Genomic Evolution in *Pseudomonas fluorescens* as a Result of Gradual Temperature Changes

Spencer A. Lee
salee@tlu.edu or spenceralee@me.com

Robert M. Jonas
Texas Lutheran University Department of Biology, Seguin, TX

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Abstract

As climate change continues to affect global temperatures, organisms will need to not only adapt but evolve to survive the changing climate conditions. Temperature selection experiments were performed on *Pseudomonas fluorescens* to select for growth at lower temperatures. The *P. fluorescens* temperature selection experiment selected for cells that can grow at a new minimum temperature which is over 20˚C lower than the optimal growth temperature (25-30˚C). Previous experiments established the low end of *P. fluorescens's* growth temperature as 4˚C. The genomes of the newly selected and reference strains of *P. fluorescens* were sent for sequencing, and the results showed differences in protein sequence between the two strains. This experiment is a model for evolution as a result of gradual temperature change (similar to climate change) over generations, and the resulting genomic changes recorded show which protein families could evolve as an organism adapts to a gradually changing temperature.

Introduction

Climate change is a looming threat to ecosystems and life on Earth. Temperatures are predicted to increase by 4˚C across the world within the next 80 years (14). Climate scientists predict that climate change could move ecological zones over 600 km north of their current locations (14). This shift will force organisms to either move with the ecological zone, evolve to survive in new conditions, or perish as a result of a changing climate (14). This data demonstrates that climate change and evolution due to climate change are unequivocally relevant to all species on Earth. Species living in the northern part of North America, Europe, and Asia, as well as species on mountains are most likely to be affected by the shifting of ecological zones due to climate change (14). In addition to average temperatures increasing, the range of temperatures is expected to change as well with extremes becoming more extreme (11). A dynamic model of *Drosophila* survival and evolution at different temperatures explained that population collapse can arise when temperatures approach an organism's heat tolerance (11). In addition, temperature selection can arise when an organism is at a temperature that is a few degrees away from its thermal limits (11). Temperature selection experiments involve subjecting organisms to temperatures outside of their ideal temperature range and measuring the changes between the initial and final organisms. Studies utilizing temperature selection may be instrumental in determining the genetic effects of climate change (2).

Bacteria are excellent candidates for temperature selection studies because of their rapid generation time, easy growth conditions, and simplicity (in comparison to multicellular organisms). A bacterial temperature selection experiment with *Escherichia coli* showed that bacteria are capable of evolving to increase their fitness significantly at the set temperature within a period of fewer than two months (400 generations) (2). Throughout the temperature selection process, the bacterial population gained fitness-improving mutations in their genomes (2). Despite

the addition of prevalent mutations in the bacteria's genomes, the evolved bacteria did not lose their fitness in their ancestral environment (2). Bacteria adapting to low temperatures use various methods including sensing the temperature, adapting the structure of their enzymes, changing the composition of their membrane, using heat shock proteins, and using cold shock proteins to allow themselves to survive at a low temperature (6). It can be hypothesized from this information that genes relating to these factors are the ones most likely to be affected as an organism undergoes a temperature selection experiment or climate change. While there is no general pattern for amino acid composition of cold-adapted enzymes when compared to others of the same function, it is commonly shown that the amino acid composition of enzymes is different in cold-adapted and non-cold adapted enzymes (6). Enzymes are found in pathways relating to sensing environmental conditions and membrane composition maintenance as well as in metabolic pathways (6).

The *Pseudomonas fluorescens* species complex contains at least 52 species of Gram-negative motile rods (12). *P. fluorescens*, a psychrophile, has an optimal growth temperature of 25-30 ˚C but has been shown previously to be able to grow at temperatures ranging from 4-37 ˚C (8,12). *P. fluorescens* contains a relatively large genome (between 6.1-7.1 million bp) when compared to other bacteria like *E. coli* (4.6 million bp) (3,7,10). Genomic analysis of one strain of *P. fluorescens* showed that it had approximately 90 rRNA/tRNA-coding genes and over 5,500 genes that coded for proteins (7). This is nearly 1,300 more protein-coding genes than *E. coli* K-12 (3,7). Analysis of a different strain of P. fluorescens showed over 6,100 open reading frames within its genome (10). A large portion of its genome is devoted to genes dealing with motility, chemotaxis, and other environment-sensing and interacting genes (7). The large genome of other members of the genus *Pseudomonas* are theorized to allow the bacteria to survive in a wide range of environmental conditions and arose as a result of evolutionary adaptations (13). It was also proven that as bacterial genome size increases, more genetic real estate is dedicated to regulatory proteins (10,13). Its large genome and high number of regulatory genes allow *P. fluorescens* to be a plausible model organism for the environmental evolution of multicellular organisms.

P. fluorescens has been the subject of both temperature adaptation and experimental evolution experiments. One experimental evolution experiment subjected the bacteria to media with varying amounts of carbon substrates and determined the evolved and original strains' fitness in both the simple and complex media (1). Experiments involving low temperatures consistently reached a minimum temperature of 4-5 ˚C, but not lower, in their procedures (5,9). It is unknown whether the temperatures chosen are the true lower limit of the bacteria's growth range or whether this temperature is the lowest temperature suitable for laboratory experiments as the generation time reaches around 9 hours (5). In this experiment, the main possible mechanisms of genomic change are adaptation, mutation, and genetic drift. The purpose of this experiment was not only to determine the minimum growth temperature of P.

fluorescens but also to determine which genes evolve as an organism is subjected to extreme temperatures.

Materials and Methods

Media

Nutrient agar plates were made using Difco™ nutrient agar. 23 g/l of nutrient agar was suspended in deionized water. It was heated with frequent agitation and boiled for 1 minute to completely dissolve the powder. Nutrient broth was made using Difco™ nutrient broth. 8 g/l of nutrient broth powder was suspended in deionized water. It was heated with frequent agitation and boiled for 1 minute to completely dissolve the powder. Approximately 5 mL was distributed into test tubes, which were sterilized by autoclaving. Additional plates and test tubes were made as needed using the same methods.

Experimental Methods

A lab stock of *Pseudomonas fluorescens* strain ATCC® 13525 was grown at approximately 18 ˚C (the room temperature of the lab in February) and stored in a 4 ˚C refrigerator. Glycerol stocks of the stock strain were created by putting 500 µL of 50% glycerol and 500 µL of bacterial culture in a cryovial. All stocks were stored at -70 ˚C. Two test tubes containing 5 mL of sterile nutrient broth were each inoculated with one colony of *P. fluorescens* from the plate grown at 4 ˚C. The tubes were labeled, and a small stir bar was put into each tube. The temperature of the tubes was recorded every day until the end of the experiment. The tubes were placed into a beaker along with a thermometer. The beaker was placed into a Styrofoam container on a magnetic stirrer. Glycerol stocks were created of every other passaged culture and every four passages, the culture was streaked on a nutrient agar plate to check for uniformity and lack of contamination. *P. fluorescens* develops flocculent growth patterns creating small clusters that can be described as visible aggregates. All cultures were grown until they contained significant amounts of visible aggregates. New cultures were inoculated from the previous culture. The first culture was grown at the room temperature of the lab in May (~ 23 ˚C). The temperature was decreased by 1-1.5 ˚C daily until there was no more growth observed and the broth began to freeze despite agitation. The temperature of the culture of *P. fluorescens* was recorded multiple (3 or more) times daily at morning, noon, night, and other times the researchers were available.

Sequencing/Basic Bioinformatics

Samples of both the original and final cultures were sent to The Sequencing Center for DNA extraction, whole-genome sequencing, and sequence alignment to the reference genome. The DNA from both cultures was extracted using the Illumina DNA Prep and Illumina MiniSeq mid-output reagent kits. The DNA was paired-end sequenced using 150 bp reads. The resulting sequences were aligned against the genome of *P. fluorescens* strain ATCC® 13525, and variant/SNP analysis were performed using the Geneious Prime application using default settings. The results were then obtained from The Sequencing Center (Fort Collins, CO).

Additional Bioinformatics

The resulting variant/SNP data from the initial and final cultures were compared against each other manually to determine where the sequences differed between the initial and final strains. The variant/SNP data was compared manually due to the lack of available sequencing software. The genes containing an altered primary structure, location of nucleotide change within the genome, and the amino acid change were all recorded. The number of amino acids changed per protein (aa/p) was recorded and the overall results were analyzed.

The average daily temperature of the culture of *P. fluorescens* was calculated. The day of the experiment, the average culture temperature on that day, and the number of the culture growing on that day are shown below in Figure 1. The culture number indicates the number of passages since the original plated P. fluorescens. The equation of the trendline confirms that the temperature of the culture was decreased by 1-1.5 ˚C daily. Growth of the culture was observed at temperatures below 4 ˚C.

Results

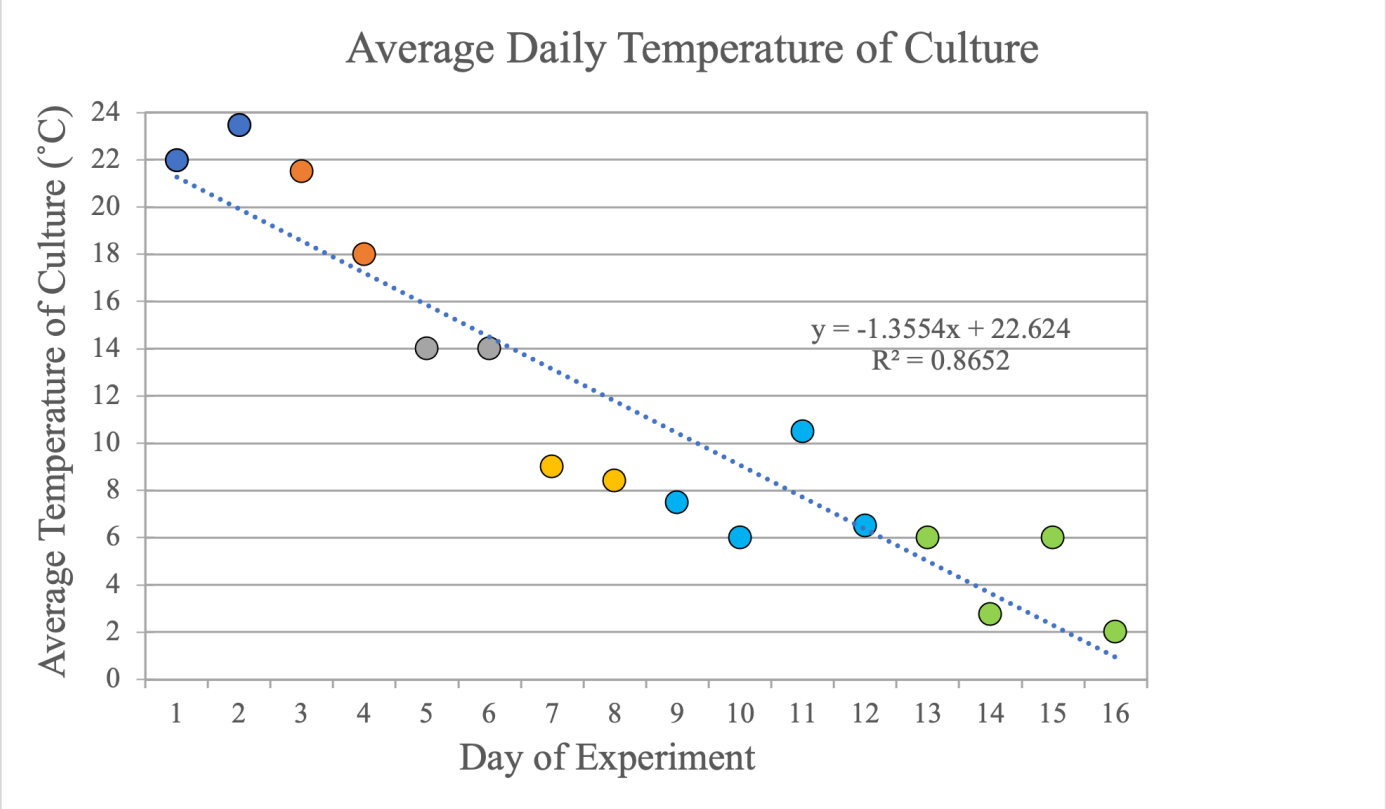

Fig 1: Average daily temperature of each culture throughout the experiment (Left to right: blue dots, culture 1; orange dots, culture 2; grey dots, culture 3; yellow dots, culture 4; cyan dots, culture 5; green dots)

The analyses from both the initial and final strains from the experiment were compared and a list of the genes that contained altered amino acid sequences was compiled. The altered genes were then grouped based on their function or the location of the resulting protein as shown in Table 2. The altered genes list contained 53 enzyme-encoding genes, 17 transport or membrane affiliated genes, 17 transcription factor related genes, 8 motility or flagellum related genes, 7 stress related genes, 3 genes that produced products involved in signaling pathways, 2 environmental factor genes, and 31 genes that produced proteins with other or unknown functions with some falling into multiple categories.

Protein Function or Location	Altered Genes
Enzyme	GNAT family N-acetyltransferase (3), tRNA-dihydrouridine synthase, alpha/beta hydrolase, tRNA (adenine(22)-N(1))-methyltransferase TrmK, class II aldolase and adducin N-terminal domain-containing protein, 3-dehydroquinate dehydratase, NAD(P)/FAD-dependent oxidoreductase, carbonic anhydrase, iron-containing redox enzyme family protein, thiolase family protein, PLP-dependent aminotransferase family protein, patatin-like phospholipase family protein, IS5/IS1182 family transposase, *atuC*, acetyl/propionyl/methylcrotonyl-CoA carboxylase subunit alpha, cobyrinate a,c-diamide synthase, C40 family peptidase, cytosine permease, amino acid dehydrogenase, FMN-dependent NADH-azoreductase, ABC transporter ATP-binding protein/permease, FAD/NAD(P)-binding protein, CPBP family intramembrane metalloprotease, *dnaX*, N-acetylmuramidase family protein, putative zinc-binding peptidase, *ligA*, gamma-glutamylcyclotransferase, 1-acylglycerol-3-phosphate O-acyltransferase, transglutaminase family protein, class I SAM-dependent methyltransferase, radical SAM protein, *mltG*, *rne*, HNH endonuclease, HAD-IB family hydrolase, AAA family ATPase, helix-turn-helix transcriptional regulator, diguanylate cyclase, antibiotic biosynthesis monooxygenase, GAF domain-containing sensor histidine kinase, aldehyde dehydrogenase family protein, VOC family protein, CPBP family intramembrane metalloprotease, DHH family protein, *lldD*, *pap*, *aroC*, *mnmC*, RidA family protein
Transport/Membrane	*chrA*, DMT family transporter (2), IS5/IS1182 family transposase, transporter substrate-binding domain-containing protein, *OmpP1/FadL* family transporter, nuclear transport factor 2 family protein, NCS1 family nucleobase:cation symporter-1, MFS transporter, carboxylate/amino acid/amine transporter, transporter substrate-binding domain-containing protein, drug:proton antiporter, *sctC, araG, mltG*, inorganic phosphate transporter, CPBP family intramembrane metalloprotease
Transcription Factor	GNAT family N-acetyltransferase (3), *LysR* family transcriptional regulator (6), *MarR* family transcriptional regulator, sigma-54-dependent transcriptional regulator, helix-turn-helix domain-containing protein, winged helix-turn-helix domain-containing protein, AAA-associated domain-containing protein, transcriptional regulator, AAA family ATPase, helix-turn-helix transcriptional regulator
Motility/Flagellum	FimV family protein, chemotaxis protein CheW, flagellar motor protein, chemotaxis protein CheA, flagellar hook-length control protein FliK, *fliD*, flagellin, flagellar hook-associated protein 3
Stress Reaction	GNAT family N-acetyltransferase (3), type II toxin-antitoxin system RelE/ParE family toxin, cold-shock protein, cold shock domain-containing protein, sigma-54-dependent transcriptional regulator
Environmental Factor Related	methyl-accepting chemotaxis protein, type II toxin-antitoxin system RelE/ParE family toxin
Signaling	Hpt domain-containing protein, diguanylate cyclase, GGDEF domain-containing protein
Other/Unknown	hypothetical protein CDS (23), ATP phosphoribosyltransferase regulatory subunit, DUF1028 domain-containing protein, DUF2790 domain-containing protein, DUF2177 family protein, DUF2167 domain-containing protein, leucine-rich repeat domain-containing protein, Bro-N domain-containing protein, DUF1003 domain-containing protein

Table 1: Genes in which coding sequences (CDS) differed between initial and final strains and their corresponding protein function or location (numbers in parentheses denote multiple protein CDS in a family that have altered sequences)

The bioinformatics yielded the difference in protein primary structure between the initial and final bacterial strains. The number of proteins with an altered primary structure and the number of amino acids changed per protein (aa/p) were recorded. The relative number of proteins with altered primary structures and average amino acids changed per protein are shown by group in Figure 2. In total, there were 2459 amino acids changes recorded across 138 genes that showed amino acid changes.

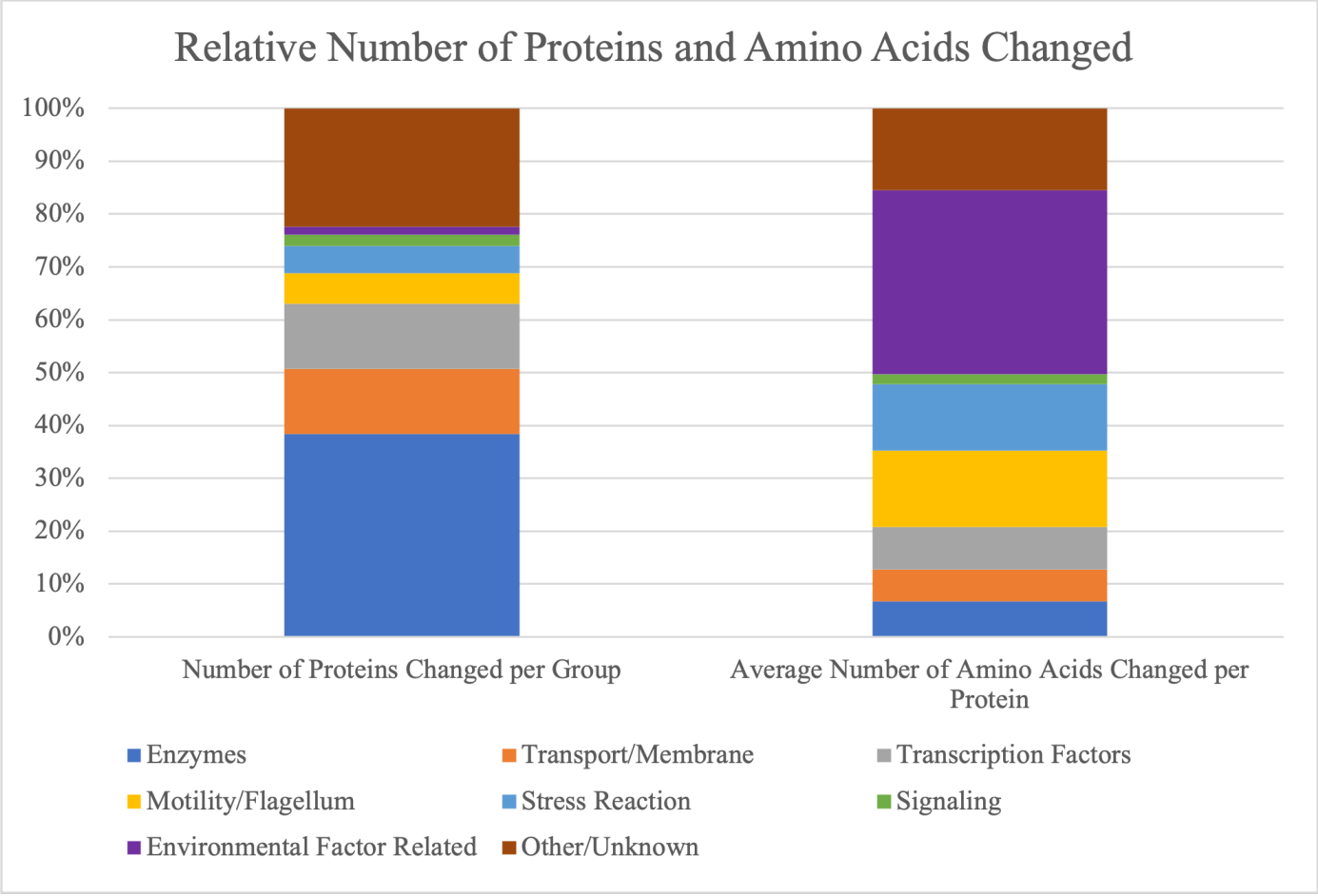

Fig 2: Relative Number of Proteins and Average Amino Acids Changed

The enzyme group was the most numerous with 53 proteins changed, but its average amino acid change per protein was one of the lower values at 12. The environmental factor related gene group had the fewest genes change (2) but they changed, on average, by the largest amount (63 aa/p). The amino acid changes per protein are shown by group in the box and whisker plot in Figure 3.

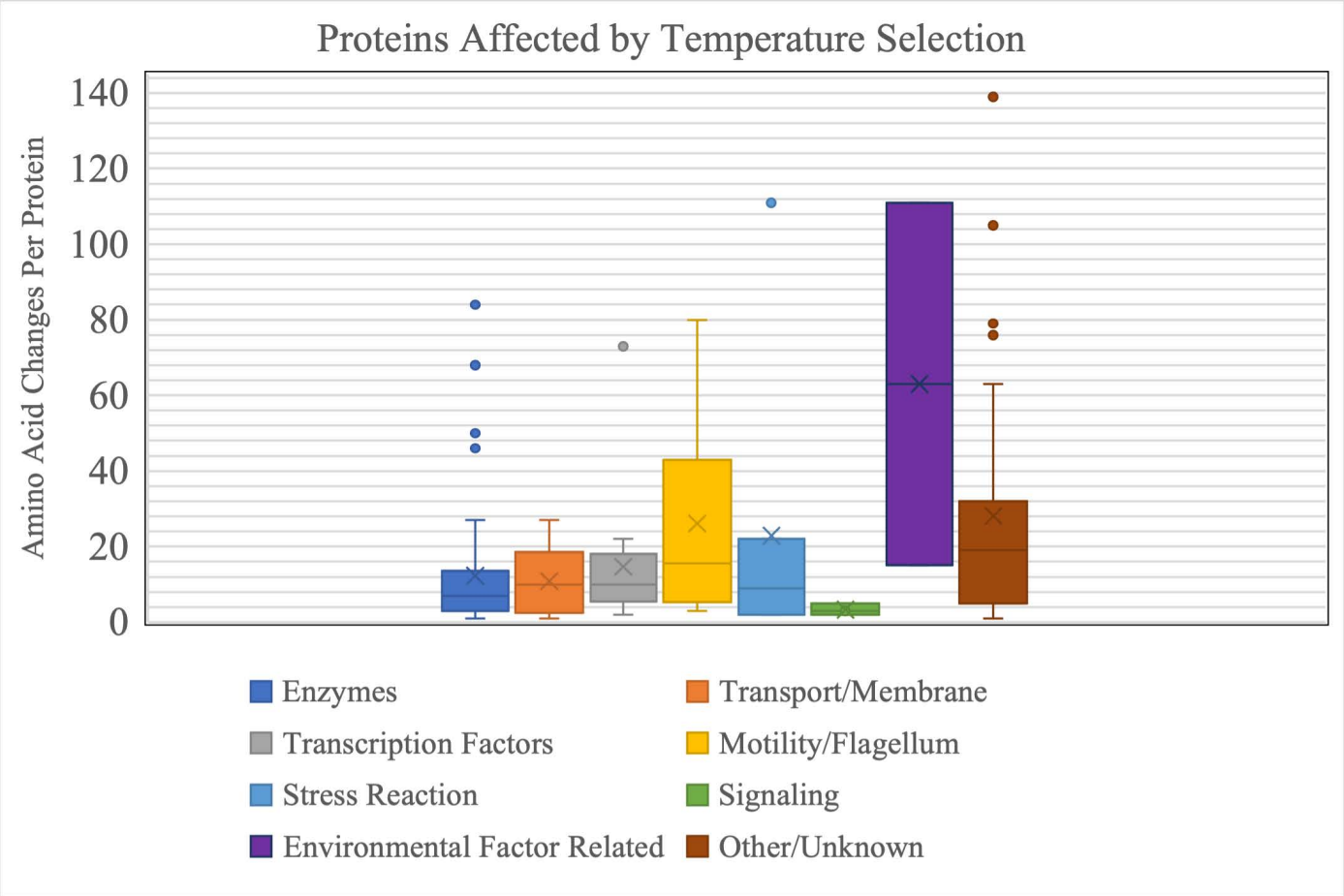

Fig 3: Proteins Affected by Temperature Selection: [x = 17.85 aa/p, s = 24.22 aa/p (Enzyme group mean = 12.26 aa/p, Transport/Membrane group mean = 10.82 aa/p, Transcription Factors group mean = 14.65 aa/p, Motility/Flagellum group mean = 26.13 aa/p, Stress Reaction group mean = 22.86 aa/p, Signaling group mean = 3.33 aa/p, Environmental Factor Related group mean = 63 aa/p, and Other/Unknown group mean = 28.10 aa/p)]

Discussion

This experiment determined a new minimum growth temperature of *P. fluorescens*, and genome sequencing and resulting analysis yielded informative data about the genomic changes resulting from evolution due to changing temperature. Bacterial growth at less than 4-5 ˚C during this experiment (2-2.75 ˚C) establishes a new minimum growth temperature of *P. fluorescens* and overwrites the previously established lower limit of *P. fluorescens's* growth (8,12). Low-temperature adaptation by bacteria is accomplished by sensing the environment, altering enzymatic structure to retain functionality, modifying membrane composition to maintain proper fluidity, and using heat shock and cold shock proteins (6).

The experimental data confirmed the prediction that genes with structural changes would fall into the categories of enzymatic genes, regulatory genes (transcription factors and signaling), genes affecting the membrane and associated proteins, and environmental related genes with additional categories of motility and unknown genes also being affected. While the exact mechanisms of change in this study are not known, they are believed to be adaptive. Laboratory medium lacks the selective pressures of the nature, so some genetic changes could occur in that environment that may not necessarily correlate to evolution in nature. A more ideal experimental set-up would passage cells equivalently at different temperatures to ascertain which alterations in the genome are directly temperature dependent. Due to the ubiquity of enzymes in a biological system and their need to change structure to retain their function at lower temperatures than optimal (6), numerous enzymatic genes contained changes in their structure as predicted. Only subtle changes in primary structure were needed for the enzymes to function properly at a lower temperature, which is why the enzyme group was the most numerous but one of the groups with the fewest amino acid changes per protein (as shown in Figures 2 and 3). The genes related to environmental factors contained the fewest genes changed, but they changed by the largest amounts (also shown in Figures 2 and 3). This is hypothesized to be due to the evolution of the bacteria at their minimum growth temperature. The protein coded by an environmental response gene has a specific structure that allows it to respond to environmental changes. If the structure of many of the environmental response genes changed, the bacterium would not be able to react properly to its new environment. That is why we predict that category had fewer genes with amino acid changes. The median protein length in bacteria is 267 amino acids (4), so the average change of approximately 18 aa/p means that if the gene (and resulting protein primary structure) is altered, 6.7% of the amino acids in a protein are changed. The percent change was not calculated for specific genes as the bioinformatics largely listed the genes by their family and was not specific. As predicted, numerous genes relating to the membrane changed their amino acid sequences. For example, the mltG gene codes for a multifunctional fusion protein that functions as an endolytic peptidoglycan terminase and catalyzes the phosphorylation of dTMP to dTDP (15). This gene changed

in 25 amino acid residues. As a gene coding for an integral membrane protein with enzymatic functions, *mltG's* change in structure shows that membrane-related genes are affected by temperature-dependent evolution. Motility is more difficult at lower temperatures. This difficulty is exacerbated close to, and at, the freezing point of water because of the aqueous nature of biological systems. Because of this, it is understandable that nearly 10 genes relating to motility would change to alter the sequence of the resulting proteins. This also, explains why stress-related genes were also affected by the temperature selection. The original bacterial strain's genome changed drastically over the course of the experiment, as shown by the sequencing and bioinformatics results. Therefore, the final bacterial strain was deemed independent from the initial strain and was given the strain identifier "DS5683" with the full strain name being *Pseudomonas fluorescens* DS5683.

This experiment shows that an organism subjected to experimental evolution due to a gradual temperature change will accumulate mutations in enzymatic genes, membrane-related genes, regulatory genes, motility genes, stress-related genes, and environmental-related genes. Because enzymes have an ideal temperature they function at, a change in environmental temperature requires a change in the primary structure to allow the enzyme to maintain its functionality. These results can be extrapolated to model genetic changes as a result of gradual temperature changes. As climate change increases global temperatures, it is hypothesized that the most numerous genes affected will be enzymatic genes and the most numerous structural changes will occur to environmental-related genes. In addition to those genes, regulatory genes, motility genes, and stress-related genes are also predicted to be altered due to climate change. Even though the temperature change due to climate change is not expected to cause all species to go extinct, the results of this experiment predict that genomic changes are expected to become prevalent in species experiencing the temperature change

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Declarations

Funding:

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Competing Interests:

The authors declare no conflict of interest.

Ethics Statement:

No approval of research ethics committees was required to accomplish to this study because microbe use is not regulated by ethical committees.

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