Bacillus cereus & Bacillus pumilus Harvested From Copper Roof Inhibit Growth of *Other Organisms*

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Abstract

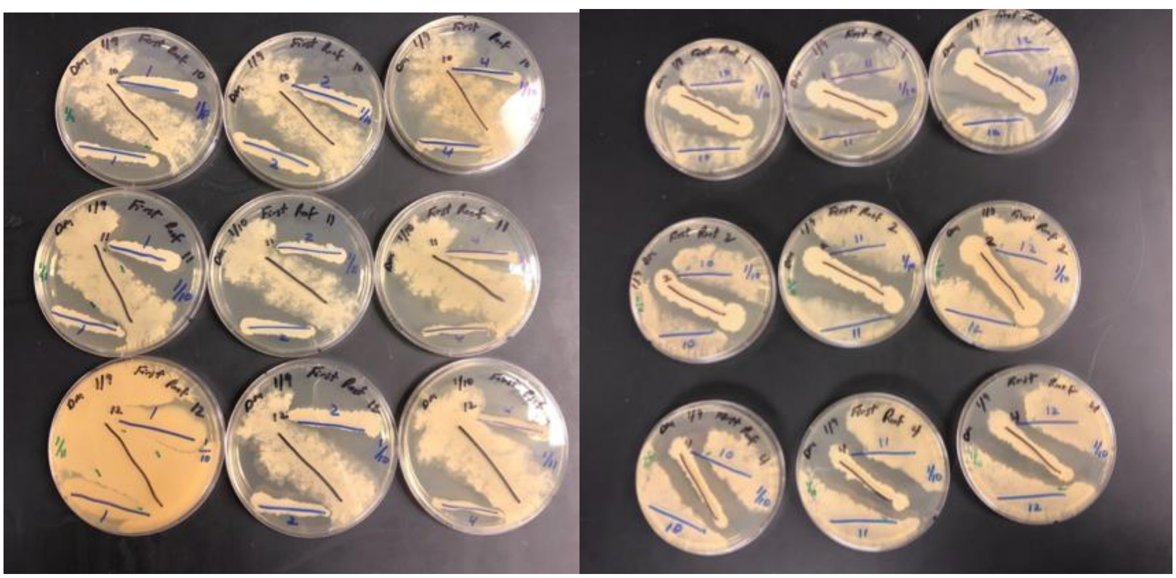
The goal of this project was to isolate bacteria from unusual places, as these bacteria may have distinct adaptations to allow them to grow in challenging environments. Bacteria samples were obtained from the copper roof of Simons Hall in Collegeville, Minnesota during November 2018. These samples are of interest because bacterial growth is typically inhibited by copper. Once isolated and grown in culture, some of the collected bacterial samples displayed the ability to out-compete other bacterial samples. A polymerase chain reaction was used to identify bacteria samples 1 and 2 as *Bacillus cereus* and bacteria 4 as *Bacillus pumilus*. Growth curve experiments show that these isolates are capable of inhibiting other bacterial species. The results from our growth curve experiments depict similar inhibitory effects on unknown bacteria samples during all stages of the growth curve. Our results support previous studies which suggest *Bacillus* have the capability of inhibiting or killing other organisms within their environment.

Introduction

Previously, it has been thought that bacteria are unable to survive for more than a few hours on copper surfaces. Being that bacteria may have the ability to survive on stainless steel surfaces for weeks, many hospitals and labs have implemented the use of copper surfaces in an effort to eliminate possibilities for contamination or spread of unwanted bacteria. As reported by Montero et al. (2019), copper killed more than 99.9% of these microorganisms after one hour of contact, as well as after consecutive inoculations over 24 hours. A novel composite coating with copper particles has been implemented in health-care settings to utilize these antimicrobial effects.

However, some bacteria may have unique adaptations allowing them to survive on copper surfaces and/or resist antibiotics, giving them a competitive advantage. According to Rodrigues, Ossa Henao, Oliveira, & Ottoboni (2018), some bacteria isolated from a copper mine environment included *Acidovorax, Acinetobacter, Bacillus, Brevundimonas, Stenotrophomonas, Kocuria, Roseomonas, Pseudomonas.* They state that *Bacillus* was the most abundant and diverse. Additionally, Rafii, Williams, Park, Sims, Heinze, Cerniglia & Sutherland (2009) explain that *Bacillus* is one type of bacteria that is able to grow in the presence of the antibiotic Cetiofur. *Bacillus* is able to produce beta lactamase and grow in the presence of other beta lactams. Thus, it is suggested they have the ability to cleave penicillins, carbapenems, and other b-lactams.

A sample was isolated from a copper roof in Collegeville, Minnesota and through polymerase chain reaction, *Bacillus cereus* and *Bacillus pumilus* were identified. These two strains of bacteria were then tested against other unknown bacteria found on the copper roof and have given us reason to believe that *Bacillus cereus* and *Bacillus pumilus* may possess the ability to inhibit or even kill other bacteria (figures 1 & 2). Research by Sharma, Dang, Gupta, & Gabrani (2018), involved isolation and characterization of the bacteriocin from *Bacillus subtilis.* It explains that the *Bacillus* bacteriocin displayed antimicrobial activity against both gram-positive and gram-negative bacteria. Further, it suggests higher doses are required to inhibit the growth of gram-negative bacteria due to the outer membrane. The bacteriocin also displayed stability across wide ranges of temperature and pH, and this was proposed to be due to unusual amino acids in the antimicrobial substances. The mechanism for bactericidal action of the bacteriocin was reported to be pore formation on the bacterial cell membrane, compromising it’s integrity. This is supported by Babasaki, Takao, Shimonishi & Kurahashi (1985), who introduced an antibiotic, Subtilosin A, which was produced by *Bacillus subtilis* 168. The antibiotic was extracted and purified through gel filtration and thin-layer chromatography. This article explains that Subtilosin A had bactericidal activity against some gram-positive bacteria. It is also mentioned by Abriouel, Franz, Omar, & Galvez (2011), that *Bacillus* bacteriocins are being investigated as food preservatives and that results are being reported in human health fields including the control of pathogenic bacteria such as MRSA, *G. vaginalis*, and *C. difficile.* Furthermore, Bottone & Peluso (2003) discuss *Bacillus* species ability to produce 167 biological compounds capable of inhibiting bacteria, fungi, protozoa and viruses. They describe a compound produced by *Bacillus pumilus* with Fungicidal activity able to inhibit spore germination and hyphal elongation in Mucoraceae and *Aspergillus* spp. We expected to observe that *Bacillus cereus* and *Bacillus pumilus* were emitting an inhibitory molecule during a specific time on their growth curves.



Figures 1 & 2 . The initial inhibitory behavior of unknown bacteria that led to the hypothesis for this study.

Methods

Bacteria was collected in November of 2018 from Simons Hall's copper roof at Saint John's University in Collegeville, Minnesota. Bacteria was then placed on agar plates and grown. Three target bacteria were isolated from the plates to allow for PCR tests to be performed. PCR tests provided an isolated portion of the 16S gene. The PCR test results were then utilized for gel electrophoresis. Purified DNA was sequenced through GeneWiz using the Sanger method. The results were analyzed to identify the microbial isolates. The PCR results were then inserted into BLAST to determine the genus/species of the isolated bacteria. Bacterial growth curves were created for each of the three bacteria with the utilization of the spectrophotometer.

The isolated bacteria were then tested against three other unknown bacteria in a series of two experiments. In the first experiment, the unknown bacteria were spread on TSA plates using sterile Q-tips in order to create a lawn of unknown bacteria a couple of days before 25 uL of the isolated bacteria were added to the plates using a micropipette. An absorbance was recorded for each of the isolated bacteria and a growth curve was created. At each point in time when the absorbance of the isolated bacteria was recorded, the bacteria was simultaneously added to the TSA plates. The TSA plates were stored in an incubator at 20℃. The combination of plates at each time in the growth curve allowed for understanding of which phase of the growth curve that the isolated bacteria were able to affect the unknown bacteria and create a zone of inhibition.

In the second experiment, the unknown bacteria were spread on TSA plates using sterile Q-tips in order to create a lawn of unknown bacteria directly before 25 uL of the isolated bacteria were added to the plates using a micropipette. An absorbance was recorded for each of the isolated bacteria and a growth curve was created. At each point in time when the absorbance of the isolated bacteria was recorded, the bacteria was simultaneously added to the TSA plates. The TSA plates were stored in an incubator at 20℃. This second experiment provided further understanding of whether or not the isolated bacteria was capable of affecting the unknown bacteria if it had time to establish itself or if they were both introduced to the TSA plate environment around the same time.

Results

PCR results processed in BLAST identified bacteria 1 & 2 as *Bacillus cereus* and bacteria 4 as *Bacillus pumilus.*

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
	Bacillus cereus strain SKH 16S ribosomal RNA gene, partial sequence	1840	1840	96%	0.0	99.60%	KJ685393.1
	Bacillus sp. G96 strain g96 16S ribosomal RNA gene, partial sequence	1838	1838	96%	0.0	99.60%	JQ661039.1
	Bacillus cereus strain WJH 16S ribosomal RNA gene, partial sequence	1834	1834	96%	0.0	99.50%	MK675099.1

Figure 3. BLAST results for bacterias 1 and 2.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
	Bacillus pumilus strain 17 16S ribosomal RNA gene, partial sequence	1812	1812	97%	0.0	99.20%	MK621233.1
	Bacillus sp. (in: Bacteria) strain HPS2 16S ribosomal RNA gene, partial sequence	1812	1812	97%	0.0	99.20%	MK602388.1
	Bacillus sp. (in: Bacteria) strain BGS1 16S ribosomal RNA gene, partial sequence	1812	1812	97%	0.0	99.20%	MK602371.1

Figures 4. BLAST results for bacteria 4.

Measurable zones of inhibition were apparent for *Bacillus cereus* and *Bacillus pumilus* on the unknown bacteria lawn throughout all stages of their growth curves. Zones of inhibition are similar in size, regardless of whether lawns were made one day prior or immediately before *Bacillus* species were introduced.

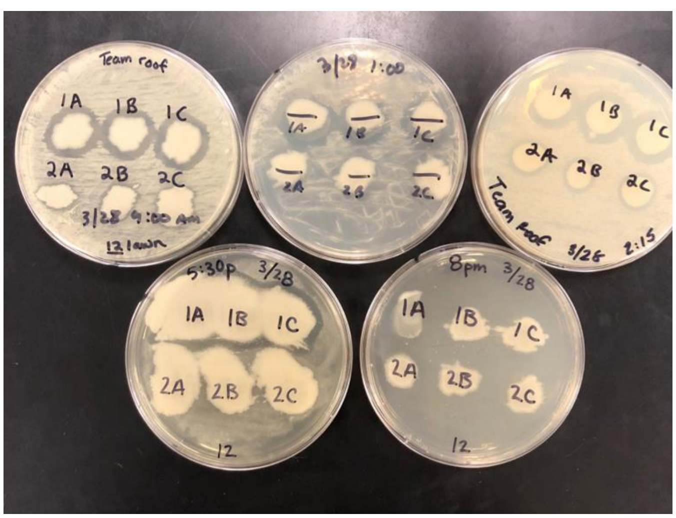


Figure 5. TSA plates of *Bacillus cereus* on unknown bacteria lawn at various stages of their growth curves. In figure 5, lawns were made immediately before *Bacillus cereus* was added.

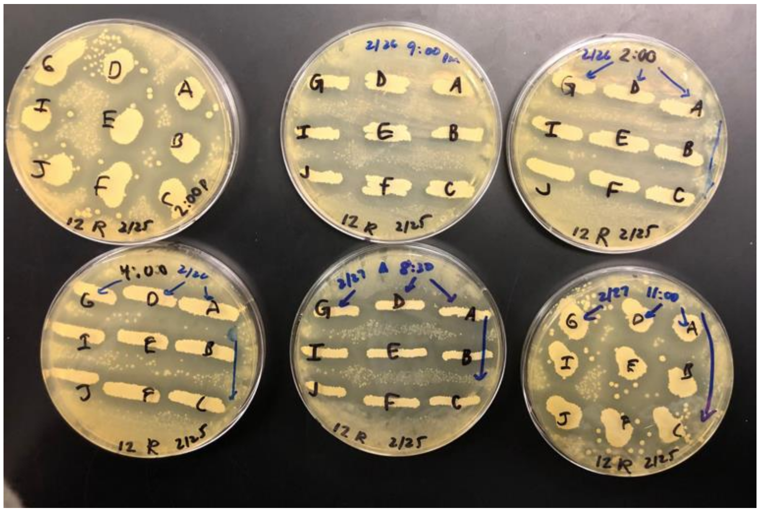


Figure 6. TSA plates of *Bacillus pumilus* on unknown bacteria lawn at various stages of its growth curves. In figure 6, lawns were made one day prior to when *Bacillus pumilus* was added.

A growth curve depicts the results of Absorbance (600 nm) taken every few hours. Absorbance testing began at hour 3.5 and ended at hour 29 with 7 measurements taken. The absorbance values for *Bacillus cereus* ranged from approximately 0.22 to 1.6, while the values for *Bacillus pumilus* ranged from approximately 0.3 to 1.6. The outlier was taken from the average of bacteria 1 and 2 during its growth curve measurements.

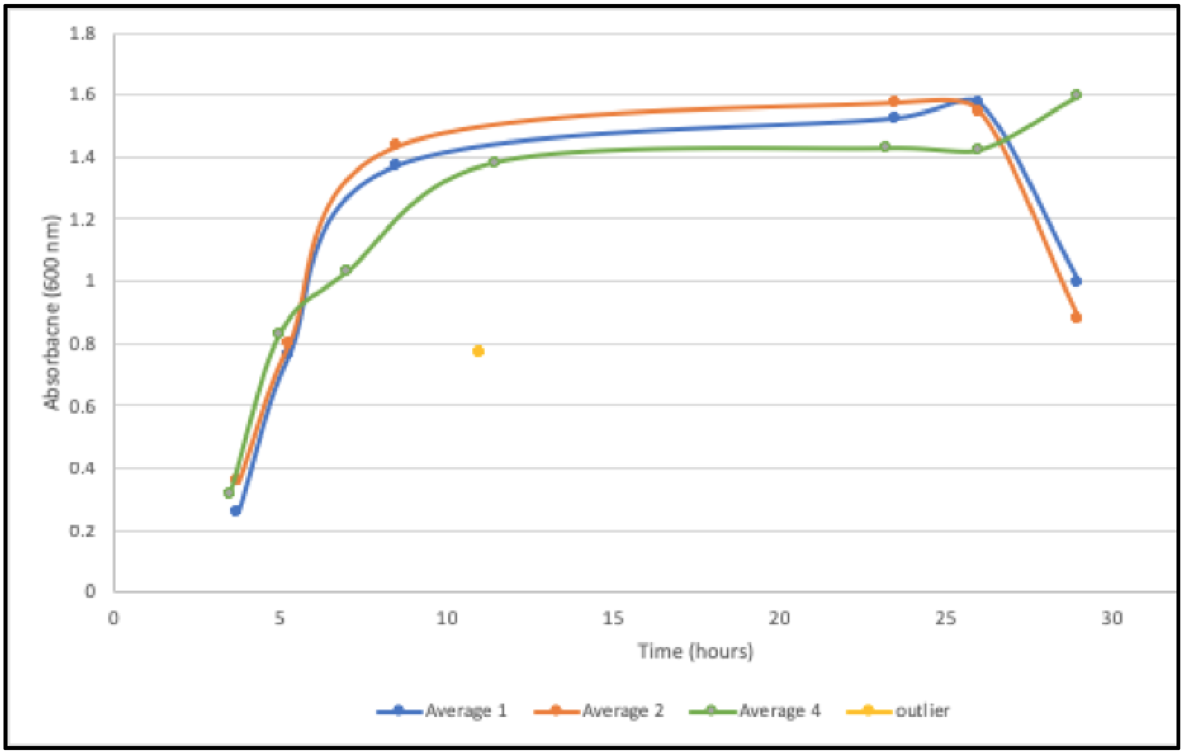


Figure 7. Average growth curves for *Bacillus cereus* (1, 2) and *Bacillus pumilus* (4) using a spectrophotometer at Saint John's University.

Discussion

Bacillus subtilis are known to inhibit gram-negative bacteria, yeasts, fungi, and some gram-positive bacteria through peptides known as bacteriocins. This inhibitory behavior suggests it may be beneficial to harvest and identify the compound(s) *Bacillus cereus* and *Bacillus pumilus* are producing because they may have similar effects on bacteria. Our results suggest *Bacillus cereus* and *Bacillus pumilus* do inhibit the growth of unknown bacteria samples throughout all stages of the growth curve (Figure 7). This is further supported by the zones of inhibition that showed decreased growth of the lawn (Figures 5 & 6). This potentially provides a competitive advantage for *Bacillus* in resource-limited environments such as on the copper roof they were isolated from. Additionally, it would be useful to identify what inhibitory compound(s) are being produced and what specific organisms they inhibit. Babasaki et al. (1985) extracted and purified an antibiotic from *Bacillus subtilis* through gel filtration and thin-layer chromatography. They explain that Subtilosin A had bactericidal activity against some gram-positive bacteria.

During experiments for obtaining the growth of bacteria 1 and 2, suspicious data was obtained during the time period of 11 hours. This was considered an error and the data was labeled as an outlier (Figure 7). The outlier data point could have come from contamination during the blanking procedures for the spectrophotometer, from debris on the cuvette containing the bacteria during blanking, or from improper orientation of the cuvette during that specific reading.

Future research should include the identification of the unknown bacteria lawn through PCR and BLAST techniques as well as production of a growth curve to help determine compounds released by the unknown. This would allow for further understanding on the competitive ability of the lawn used. As seen in the study by Sharma et al. (2018) explains that the *Bacillus* bacteriocin displayed antimicrobial activity against both gram-positive and gram-negative bacteria. Higher doses were required to inhibit the growth of gram-negative bacteria due to the outer membrane. Further studies could also test *Bacillus cereus* and *Bacillus pumilus* against gram-positive and gram-negative bacteria and determine appropriate inhibitory concentrations. Additionally, *Bacillus cereus* and *Bacillus pumilus* could be tested against various destructive bacteria that are able to live on copper surfaces within hospitals to determine their ability to inhibit the growth of these bacteria. Furthermore, once the inhibitory molecule is isolated, it would be beneficial to undergo mass spectrophotometry testing in order to determine what compounds contribute to the makeup of the inhibitory molecule. Along with the completion of mass spectrophotometry, it would be beneficial to determine the inhibitory molecule’s stability across differing pH values and temperatures in order to determine biochemical compatibility as a pharmaceutical agent.

References

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