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Tracking Disparate Colony Morphological Trends with Thermus scotoductus

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Honors Thesis

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Abstract

When plating most mesophilic bacteria, the colony shape, size, and color tends to be uniform when a single strain is present. When plating defined cell densities of *T. scotoductus*, however, the colonies were not of uniform size and shape while it grew on the surface. In this project, we sought to observe trends in colony morphology (shape and size) changes using the thermophile *Thermus scotoductus* on nutrient rich agar plates at 60°C. A general planktonic growth curve was also created to help characterize the activity of this bacterium. This project was our first attempt to characterize if this unusual phenomenon is statistically significant at a 95% confidence and ultimately a distance between colonies to colony size correlation is predictable.

Dedication or Acknowledgements

I would like to thank the University of Dayton Honors Program for providing me with this incredible opportunity. I would also like to thank Dr. Biffinger for being my mentor.

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Introduction

Only about 2% of all known microorganisms can be grown in lab. Even smaller still are the number of organisms that can grow under conditions that are outside 20-37°C and between a pH of 6.0-8.0. These organisms are classified as extremophiles (organisms that grow in extreme environments) and have adapted their metabolic and transcriptional pathways to survive in environments where most microorganisms cannot survive. Not only can extremophiles survive in these environments, such conditions are required for survival. A specific class of extremophiles, known as thermophiles, are of particular interest. One such thermophile is *Thermus scotoductus* strain SA-01, discovered in a geothermal heating system in Southern Iceland. *T. scotoductus* is a facultative anaerobe with an optimal growth temperature of 65^0C.[1] In a study looking at the microbial diversity in water heaters of American homes, SA-01 was found to be the most abundant by far compared to the other five *Thermus* strains observed[2]. *T. scotoductus* SA-01 has drawn much interest in the field of bionanotechnology.[3] It is mainly researched for its metal-reducing properties, as it can reduce gold, Iron (III), Chromium (IV), and Uranium (VI). Our project however, looks at a different aspect of this fascinating microorganism.

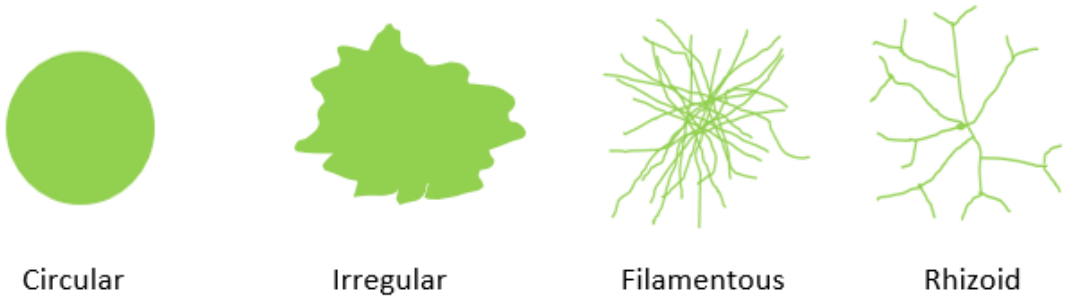

Figure 1: Above is a list of different colony morphologies. Many *T. scotoductus* colonies were of the circular type.

Colony morphology is a key feature of microbial activity on surfaces. There are several types of common colony morphologies, illustrated in figure 1 possible from cells grown on a surface and these individual colony morphologies are used primarily to identify the microorganism. Much is understood about how colonies may be classified, but little is understood when it comes to the discussion of how colonies are formed and how colonies of the same species interacts with other colonies on the same plate.

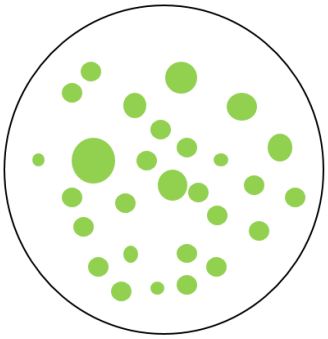

Figure 2: Disparate colony size between colonies of the same species.

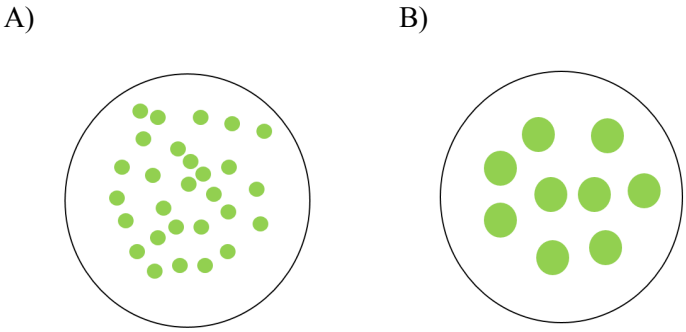

Figure 3: Schematic showing difference between A) microcolonies and B) macrocolonies

Figure 2 illustrates different colony sizes on a single plate. One study observed how colony morphology impacted the bacterial ability to grow on food, finding that the colony type influenced how they grew because of the limited diffusion characteristics of carbon sources in

agar matrices. The study mainly differentiates between micro-colonies (0-200µm in diameter), shown in figure 3a, and macro-colonies (300-400 µm in diameter), shown in figure 3b. The study also proposed different In general, bacterial colonies are usually the same size, color, and shape when they are the same strain on a single plate. If one were to spread an excess amount of bacteria, the result on the plate would be the embedding of bacteria in a chemical matrix known as a biofilm (shown in figure 4).[4] It would make sense that if two different bacterial species were inoculated on the same plate, one would observe colonies of varying size, shape, and color. It would also be rational to expect competition, resulting in one species "winning out" over the other (shown in figure 5)[5]. The phenomenon to be discussed in this project is an apparent case of one colony competing with other colonies of the same species. Such a result is certainly unexpected, and does not appear advantageous. In characterizing colonial growth, we sought to see if any statistically significant correlation between colony size and distance could be found.

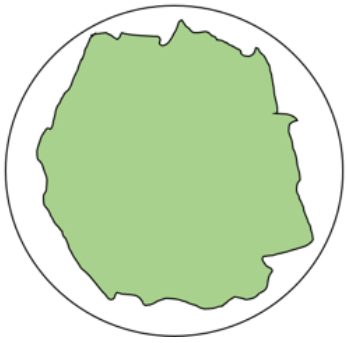

Figure 4: Illustration of a Biofilm of a single strain without competition

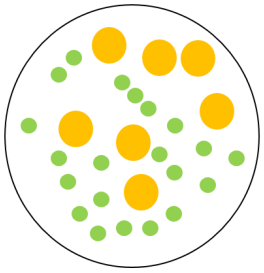

Figure 5: Illustration of a plate containing two different strains exhibiting competitive interactions.

Materials and Methods

Solution 1

Nitsch's Trace Element Solution	
Salt	**Amount**
H_2SO_4	0.25 mL
$MnSO_4$	1.123 g
$ZnSO_4 \bullet 7H_2O$	0.258 g
H_3BO_3	0.25821 g
$CuSO_4 \bullet 5H_2O$	0.00841 g
$Na_2MoO_4 \bullet 2H_2O$	0.01297 g
$CoCl_2 \bullet 6H_2O$	0.02501 g
ROS Water	500 mL

Solution 2

Castenholz Salts	
Salt	**Amount**
Nitrilotriacetic acid	0.1g
Nitsch's Trace Element Solution	1.0 mL
$FeCl_3$ Solution (0.03%)	1.0 mL
$CaSO_4 \bullet 2H_2O$	0.06 g
$MgSO_4$	0.0870 g
NaCl	0.008 g
KNO_3	0.105 g
$NaNO_3$	0.7 g
$Na_2HPO_4 \bullet 2H_2O$	0.208 g
Agar (for petri dishes only)	15.0 g
ROS Water	500 mL
pH	8.2

Solution 3

TYE	
Salt	**Amount**
Tryptone	5.00 g
Yeast Extract	5.00 g
ROS Water	500 mL

Solution 4

Phophate Buffered Saline (PBS)	
Salt	**Amount**
NaCl	4.0 g
KCl	0.1 g
Na_2HPO_4	0.72 g
KH_2PO_4	0.13 g
ROS Water	500 mL
pH	7.2

Table 1: This table lists the components of the three solutions required to make the Castenholz media (Solutions 1-3) and the PBS (Solution 4).

Media Preparation

Each media solution was prepared according to the Castenholz TYE media protocol (https://www.atcc.org/Products/All/700910.aspx#culturemethod) from the American Type

Culture Collection (ATCC). Three different solutions were produced to form the complete growth medium and mixed after sterilization.

The specific solutions prepared are listed in Table 1. In preparing the final media, the Castenholz Salt Solution, 18MΩ water, and TYE solution were mixed in a ratio of 5:4:1, v/v respectively. The 0.03% $FeCl_3$ solution was created using FeCl3-6H2O in 18MΩ water and was sterilized using a syringe-filter (Durapore PVDF membrane, 0.22µm pores). The PBS (formulation) and Castenholz Salt solution were both sterilized using a sterile filter tower (). In preparing the petri dishes, the TYE solution and the Castenholz salt/Agar solution were both sterilized simultaneously at 121^0C for 30 min. PBS was not a component of the media, but was used in the serial dilution experiments.

T. scotoductus growth from frozen stocks and culture maintenance

Thermus scotoductus SA-01 was purchased from American Type Culture Collection (ATCC 7000910) and stored as 20% glycerol stocks. Too begin a culture, the stocks were thawed, and 200µL of culture was added to a 15 mL falcon tube containing Castenholz media. The culture was then incubated aerobically at 62^0C.

Serial Dilution Protocol and plating of T. scotoductus.

To begin, six sterile Eppendorf centrifuge tubes were placed in a heating block set to 65^0C. 900 µL of filter-sterilized PBS was added to each centrifuge tube. A seventh centrifuge tube was filled with 1000µL of culture. The culture tube was vortexed in order to resuspend the cell pellet. 100 µL of culture was transferred to tube 1 using a 200 µL micropipette, and tube 1 was vortexed. 100 µL of the new tube 1 solution was transferred to tube 2, and tube 2 was vortexed, etc. This process continued all the way to tube 6, creating a set of dilutions from 10^{-1} through 10^{-6}.50µL of tubes 3, 4, 5, and 6 (10^{-3},10^{-4}, 10^{-5}, and 10^{-6}) were each plated onto petri dishes. Duplicate plates

were made for each dilution. The plates were placed in an incubator set to 62^0C. The plates were incubated in Ziploc bags with moist paper towels in order to prevent the agar from drying out.

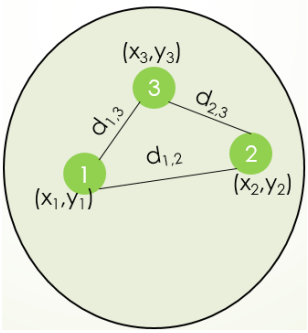

Figure 6: Illustration of how the distances between colonies and colony radii were calculated

Plate Imaging

All plate images were taking using UVP plate-imaging software. The software was used to count colonies in the 10^{-4} plates of each duplicate, and determine colony size and shape. Calibration markings were used while taking the image. The average colony size was taken by dividing the diameter by two, giving the colony radius in pixels. This was then converted to millimeters using a conversion factor of 0.26458 mm/px. The plate imaging software was also used in order to obtain the coordinates of each colony. The distance between one colony and every other colony in the region was obtained using the distance formula (equation 1). The average and standard deviation of size was taken for the following distance ranges: 0-25mm, 25-50mm, 50-75mm, 75-100mm, and greater than 100 mm (results shown in table 2).

$$d = \sqrt{(x_1 - x_2)^2 + (x_1 - x_2)^2} \qquad \text{(Equation 1)}$$

DNA isolation protocol

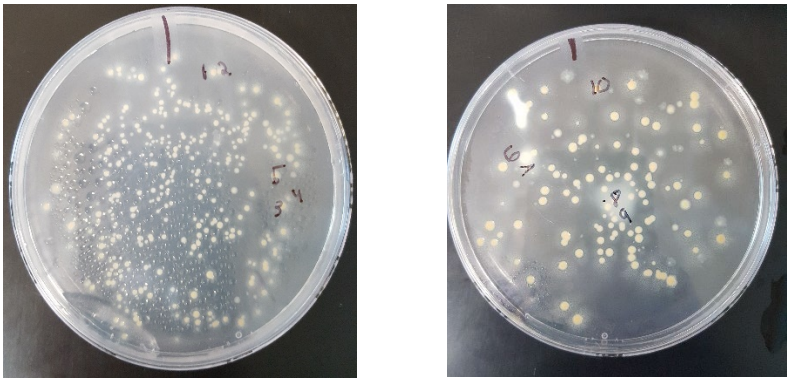

Figure 7: Images of the plates with markings indicating the colonies that were removed for DNA isolation and PCR analysis.

As shown in Figure 7, ten colonies were labeled on each of the 10^{-4} plates. Each colony was transferred to a 15 mL falcon tube with 5 mL of Castenholz media. After the cultures were grown, they were taken for DNA isolation using a BioWorld DNA isolation kit. First, 1 mL of culture was centrifuged at 14,000 rpm for 1 min. The supernatant was discarded. The pellet was then resuspended in 200 µL of solution I. 200µL of solution II was then added, and the solution was allowed to mix for approximately 5 min. The Eppendorf tube was then centrifuged at 14,000 rpm for ten minutes. The supernatant was transferred to a fresh tube using a micropipette. 900µLof 100% ethanol was then added. The resulting solution was mixed and then centrifuged at 14,000 rpm for ten minutes. 100 µL of iced 75% ethanol was added, and the pellet was again resuspended and centrifuged for 30 seconds. The Eppendorf tubes were left to air dry overnight on top of a Kimwipe. This procedure was not finished due to time constraints.

PCR primer design

PCR primers were designed using ncbi blast for the 16S rRNA partial sequence (reference EU330195.1). Primer stability was analyzed using the IDT Oligoanalyzer tool. The forward primer was (5'-GGACTAAAGGGTGAATAGCC-3'), and the reverse primer was (5'-GCTATTACCTGGTTACCGTC-3'). These primers were purchased. Due to time constraints, the PCR data was not collected for this report.

Statistical Analysis

Microsoft excel was used to calculate the correlation coefficient (equation 2), giving a value between -1 and 1. A value of -1 corresponds to a perfectly straight line with a negative slope, while a value of +1 indicates a perfectly straight line with a positive slope. The correlation coefficient was converted into a t-statistic using equation 3. The computed t-value was compared to the appropriate critical t-value for a 95% confidence interval.

$$r = \frac{\sum(x-\bar{x})(y-\overline{y})}{\sqrt{(x-\bar{x})^2(y-\bar{y})^2}} \qquad \text{(Equation 2)}$$

$$t = r\frac{\sqrt{n-2}}{\sqrt{1-r^2}} \qquad \text{(Equation 3)}$$

Results and Discussion

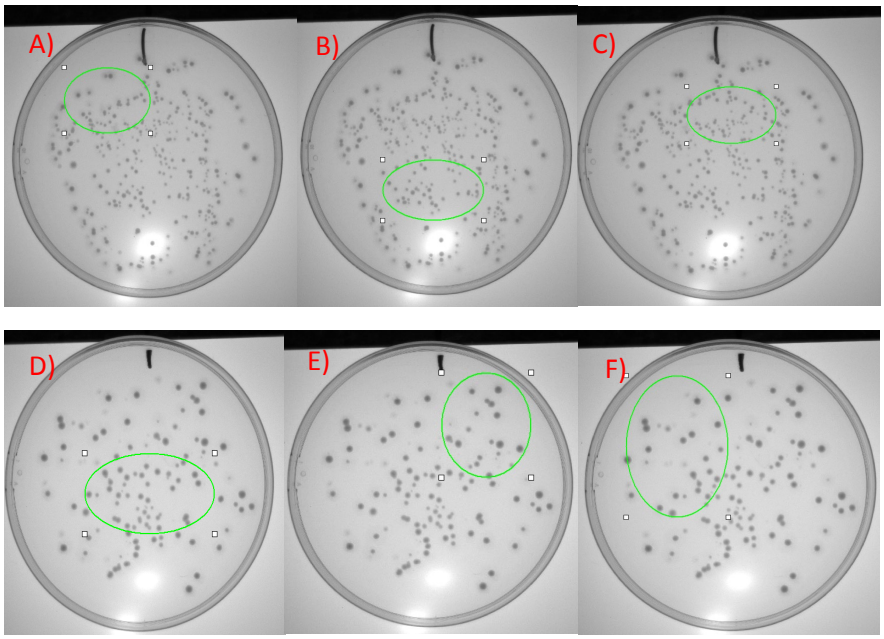

Figure 8: A) Replicate 1 Region 1, B) Replicate 1 Region 2, C) Replicate 1 Region 3, D) Replicate 2 Region 1, E) Replicate 2 Region 2, F) Replicate 2 Region 3

Figures 8 shows the regions labeled on each 10^{-4} plate of the two replicates. Table 2 shows the average and standard deviation for each distance range. In many cases, the standard deviation was much higher than the average, indicating a high degree of variability. For example, one cannot say the radius of a colony at a distance of 25-50mm is likely to be 3.2mm. One table of the correlation coefficient and t statistic results is shown as a sample (shown in table 3). In many cases, the calculated t value was in fact smaller than the critical t value, indicating a statistically significant correlation between size and distance. However, the values of the correlation coefficients are very small, indicating the relationship is most likely not linear. Going forward, it would be reasonable to perform other statistical analyses for a more complex model.

Distance from colony	Average Radius (mm)	Compounded Standard Deviation of Radius (mm)
<25	2.997	3.719
25-50	3.230	3.135
50-75	2.630	3.250
75-100	2.388	2.291
>100	1.802	0.9049

Table 2: Size Distributions for Replicate 1 Region 1

Colony Number	Correlation Coefficient	Absolute Value of t-value	Degrees of Freedom	Critical t value	Statistically Significant?
1	-0.5055	3.366	34	2.032	No
2	-0.4254	2.781	36	2.028	No
3	-0.02199	0.1122	27	2.052	Yes
4	-0.2327	1.415	36	2.028	Yes
5	-0.1899	1.144	36	2.028	Yes
6	-0.3028	1.879	36	2.028	Yes
7	-0.07839	0.4517	34	2.032	Yes
8	-0.2439	1.445	34	2.032	Yes
9	-0.4323	2.670	32	2.037	No
10	-0.2946	1.688	31	2.040	Yes

Table 3: Statistical analysis of Replicate 1 Region 1

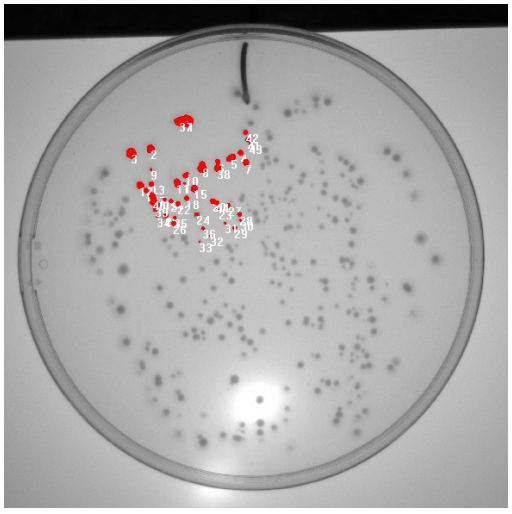

Figure 9: Colony count of Replicate 1 Region 1 using UVP software

As to causes of this phenomena, two causes I wish to posit are genome plasticity and quorum sensing. In studying the genomics of *T. scotoductus* SA-01, it has been found that it is

extremely prone to mutation, taking up DNA, and expelling DNA in order to survive[6]. Could this high rate of mutation cause *T. scotoductus* to be unable to recognize itself, resulting in competition rather than cooperation? Quorum sensing is a phenomena where bacteria work together to achieve the same effect using chemicals known as autoinducers. These chemicals are always given off by bacteria. When there is a certain bacterial concentration in a given area, the autoinducer reaches a threshold concentration, leading to expression of a certain gene, whether it be for biofilm formation, expression of virulence factors, or both[7]. The specific autoinducer found in *T. scotoductus* is autoinducer-2, shown in figure 10 [8]. Perhaps instead of creating a biofilm, *T. scotoductus* limits growth of colonies in order to use available nutrients in a more efficient manner.

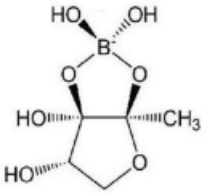

Figure 10: Chemical Structure of Autoinducer-2

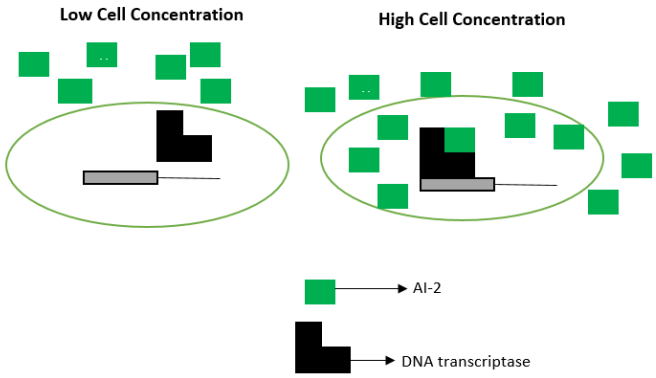

Figure 11: Illustration of a potential mechanism for autoinduction within quorum sensing behavior

Future work in this area would involve making a more complex statistical model, and seeking out the phenomenon in other organisms, including both thermophilic and mesophilic organisms. One statistical method that may be useful is the creation of a stochastic model, used in cases for a phenomenon that is seemingly much more random.[9] The 16S PCR primer analysis also needs to be completed to confirm that none of the observed phenomena are due to contamination. We can mix the isolated DNA with the primers, and then amplify the binded segments. We would then run gel electrophoresis and send the resulting band out for sequencing to ensure it matched *T. scotoductus* SA-01. Such a method is very useful for bacterial identification.[10] Contamination is highly unlikely, but precautions must still be taken. As literature on both thermophiles and colony morphology is sparse, much more can be done to elaborate on these fields.

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