the following people.

My deepest thank you to **Dr. Gary Huffnagle, Ph.D**, for being a leader amidst an unprecedented global pandemic. He has had a decent stake in my academic career here at Michigan as my degree advisor, professor, and principal investigator. In our time working together, he has shown me how powerful the combination of creativity and practicality can be. I extend a great deal of gratitude to **Nicole Falkowski** for her incredible mentorship, oversight, and support over the course of this project. Her careful eye and attention to detail were critical for this thesis to take flight. I am also grateful for her patience with me. I had the pleasure of working with **Dr. Kathryn Nawrocki, Ph. D.**, at the start of what I thought was my thesis. She was instrumental in showing me how to tackle complex scientific problems with humor and reasonable expectations. I would like to express my appreciation to my readers: **Dr. John Erb-Downward, Ph.D**, and **Dr. Tom Moore, Ph. D**. Thank you for taking time out of your busy lives to read my work and be an important part of my degree fulfillment. An extra thanks to Dr. E for letting me complete the preliminary framework for this thesis in his classroom. By introducing advanced caveats and considerations early on, he improved the reported work on multi-genic analysis and gene/protein homologies.

I'd like to thank **Mario Ficaro** for her technical help in data collection and **Natalie Thompson** for her thesis work involving the original isolation of each of the unknown milk isolates. I'd also like to recognize **Spenser Jett**, **Tristan Murray**, **Jack Eichman**, **Alexis Brewster**, and **Katherine McCollum** for their collaborative efforts to analyze the results from multi-genic identification and metabolic gene and protein homologies with me.

As I move into personal territory, I'd like to first thank **Nicole Haggerty** for being my right hand woman. With all the roles she juggles, she always manages to make time for our friendship. My time at Michigan would not have been the same without her. **Sophie Allen**, **Micky Esteban**, and **Cailin Ferguson**: thank you for being the first female-only support system I've ever had. You all have been the ones to empower me at moments when I needed it the most. I am also grateful to **Yoshiko Iwai** for her unwavering support from near and far. In our ten years of knowing each other, her limitless dedication and drive have always inspired me to challenge my expectations of myself. A very special thanks to **Keegan McGuire** for having profound belief in my capabilities, even at the times when I lost sight of them. Easy or hard, he has been there to lift me up and remind me of my motivation and purpose. This work would not be complete without recognizing my parents, **Chris** and **Cris Senkowski**. Their hard work, years of parenting, and words of wisdom over the phone have supported me through my life thus far and made me the person I am as I write this. Thank you for believing in me, seeing my potential, and helping me realize my dreams.

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