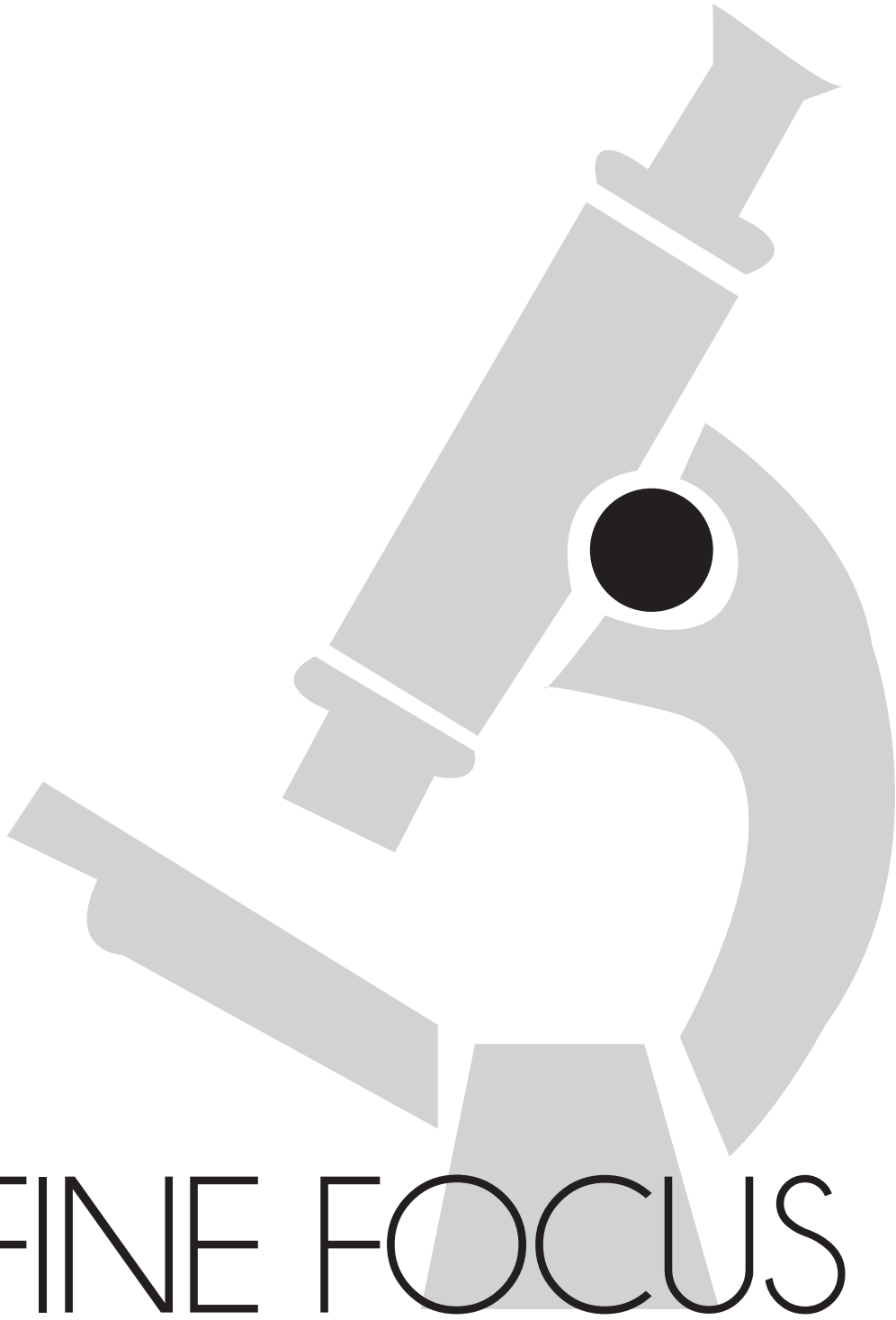


FINE FOCUS

AN INTERNATIONAL MICROBIOLOGY JOURNAL
FOR UNDERGRADUATE RESEARCH

MISSION

We publish original research by undergraduate students in microbiology. This includes works in all microbiological specialties and microbiology education.

SCOPE

We are an international journal dedicated to showcasing undergraduate research in all fields of microbiology. *Fine Focus* is managed entirely by undergraduate students from production to print but utilizes an External Editorial Board of experts for double-blind peer review of manuscripts.

CONTACT INFORMATION

Call: +1-765-285-8820
Email: finefocus@bsu.edu
Facebook: Fine Focus Journal
Twitter: @focusjournal
Online: finefocus.org

TABLE OF CONTENTS

PERSPECTIVE

OBJECTIVE LENS

JOHN L. MCKILLIP, PH.D
MANAGING EDITOR, FINE FOCUS
ASSOCIATE PROFESSOR OF BIOLOGY,
BALL STATE UNIVERSITY

The Campus Ambassador Program (CAP) was mentioned in this section of our last issue, and continues to be cultivated by our student editorial and marketing teams as a very important STEM outreach initiative. Last autumn, the effort was led single-handedly by student Monica Neeb, who had a minor in marketing/management and earned credit for this outcome. The CAP idea is meant to extend our network with carefully selected partner institutions. Monica started with the University of Detroit Mercy (UDM), and developed a CAP contract, formalizing the arrangement with UDM Biology Chairperson Dr. Stephanie Conant. This CAP partnership will allow Fine Focus resources (print journals & promotional materials) to be made available to faculty and students, as well as ensuring students at each partner institution are aware of publishing opportunities for undergraduates in microbiology research and facilitating submission of their manuscripts. Overall, the model of the CAP is meant to encourage high quality manuscripts from traditionally underrepresented students in the sciences, and to teach them the fundamentals of peer review. At the same time, the CAP partner universities will serve as feeder institutions for manuscripts in the long-term. In addition to UDM, we have initiated CAP arrangements for two international universities: the Technical University of Dresden (Germany), and the University of Akureyri (Iceland). Each Fine Focus CAP site will also have a heavy emphasis on general aspects of professional activities relevant to all areas of STEM, not just microbiology. We envision each CAP site to be best implemented as a recognized student organization on each campus.

Each Fine Focus CAP student organization will establish a constitution and by-laws (using the existing template at Ball State University Fine Focus as a guide), officers, and will be comprised of students from all STEM disciplines. Although the frequency and specific format for CAP meetings and activities will vary slightly at each institution, the activities could include:

- Cultivate new ideas on how Fine Focus might work with the American Society for Microbiology (ASM, our existing primary community partner) to promote diversity and inclusion in the sciences
- Promote the submission of eligible manuscripts to Fine Focus using print and other promotional materials (pens, business cards, pamphlets, t-shirts, and print journals) sent from Ball State University
- Negotiate the campus library at each institution to carry Fine Focus in the stacks.
- Work with student leaders and undergraduate researchers to advocate for resources, events, policies that support the practice of undergraduate research
- Work with their campus URO/URC to help deliver needed student programming
- Provide information on best practices in undergraduate research and creative activity to their department and campus leaders
- Meet with state and federal elected officials to advocate for undergraduate research at professional conferences in their discipline

If you are interested in learning more about how your institution could become a potential CAP partner, please get in touch with me; I would be happy to discuss additional details. We

If you are interested in learning more about how your institution could become a potential CAP partner, please get in touch with me; I would be happy to discuss additional details. We always welcome your suggestions and feedback by email at finefocus@bsu.edu

– JLM

FACULTY PERSPECTIVE

REBUILDETROIT AND FINE FOCUS COLLABORATION TO FOSTER SUCESSFUL STEM EDUCATION OPPORTUNITIES

STEPHANIE CONANT, PH.D
ASSOCIATE PROFESSOR & CHAIR,
DEPARTMENT OF BIOLOGY,
UNIVERSITY OF DETROIT - MERCY

Overview of the NIH ReBUILDetroit Program

The Building Infrastructure Leading to Diversity (ReBUILDetroit) NIH grant was awarded in 2014 as a Consortium-wide effort to expand and develop avenues for students from underrepresented and socio-economically disadvantaged backgrounds to experience an undergraduate education designed to expose them to biomedical research and career opportunities in STEM fields. The Consortium consists of faculty and staff from the University of Detroit Mercy (UDM) and Wayne State University, with the overarching goal to provide collaborative academic, pedagogical, and research-based opportunities for students that are accepted as ReBUILDetroit Scholars. ReBUILDetroit is one of only 10 NIH-funded BUILD awards in the country and has been an exciting and transforming opportunity for both students and faculty across the Consortium. The focus and timing of this grant support aligns nicely with the re-envisioning and revitalization of Detroit as a hub for urban invention and opportunity.

Benefits of the NIH ReBUILDetroit program at UDM and consortium institutions

The components of this NIH funded program for students and faculty at all institutions are numerous and diverse but include a pre-college summer enrichment program, cross-institutional collaborations, curricular re-design, and faculty-mentored research opportunities for ReBUILDetroit scholars as soon as the summer after their first year. In addition, UDM students are provided stipend and tuition support and intensive mentoring to help them navigate the academic and personal struggles that may come as they progress through their undergraduate education. Educational opportunities and research based pedagogy is available to scholars interested in one of three areas of research: biology, chemistry, and health disparities. ReBUILDetroit Scholars begin their first year with courses designed to provide hands-on authentic research experiences in the classroom that give them the technical and theoretical skills to approach learning and data collection with critical thinking, persistence, and confidence. Students are matched to research mentors in their summer after their first year and begin a collaborative relationship with a research mentor where they are able to further develop the skills they have learned in their research based courses and begin their transformation into productive and prolific research scientists.

Intensive mentoring, cohort building, leadership roles, and exposure to opportunities for these students in the different areas of science support academic and personal goals as well as open students' eyes to the wealth of career possibilities this unique undergraduate educational experience can lead to. All of this on top of a traditional degree-granting curriculum in their academic department of choice means that the ReBUILDetroit Scholars are immersed in a unique and supportive environment to guide them through the trials and tribulations of the college experience all the while giving them a great opportunity for experience and growth as future research scientists.

Over the last four years UDM and its ReBUILDetroit Scholars have learned a tremendous amount. Exposure to the process of how to choose a mentor that fits your learning style and goals, how to think like a scientist, and how to identify as a contributing member of a research team are new ideas and objectives for ReBUILDetroit Scholars. These skills are a model to ultimately build and sustain diversity in biomedical and health research professions in Detroit and around the country. Faculty have been provided mentoring training and experiences, pedagogical training for course-based undergraduate research experiences (CUREs), as well as professional training and guidance for advising and working through unique issues that students from diverse and disadvantaged groups may bring to their undergraduate education. These types of personal and professional development opportunities disseminate throughout the student body at

our university and make for stronger and more effective faculty to serve our students. Students in the ReBUILDetroit program at UDM have met with leading scientists and researchers in many fields of study from around the world. They have traveled to and presented at scientific conferences in Michigan, California, Georgia, Louisiana, and Washington DC, just to name a few. These travel and presentation opportunities are few and far between in a traditional undergraduate education so all academic institutions participating in ReBUILDetroit are excited and humbled by the opportunity to expose undergraduate researchers to as many avenues of growth as possible. Participation in the ReBUILDetroit NIH program has therefore expanded student horizons and their view of possibility.

UDM ReBUILDetroit/Fine Focus Collaboration

When we at UDM were approached by Dr. John McKillip, faculty mentor and Managing Editor of Ball State's *Fine Focus* publication, to collaborate on a project designed to explore and comment on diversity and its role in undergraduate educational opportunities in STEM fields, along with providing an opportunity for UDM ReBUILDetroit Scholars to gain experience in the process of double-blind peer-review and assembling a scientific publication we jumped at the opportunity. Initially the collaboration was most attractive as an avenue to expose young scientist in training at UDM to another aspect of being successful in biomedical research, peer-review and publishing of scientific manuscripts. Scholars have participated in generation and analysis of data, and even presentation of data in a conference setting however they had not delved into the process of getting that data published in a peer-reviewed publication; a hallmark of biomedical research success. Ten of our highly motivated ReBUILDetroit Scholars, Ball State student editors, staff, and faculty from both UDM and Ball State met four times over the course of the Fall 2017 semester for intensive manuscript review sessions and student interview opportunities. The *Fine Focus* team of students interviewed and recorded personal accounts of success and struggle that our UDM Scholars have experienced in their pursuit of a career in the biomedical sciences. These candid and well-These candid and well-structured interviews drew out the most honest and humbling accounts of persistence, self-awareness, and tenacity from our UDM Scholars and opened even our eyes here at UDM to the potential that our students possess.

What we found when our two schools provided these opportunities for student interactions was a deep reflection from students from both universities as to what has led them to scientific fields of study, what challenges they have faced, and how they have overcome these challenges and walked away with a better understanding of themselves and the strength it takes to reach the goals they have set for themselves both personally and professionally. Student perspectives published in the Spring 2018 issue (finefocus.org) label this collaboration as "insightful", "enjoyable", and "fullfilliing". These descriptions, I believe, hold true for faculty and staff involved as well. As a faculty member advising and guiding ReBUILDetroit Scholars through academic programs and research opportunities I am thoroughly impressed and proud of the mature and open attitude that all students demonstrated throughout this collaboration. Students, faculty, and staff from both universities were able to share meals, stories, goals, and a lot of laughs over the course of these campus visits leading to a bond and respect that we at UDM hope will continue for years to come. We at UDM believe that opportunities such as this collaboration with the *Fine Focus* team and others like it show that the mission of the ReBUILDetroit grant and collaborative opportunities such as this one are vital to the future of science education and providing quality professionals in all areas of STEM.

“

FACULTY PERSPECTIVE KRISTEN PICARDO, Ph.D

ASSOCIATE PROFESSOR, BIOLOGY DIRECTOR, CENTER FOR STUDENT SCHOLARSHIP, RESEARCH, AND CREATIVE WORK ST. JOHN FISHER COLLEGE

On a recent flight I was seated next to an undergraduate Ivy League college student. She was exhausted after having chosen to leave a stint in a military training academy and slept for a solid hour, mouth wide open. When she awoke, we assumed the typical mentor–student chit–chat even before she realized I was a biology college professor and she an undergraduate biology major grappling to plan her future. She was confused and needed to tell someone about it, and I couldn't help but be her sounding board, offering free advice – get in the lab and try a research experience!

Often times, students seek out a welcoming professor to ask about how they can get involved as a student researcher. At my small PUI, our doors are open, our classes are small, and so we enjoy plenty of student interaction and time to explore such conversations. We connect our students with our colleagues who have research expertise in the students' favorite course topic and natural connections occur.

At times this serendipitous method isn't the best approach. If a student is one of many or is not comfortable approaching a professor, it can be helpful if the college or program offers details on a website or through scheduled information sessions. Some colleges have formalized offices that serve to reach out to educate students about research opportunities on and off their home campus. Attending these sessions can be very helpful and informative and remove the hesitation some students feel about approaching a faculty mentor on their own. A survey at my own institution revealed that most of our students learn about research opportunities through talking with other students. Attending research presentations hosted by departments is also a great way to learn about opportunities.

When students approach me or when I discuss research projects ongoing in my laboratory, I do two things: 1) get them excited and 2) use language that shows them that my research is accessible, understandable, and not just for senior students. In order to succeed, I've learned that students in my lab need to be excellent communicators. I am not with them every hour they spend in the lab, and therefore I ask for meetings and pop–ins as well as electronic updates as students plan experiments and decipher their data. Sometimes, I am "busy in meetings" when I know I want my students to struggle a bit and work toward a solution to a problem they are facing in their project. In order for a student to be ready to take on a project with me, they must have the maturity to handle to recursive nature of a sometimes frustrating process. Finally, they must have time in their schedules to fully commit. The best way for students to be an attractive candidate for a project is to show their potential mentor that they have time in their schedule to fully engage with the research (e.g. minimum course load not including research,

Through mentored research experiences, students grow in their ability and confidence to creatively analyze information in new ways often leading to novel solutions to complex problems. They learn practical, transferrable skills and resiliency allowing them to grow from challenging experiences. These experiences prepare students for advanced work in their disciplines, and more importantly ignite their curiosity and strengthen their ability to adapt and utilize core skills and habits of mind that will prime them for their futures. It is no wonder that employers and graduate programs look highly on how these experiences prepare the future workforce. As I told the sleepy student, a research experience will transform and direct one's future in ways traditional classwork cannot. I look forward to reading her first manuscript someday. that will prime them for their futures. It is no wonder that employers and graduate programs look highly on how these experiences prepare the future workforce.

This piece was received as an unsolicited contribution to XX at ASM from a group of high school students in Dubai UAE. The form is that of a generally written research proposal. We opted to take it and publish it as a Letter to the Editor here, in order to serve as an example of the type of research-based assignments in STEM curricula internationally. Please direct all queries or questions to Ms. Sarah Groves, teacher using email provided.

LETTER TO THE EDITOR:

GENETIC EDITING OF SECRETORY PATHWAY OF *PENICILLIUM CHRYSOGENUM* AFTER OBSERVATION OF INCREASED SECRETORY RATES IN AN INCREASED STRESS ENVIRONMENT (MICROGRAVITY), A RESEARCH PROPOSAL BY HIGH SCHOOL STUDENTS IN DUBAI

AKIO SHIRALI [1,*], WILSON MINTER HUIJSMANS [2,*] AND MATTEO SOTTOCORNOLA [3,*]

[1] THE CRANLEIGH SCHOOL, ABU DHABI, UNITED ARAB EMIRATES

* AUTHORS HAVE CONTRIBUTED EQUALLY TO THIS WORK

MANUSCRIPT RECEIVED 12 DECEMBER 2017

CORRESPONDING AUTHORS

Akio Shirali
Student, Cranleigh AD, Abu Dhabi, UAE
Email: shiraliakio@gmail.com
Ms. Sarah Groves
Science Teacher, the Cranleigh AD, PO BOX 126888 Abu Dhabi, UAE
Email: sgroves@cranleigh.ae
Wilson Minter Huijsmans
Student, Cranleigh AD, Abu Dhabi, UAE
Email: wilbuoy@gmail.com
Matteo Sottocornola
Student, Cranleigh AD, Abu Dhabi, UAE
Email: m.sottocornola@icloud.com

KEYWORDS

- *Penicillium Chrysogenum*
- CRISPR cas-9
- miniPCR
- RTQ-PCR
- microgravity
- beta – lactam antibiotics
- pcbAB
- pcbC
- penDE

ABSTRACT

In this study, we aim to amplify the secretory pathway of *Penicillium Chrysogenum* within the ISS or similar simulated microgravity using the miniPCR and/or RTQ-PCR and then optimizing *Penicillium Chrysogenum* function using CRISPR cas-9 (Clustered Regularly Interspaced Short Palindromic Repeats), a new technology in the genetics which can help in gene alteration for better drug production. The secretory pathway of *Penicillium Chrysogenum* is controlled by genes pcbAB , pcbC and penDE

INTRODUCTION

The secretory pathway of microorganisms have shown promising results in regard to increased secretory rates when simulated by microgravity. Therefore, in microgravity, this microorganisms can be used to obtain higher amount of microorganisms' secon`dary metabolites. Beta lactam bacteria are of great biotechnological interest due to their ability to secrete bioactive antibiotica as secondary metabolites. Secondary metabolites are organic compounds produced through the modification of primary metabolite synthesis. Secondary metabolites do not play a role in growth, development, and reproduction like primary metabolites do, because they are typically formed during the end of log phase or near the stationary phase of growth. The secretory pathway of *Penicillium Chrysogenum* is controlled by genes pcbAB, pcbC and penDE. These genes control the production of penem, a type of beta lactam antibiotics. Space flight experiments have been suggested to affect cellular processes in microorganisms. For instance, preliminary reports on the effects of spaceflight on secondary

metabolism. In our study, we plan to fill the gap between same quality higher yields versus same quality lower yields of secondary metabolites during space flights. Furthermore, *Penicillium Griseofulvum* is found to have a penicillin gene cluster similar to that of *Penicillium Chrysogenum*. No other species among the studied fungi were found to produce penicillin or to possess the penicillin biosynthetic genes, except *P. verrucosum*, which contains the pcbAB gene but lacks pcbC and penDE. Hence *Penicillium Chrysogenum* is our fungi of choice. The process of regulation of penicillin biosynthesis has been studied for many years. Specifically, the improvement of *P. chrysogenum* strains to obtain higher penicillin yields is a main intense objective in industrial research. To simulate the microgravity environment on earth, several models have been developed and applied to examine the effect of microgravity on secondary metabolism. The purpose of our study is to use *Penicillium Chrysogenum* for production of higher penicillin yields in microgravity environment.

METHODS

We divided the method of the experiments into two parts:

1– Freeze and fly: A simple experiment where the *Penicillium Chrysogenum* is monitored in a low gravity environment on earth. This acts as a proof of concept experiment.

2– Further plan: Use of data obtained by the freeze and fly experiment to edit the microbe gene structure using CRISPR technology in order to produce a permanent artificially induced stress condition to increase penicillin production rate by *Penicillium Chrysogenum*.

1- FREEZE AND FLY EXPERIMENT:

The "Freeze and fly experiment" will be a proof of concept and a chance to gather information on how the expression rate of genes is different to the ones on plain gravity. Genes for the biosynthesis of secondary metabolites are arranged in clusters together with genes for resistance to the toxic action of secondary metabolites on the producer organisms. Likewise, mRNA templates of the Penicillin biosynthesis cluster of *P. Chrysogenum* – pcbAB, pcbC , and penDE will be used on the miniPCR. These templates will be prepared and purified on earth. The frozen mRNA templates will be sent on the ISS to run the miniPCR. The freeze and fly will be used with the three genes (*pcbAB* , *pcbC* and *penDE*) and their primers (from Ref 15):

pcbAB:

FWD: GAA GAC GTC ATA CTT ATT CTC TG
REV: CGG CAT CGG ATA AAG AGA TCT GG

pcbC:

FWD: GAT TGG CGC TCC TCG TTC ACC REV: CCA TTA TTT TTC TAG TCG ACA TGG CAT CGA TTC CCA AGG CCA ATG TCC CC

penDE:

FWD: CCC GCA GCA CAT ATG CTT CAC ATC CTC TGT CAA GGC

REV: ATG ACA AAC ATC TCA TCA GGG

How can this help the world? Our Idea is there to fill any of two voids:

1. The idea can be used to increase the production of stock which then can help

decrease the problem of a low inventory. This will make sure that in a financial sense, the constant fear of not being able to accommodate the high demand with a low supply can be cancelled or at the least reduced.

2. The increase in the stock of medicine can be help full as any charity buys the medicines at either a lower cost or in a greater quantity at the same cost which will help the underprivileged to help procure a medicinal cure to ailments that they may suffer from.

We hope that our work can have a massive impact on the lives of others, as the blessing and happiness of a person is far greater than any sort monetary gain that anyone or we could gain.

Considering the method that we have tried to introduce here we believe that this will have many implications on the pharmaceutical business as there are many types of medicines procurable from bacterial secondary metabolites. Here are some examples:

1. Cancers– the chemotherapy drugs come from secondary metabolites of plant.

2. Diabetes Type 1 – Humulin, the most successful medicine to treat type 1 diabetes is the recombinant insulin produced in bacteria Escherichia coli and yeast.

The experiment will be done in a miniPCR in duplicate, with 8 sample reactions as following:

Sample 1 – Control: no polymerase	Sample 5 – Primers pair: pcbC
Sample 2 – Control: no template	Sample 6 – Primers pair: pcbC
Sample 3 – Primers pair: pcbAB	Sample 7 – Primers pair: penDE

Table 1: PCR experiment will be run on the genes pcbAB, pcbC and penDE from Penicillium Chrysogenum

While this is being done in microgravity there would be another batch from the same strain of Penicillium chrysogenum would be monitored on normal gravitational conditions.

2- FURTHER PLAN EXPERIMENT:

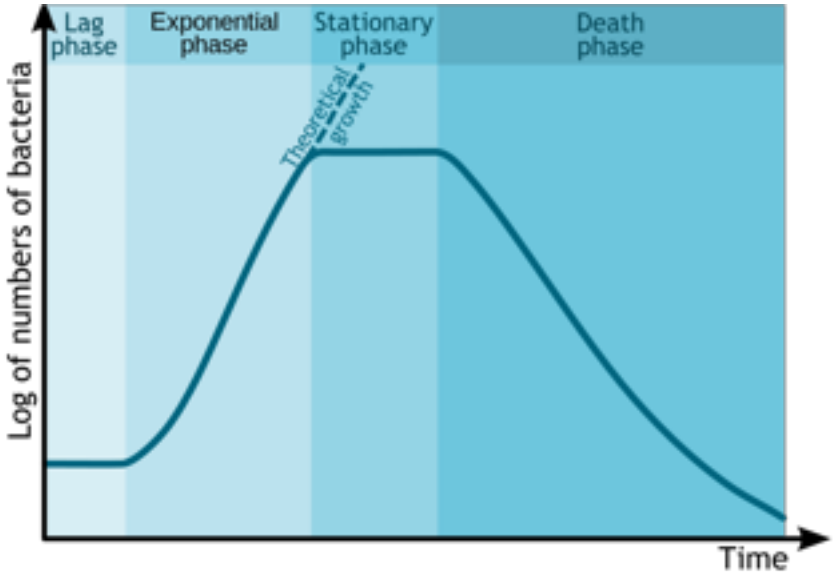
The expression rates of the three genes on earth would be compared to their expression rates in microgravity. This would be done using a reverse transcription method form their RNA to create a cDNA, a technique known as RT PCR. When this is done, we plan to create a draft plasmid containing an edited version of the DNA to account for the changes that have been made in the expression rates in microgravity. This plasmid would then be implanted into a cell which can express the plasmid; like F+ cell. Once F+ cell is placed with a group of F– cells, the DNA as a plasmid can move from the F+ cell to the F– cell via the help of the protein relaxosome and relaxase. This is the process of conjugation. Once complete the F+ cell could be placed in a colony with massive amounts of F– cells. This would then allow the F– cells to procure the same traits of the F+ cell, which is an increased secretory rate.

Why the stationary phase is important for secondary metabolites production?

First, we would like to reemphasize the objective of the experiment "Genetic editing of secretory pathway of *Penicillium chrysogenum* , after observation of secretory rates in increased stress environment".
This idea is based on multiple facts:

1. Stress that is induced on micro bacteria increases the rate of secondary metabolite production [1] .
2. Genetic editing is now quite widely used.
3. Other studies done on bacterium with similar genomic structures have shown promising results [2, 4] .

To understand the process of secondary metabolite production, it is important to take into account that secondary metabolite production is only temporary as a results of adaptation to stress by bacteria and fungi. This best to compare to puts of Charles Darwin, one of history's best biologist, "it is not the strongest that survives, but the species that survives is the one that is able to adapt to and to adjust best to the changing environment in which it finds itself". To ensure that the fungi cannot adapt to the stress we propose that the edited stress is added to the micro bacteria in their stationary phase (see graph 1).



Graph 1: The Bacterial growth curve [1,4] This is to ensure that the bacterium does not create offspring that are resistant to the stress because they die at the end of the graph due to lack of nutrients and food.

DISCUSSION

How can this help the world?? Our Idea is there to fill any of two voids:
1- The idea can be used to increase the production of stock which then can help decrease the problem of a low inventory. This will make sure that in a financial sense, the constant fear of not being able to accommodate the high demand with a low supply can be cancelled or at the least reduced.

2- The increase in the stock of medicine can be help full as any charity buys the medicines at either a lower cost or in a greater quantity at the same cost which will help the underprivileged to help procure a medicinal cure to ailments that they may suffer from .

We hope that our work can have a massive impact on the lives of others, as the blessing and happiness of a person is far greater than any sort monetary gain that anyone or we could gain.

Considering the method that we have tried to introduce here we believe that this will have many implications on the pharmaceutical business as there are many types of medicines procurable from bacterial secondary metabolites. Here are some examples:

1- Cancers- the chemotherapy drugs come from secondary metabolites of plant. 2- Diabetes Type 1 - Humulin, the most successful medicine to treat type 1 diabetes is
the recombinant insulin produced in bacteria Escherichia coli and yeast.

ACKNOWLEDGEMENTS

- Dr. Wael Mohammed Osman (Khalifa University, Abu Dhabi, UAE)
- Dr. Claudia Schafer (MIT, Boston, USA)
- Mr. Matthew MacNaught (The Cranleigh School, Abu Dhabi, UAE)
- Ms. Sarah Groves (The Cranleigh School, Abu Dhabi, UAE)
- Mr. Damien Ward (The Cranleigh School, Abu Dhabi, UAE)
- miniPCR (Amplyus 1770 Massachusetts Avenue Cambridge MA 02140 USA)

ABOUT THE AUTHORS

Akio Shirali
Hello, my name is Akio Shirali, I am a 13 year old student at Cranleigh Abu Dhabi. I have always been interested in the sciences and have always excelled in mathematics. One of my main motivations is my Grandmother who was a microbiologist. My area of specialty is biology.

Wilson Minter Huijsmans
My name is Wilson Minter Huijsmans and I am 15 years old. I have always excelled at Mathematics and the Sciences, specifically Physics. I have won/achieved multiple things in all these areas, with the most recent being a finalist prize in the Genes in Space UAE competition, the motivation for this paper, alongside my science obsessed teammates, the co-authors of this paper.

Matteo Sottocornola

Hello, my name is Matteo Sottocornola. I'm Italian and French and speak fluently three languages. I'm currently studying for my A levels as I am a 16 years old. My main interests lie in the fields of Physics and Mathematics although I also take interest in subjects such as biology and chemistry.

REFERENCES

1. Ana M. Calvo, Richard A. Wilson, Jin Woo Bok, and Nancy P. Keller. 2002. Relationship between Secondary Metabolism and Fungal Development. *Microbiol Mol Biol Rev*; 66(3): 447–459.

2. Hong Gao, Zhiheng Liu, Lixin Zhang. Secondary metabolism in simulated microgravity and spaceflight. *Protein Cell* 2011. 2(11): 858–861 DOI 10.1007/s13238-011-1125-z

3. Microbiology Book Series number 2: Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology (Volume 1), Publisher: FORMATEX RESEARCH CENTER, Badajoz, Editors: A. Méndez-Villas, pp.577-588.

4. Nitin Kumar Singh, Adriana Blachowicz, Aleksandra Checinska, Clay Wang, Kasthuri Venkateswaran. Draft Genome Sequences of Two Aspergillus fumigatus Strains, Isolated from the International Space Station. *Genome Announc* . 2016 Jul-Aug; 4(4): e00553-16.Published online 2016 Jul 14. doi: 10.1128/genomeA.00553-16

5. Efimov VP, Morris NR. The Lis1-Related Nudf Protein of Aspergillus nidulans Interacts with the Coiled-Coil Domain of the Nude/Ro11 Protein. *J Cell Biol* . 2000 Aug 7; 150(3): 681–688.

6. Marco A van den Berg, Richard Albang, Kaj Albermann, Jonathan H Badger *et al*. 2008. Genome sequencing and analysis of the filamentous fungus *Penicillium chrysogenum*. *Nature Biotechnology* 26:1161-1168.

7. Eric Fourest, Catherine Canal and Jean-Claude Roux. 1994. Improvement of heavy metal biosorption by dead biomasses and *Penicillium* pH control and mycelial (*Rhizopus arrhizus, Mucor miehei chrysogenum*). *FEMS Microbiol. Rev.* 14(4):325-332.

8. David J. Smith, Martin K. R. Burnham, John H. Bull et al. B-Lactam antibiotic biosynthetic genes have been conserved in clusters in prokaryotes and eukaryotes.

9. . Henry F. Chambers, Delphine Moreau, David Yajko *et al*. 1990. Can Penicillins and Other -Lactam Antibiotics Be Used To Treat Tuberculosis? *EMBO J*. 9(3):741-747.

XYLANOLYTIC PSYCHOTROPHS FROM ANDOSOLIC SEDGE FENS AND MOSS HEATHS IN ICELAND

OLIVIA J. RICKMAN[1], M. AUÐUR SIGURBJÖRNSDÓTTIR[2], ODDUR VILHELMSSON [2,3*]

1UNIVERSITY OF READING SCHOOL OF BIOLOGICAL SCIENCES, RG6 6UR, ENGLAND. PRESENT ADDRESS: THE UNIVERSITY EXETER MEDICAL SCHOOL, EXETER

2UNIVERSITY OF AKUREYRI FACULTY OF NATURAL RESOURCE SCIENCES, AKUREYRI, ICELAND.

3UNIVERSITY OF ICELAND BIOMEDICAL CENTER, REYKJAVÍK, ICELAND.

MANUSCRIPT RECEIVED 11 OCTOBER 2017; ACCEPTED 15 MAY 2018

CORRESPONDING AUTHOR

Oddur Vilhelmsson
oddurv@unak.is

KEYWORDS

- xylanolytic
- bacteria
- glycohydrolase
- moss heaths

ABSTRACT

Nine xylanolytic bacterial strains were isolated from fen and heath soils in northern Iceland. They were found by 16S rRNA gene sequencing to belong to the genera *Paenibacillus, Bacillus, Pseudomonas,* and *Stenotrophomonas.* Using a simple, plate-based semiquantitative assay with azo-crosslinked xylan as the substrate, it was determined that although isolated from cold environments, most of the strains displayed greater xylanolytic activity under mesophilic conditions, with only the paenibacilli displaying markedly cold-active xylanolytic activity. Indeed, for one isolate, *Paenibacillus castaneae* OV2122, xylanolytic activity was only detected at 15°C and below under the conditions tested. Of the nine strains, *Paenibacillus amylolyticus* OV2121 displayed the greatest activity at 5°C. Glycohydrolase family-specific PCR indicated that the paenibacilli produced multiple xylanases of families 10 and 11, whereas a family 8 xylanase was detected in *Pseudomonas kilonensis* AL1515, and a family 11 xylanase in *Stenotrophomonas rhizophila* AL1610.

INTRODUCTION

Xylanases, catalyzing the endohydrolysis of 1,4-β-D-xylosidic linkages in the hemocellulosic polymer xylan, are a widespread group of enzymes that play an important role in plant detritus degradation and are of considerable importance in many industrial processes, such as in the paper and food industries (3, 7). Unsurprisingly, xylanase-producing microorganisms are typically found on plants or in association with plant material, such as in the phyllosphere and rhizosphere (15, 32). While xylanases have been isolated from various extremophiles, in particular thermophiles (7), research on cold-adapted xylanases is relatively scarce. Several xylanases have nevertheless been isolated from psychrophiles (12, 19) and found to possess common features such as a low temperature optimum and poor thermostability due to fewer salt-bridges and less compact hydrophobic packing as compared to their mesophilic and thermophilic counterparts (7). Xylanolytic activity is among the most important polymer degradation activities in the phyllosphere and xylanolytic bacteria can thus be expected to be important players in plant detritus turnover. Furthermore, xylanases are valuable enzymes that are used in several processes within the food and paper industries. Cold active xylanases and psychrotrophic xylanolytic organisms are thus of considerable interest both to biotechnology and cold-climate ecology.

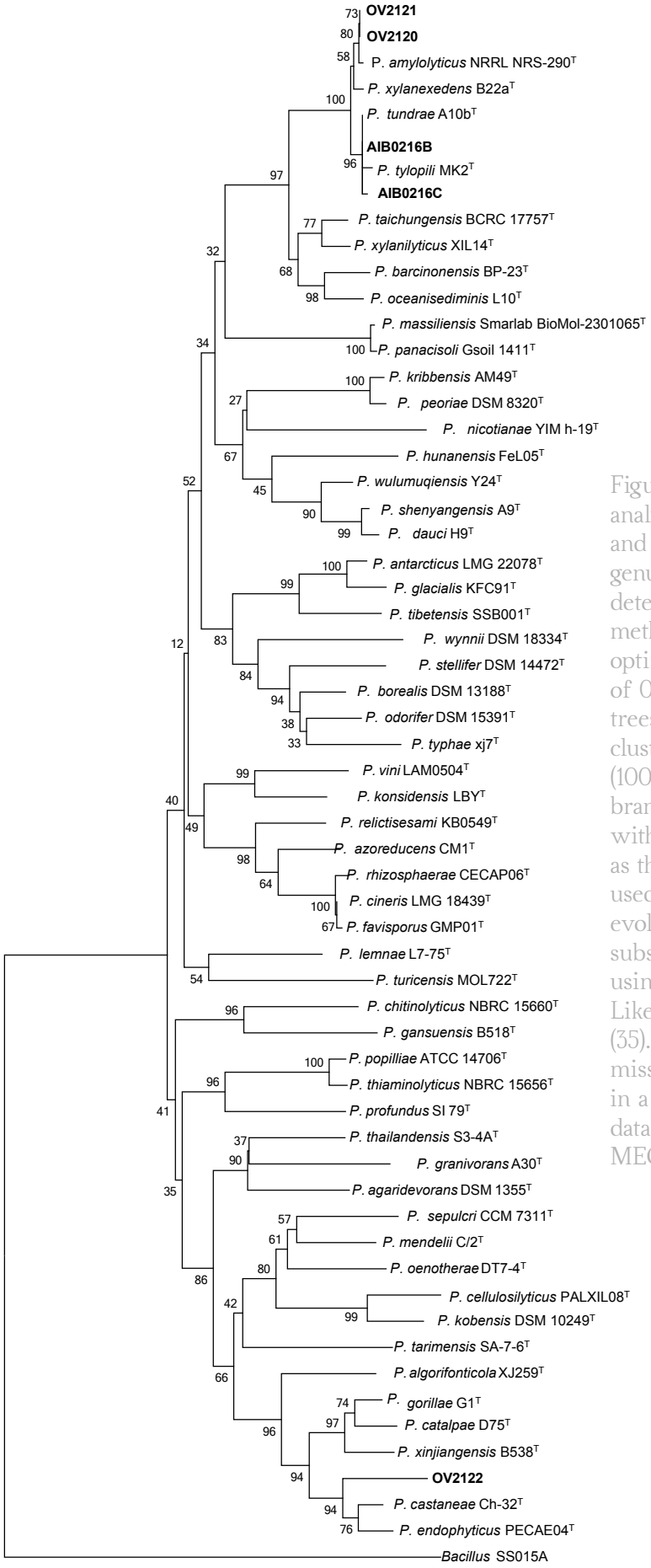


Figure 1. 16S rRNA gene phylogenetic analysis of the *Paenibacillus* isolates and selected type strains from that genus. Phylogenetic relationships were determined using the Neighbor–Joining method of Saitou and Nei (33). The optimal tree had a branch length sum of 0.8577. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (10). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances (number of base substitutions per site) were computed using the Maximum Composite Likelihood method of Tamura et al. (35). All positions containing gaps and missing data were eliminated, resulting in a total of 1369 positions in the final dataset. The analysis was conducted in MEGA6 (36).

In this study, we present nine psychrotrophic xylanolytic bacterial strains from three soil habitat types in northern Iceland, identified as paenibacilli, pseudomonads, a *Bacillus* and a *Stenotrophomonas*, and discuss the temperature-dependence of their xylanase activity and the likely glycohydrolase family of their respective xylanases.

MATERIALS AND METHODS

ISOLATION OF BACTERIA

Leptosolic and andosolic sites (Table 1) were sampled aseptically with sterile implements into sterile Falcon tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) or WhirlPak bags (Nasco, Fort Atkinson, WI, USA). To obtain microbial suspensions, soil samples were placed in a tenfold volume of Butterfield's buffer (Butterfield 1932) at pH 7.2 in a sterile stomacher bag and stomached in a LabBlender 400 (Seward, Worthing, UK) for 2 min. The slurry was then serially diluted in Butterfield's buffer to 10^{-6}. All dilutions were spread-plated in duplicate onto Plate Count Agar (PCA), Tryptic Soy Agar (TSA), and Actinomycete Isolation Agar (AIA) (Becton-Dickinson) and plates incubated in the dark at 15°C for four weeks. The plates were examined for colony morphotypes based on color, sheen, convexity and other visible features. Representatives of each morphotype were aseptically picked, streaked onto fresh media and incubated under identical conditions as the original isolation plates. In order to obtain pure cultures, the isolates were re-streaked and incubated at least once more. Stocks of purified isolates were prepared by suspending a loopful of growth in 0.5 ml 28% (v/v) glycerol and stored at -70 °C.

XYLAN DEGRADATION SCREENS AND ASSAYS

Degradation of xylan was screened for by the appearance of blue halos on an 0.5 g l^{-1} suspension of azo-cross-linked birch xylan (Megazyme,Wicklow, Ireland) in a medium that consisted of 4 g Nutrient Broth (NB) and 15 g l-1 agar (Becton-Dickinson). When used as a semi-quantitative xylanase assay, halo emergence on equal-volume (20 ml) plates was monitored twice daily and halo diameter measured with a ruler. One-way ANOVA on terminal halo diameters was used to determine effect of temperature on xylan degradation. Precultures were incubated in the presence of xylan.

PHENOTYPIC CHARACTERIZATION

Several standardized biochemical tests were performed using the API 20E test strips (Biomériaux) according to the manufacturer's instructions.

16S rRNA GENE SEQUENCING

Colonies were picked using sterile toothpicks and suspended in 25 µl colony lysis buffer (1% Triton X-100 in 0.02 mol l-1 Tris / 0.002 mol l-1 EDTA at pH 8.0). The resulting suspension was heated to 95°C for 10 min and then cooled to 4°C and 1 µl of the suspension used as a template in a standard Taq-PCR reaction (35 cycles, annealing at 51°C for 30 sec, extension at 68°C for 90 sec, denaturing at 95°C for 30 sec) in an MJR PTC-200 thermocycler (MJ Research Inc., Waltham, MA, USA). The primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1522R (5'-AAGGAGGTGATCCAACCGCA-3')

Depth (cm)	Strain no.	Isolation medium	Colony morphology
Site 1. Sandabotnaskarð (65.685°N, 16.769°W).			
Brown andosol. Sparse vegetation.			
10	AIB0216B	PCA	White, flat, dry
10	AIB0216C	PCA	White, flat, dry
10	AIB0228	TSA	Cream-colored, convex
Site 2. Svartárkot (65.336°N, 17.242°W)			
Brown and histic andosol. Moist moss heath.			
5	AL1515	PCA	Cream-colored, convex
17	AL1610	AIA	Tan, convex, glistening, small
17	AL1614	PCA	White, matte, convex, grainey
25	DO1702	AIA	Cream-colored, irregular, glistening, slimey
Site 3. Hesteyrarskarð (66.358°N, 22.930°W)			
Histic andolsol. Short sedge fen.			
5	OV2120	TSA	White, flat, dry
5	OV2121	TSA	White, flat, dry
5	OV2122	TSA	White, flat, dry

Table 1. Strains, source samples, and isolation media; Plate Count Agar (PCA), Tryptic Soy Agar (TSA) and Actinomycete Isolation Agar (AIA).

were used at a final concentration of 0.2 µM in a total volume of 25 µl of PCR mixture containing 0.15 µl of Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA).

16s rRNA GENE SEQUENCING CONT.

The primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1522R (5'-AAGGAGGTGATCCAACCGCA-3') were used at a final concentration of 0.2 µM in a total volume of 25 µl of PCR mixture containing 0.15 µl of Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA). Amplicons were visualized on an 0.8% agarose gel using SYBR Safe (Life Technologies, Carlsbad, CA, USA) and cleaned up for sequencing using 20 U µl-1 exonuclease I and 5 U µl-1 Antarctic phosphatase (New England Biolabs) at 37°C for 30 min, followed by deactivation at 90°C for 5 min. Partial sequencing of the purified amplicons was performed with a BigDye terminator kit and run on Applied Biosystems 3130XL DNA analyzer (Applied Biosystems, Foster City, USA) at Macrogen Europe, Amsterdam, the Netherlands using sequencing primers 519F (5'-CAGCAGCCGCGGTAATAC-3') and 926R (5'-CCGTCAATTCCTTTGAGTTT-3'). Sequences were examined in ABI Sequence Scanner 1.0 (Applied Biosystems, Framingham, MA, USA) and the forward and reverse complement of the reverse sequence manually aligned and merged.

PHYLOGENETIC ANALYSIS

The 16S rRNA gene sequences were assigned

to taxa based on the identification of phylogenetic neighbors as determined by a BLASTN (1) search against a database containing type strains with validly published prokaryotic names and representatives of uncultured phylotypes (18). The top thirty sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using a global alignment algorithm (22), which was implemented at the EzTaxon server (18). For further phylogenetic analysis, sequences were multiply aligned using MUSCLE (9) and bootstrapped (10) neighbor-joining trees (33) were calculated in MEGA6 (36) using the Maximum Composite Likelihood model (35).

AMPLIFICATION OF XYLANASE GENE FRAGMENTS

Genomic DNA was extracted and purified from cultures grown on Nutrient Agar (NA) plates using MoBio Microbial DNA Isolation kit (MoBio Laboratories, Solana Beach, CA) according to the manufacturer's instructions. Primers specific to xylanases of families 8 (Xyn8A, forward: 5'-CATCCTGTTCAGGAAGACAGTAGTGGGG-3', reverse: 5'-CTTGATAATCCGGAAATTGCCACTGACATGC-3'), 10 (XynFA, forward: 5'-CACACKCTKGTKTGGCA-3', reverse: 5'-TMGTTKACMACRTCCCA-3'), and 11 (PAXynA, forward: 5'-GAYTAYTGGCARTAYTGGAC-3', reverse: 5'-ATRTCRTANGTNCCNCCRTCRCT-3') were used for amplification. Reaction conditions, amplicon clean-up and sequencing were as described for the 16S rRNA gene amplification, above.

RESULTS

IDENTIFICATION OF XYLANOLYTIC ISOLATES

Isolated bacterial strains (106) from andosolic wetland and moss heath sites (Table 1) were screened for the ability to yield blue halos on azo cross-linked xylan plates at 15°C, and nine positive isolates selected for further study. The isolates were identified by 16S rRNA gene sequencing (Table 2) and found to comprise paenibacilli (5 isolates), pseudomonads (2 isolates), a stenotrophomonad and a *Bacillus*.

A pairwise distance comparison of the MUSCLE multiply aligned 16S rRNA gene sequences obtained for all nine strains (Table 3) reveals that four of the paenibacilli, strains OV2120, OV2121, AIB0216B, and AIB0216C are very closely related to one another, with on average less than 2 base substitutions over the 987 nucleotide positions tested.

A phylogenetic analysis of the paenibacilli (Fig. 1) was performed using 54 *Paenibacillus* type strain reference sequences. The reference sequences were selected based firstly on their similarity to the test strains as determined by the BLASTN algorithm on the EzTaxon server and secondly based on their completeness, enabling us to run the phylogenetic analysis over 1369 nucleotide positions. The analysis revealed that isolates OV2121, OV2120, AIB0216B, and AIB0216C form a clade with *P. amylolyticus*, *P. xylanexedens*, *P. tundrae* and *P. tylopili*, supporting the EzTaxon assignments of isolates OV2120 and OV2121 as *P. amylolyticus*, and AIB0216B and AIB0216C as *P. tundrae*. The divergence of strain OV2122 from the other four paenibacilli was further demonstrated on the phenotypic level by biochemical characterization using the API 20E strip test, in which OV2122 was distinguished by lack of ONPG hydrolysis, presence of arginine dihydrolase and gelatinase, citrate utilization, and lack of sucrose and amygdalin utilization (Table 4).

PRESENCE AND FAMILY OF XYLANASE GENES

To independently verify the presence of glycohydrolase family 8, 10 or 11 xylanase genes, PCR reactions using primer pairs Xyn8A, XynFA, and PAXynA were run on DNA extracted from pure cultures of isolated strains, as well as of the Az-Xylan screen–negative strain DO1702 as a negative control (Fig. 2). Selected product bands were extracted and sequenced (Table 5). Under the reaction conditions employed, the family 8 primer pair Xyn8A yielded apparent primer dimers in all cases, but nevertheless clear product bands at approximately 400 bp were visible for strains AL1515, OV2121, and OV2122. The AL1515 band was sequenced and found by BLASTx to contain a 359-bp product most similar to a hypothetical protein of unknown function in the Betaproteobacterium Andreprevotia chitinilytica, and thus probably constitutes an unspecific amplification of an unknown component of the AL1515 genome, or possibly a hitherto unidentified xylanase. The family 10 primer pair XynFA yielded a variety of amplicons: a large (>10 kb) product from strain AL1610, an approximately 150 bp product from strain OV2122, an approximately 250 bp product from strain OV2122, three products (3 kb, 1 kb and 700 bp) from each of strains OV2120 and OV2121, and two products (150 and 750 bp) from strain AIB0216B. Of these, three amplicons were sequenced (both AIB0216B products and the AIB0228 product), one of which was found to correspond to a *Paenibacillus* xylanase, while the others showed greatest similarity to proteins of unknown function (Table 5). The family 11 primer pair PAXynA yielded an approximately 200 bp–product from strain AIB0216C, and approximately 800-bp products from strains AL1610, OV2120, OV2121, and OV2122. The products for AL1610, OV2121, and OV2122 were partially sequenced and all displayed greatest similarity to *Paenibacillus* endo-1,4-beta xylanases (Table 5).

Isolate	Sequence length (nt)	GenBank accession	Most similar taxon at EzTaxon[1] (% similarity)
AIB0216B	1523	KX349197	*Paenibacillus tundrae* (99.87)
AIB0216C	1452	KX349198	*Paenibacillus tundrae* (99.79)
AIB0228	1436	KX349200	*Pseudomonas baetica* (99.30)
AL1515	998	KX349201	*Pseudomonas kilonensis* (99.50)
AL1610	1503	KX349202	*Stenotrophomonas rhizophila* (99.73)
AL1614	1435	KX349199	*Bacillus subtilis* (99.23)
OV2120	1459	KX349195	*Paenibacillus amylolyticus* (99.72)
OV2121	1451	KX349194	*Paenibacillus amylolyticus* (99.79)
OV2122	1457	KX349196	*Paenibacillus castaneae* (99.73)

Table 2. Identification of xylanolytic bacterial isolates by 16S rRNA gene sequencing.

[1] The isolates were identified using the EzTaxon server (http://www.ezbiocloud.net/eztaxon; Kim et al., 2012) on the basis of 16S rRNA gene sequence data.

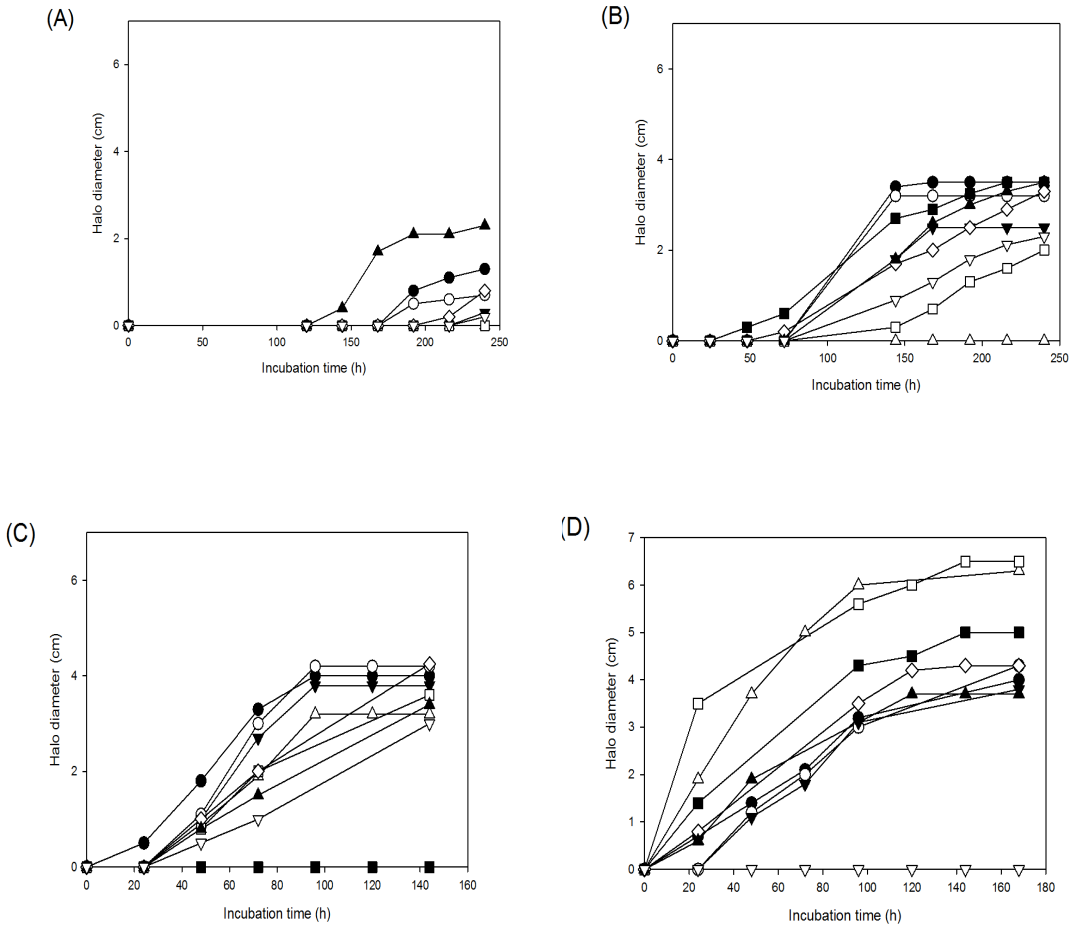
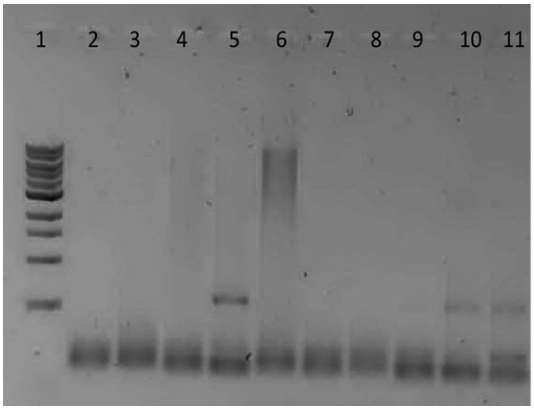
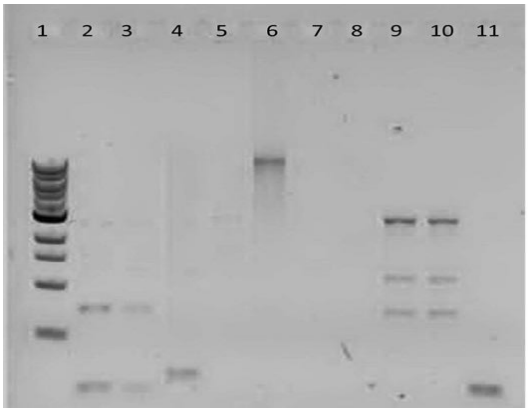


Figure 3. Xylanolytic activity as measured by the emergence and diameter of dye halos on azo-crosslinked birch xylan (Megazyme) agar media. Plates were incubated at 5°C (A), 15°C (B), 22°C (C), and 35°C (D). Values are means of measurements of triplicate plates. Error bars are omitted for clarity (standard deviations of terminal values can be seen in Table 6). Solid circles indicate strain AIB0216B, open circles strain AIB0216C, solid downwards-pointing triangles strain AIB0228, open upwards-pointing triangles strain AL1515, solid squares strain AL1610, open squares strains AL1614, open diamonds strain OV2120, solid upwards-pointing triangles strain OV2121, open downwards-pointing triangles strain OV2122.

Temperature-dependence of xylanolytic activity

The xylanase screen was adapted to a semi-quantitative assay by monitoring the emergence and diameter of the blue dye haloes on triplicate plates incubated at 5, 15, 22, and 35°C over a period of up to 10 days. The effect of incubation temperature on xylanolytic activity varied substantially among the strains (Fig. 3), with OV2121 displaying the most prominent activity at 5°C, whereas at 35°C AL1614 and AL1515 displayed the most activity. Although variation among replicates was in many cases high (Table 6), especially at lower temperatures, one-way ANOVA on terminal halo diameters revealed significant differences in xylanase activity among the strains at 35°C ($F(7,16) = 8.45$; $p<0.001$) and at 22°C ($F(8,16) = 4.45$; $p=0.005$), while at 15°C one-way ANOVA did not reveal a difference ($F(7,16) = 1.44$; $p=0.257$) and at 5°C, while evidence against the null hypothesis of there being no difference was found, it was not significant at the 95% level ($F(5,12) = 3.01$; $p=0.055$). Most of the isolates displayed higher xylanolytic activity at higher temperatures, although isolate OV2122 only displayed activity below 35°C. No activity was detected at 5°C in any of the replicates of isolates AL1515, AL1610, or AL1614.





(C)

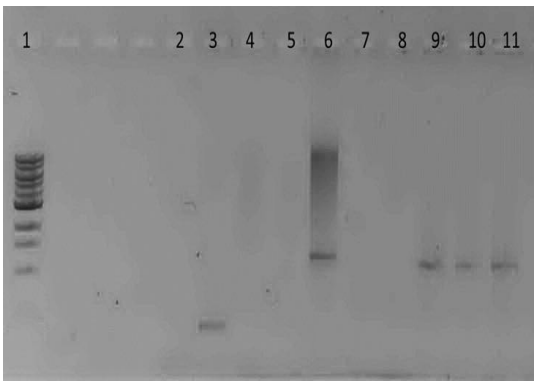


Figure 2. Agarose gels with putative xylanase gene fragment amplicons. The amplicons were obtained by Taq polymerase PCR using primer pairs specific for xylanases of glycohydrolase families 8 (A; xyn8A), 10 (B; xynFA), and 11 (C; PAxynA). All gels show a 1kb DNA ladder (NEB) in the first lane, showing bands from 0.5kb to 10kb. Lanes 2 to 11 contain amplicons from strains as follows: 2, AIBO216B; 3, AIBO216C; 4, AIBO228; 5, AL1515; 6, AL1610; 7, AL1614; 8, DO1702; 9, OV2120; 10, OV2121; and 11, OV2122.

	OV2121	OV2120	OV2122	AIB0216B	AIB0216C	AL1614	AIB0228	AL1515	AL1610
OV2121	0.000								
OV2120	0.000	0.000							
OV2122	0.068	0.068	0.000						
AIB0216B	0.002	0.002	0.068	0.000					
AIB0216C	0.002	0.002	0.068	0.000	0.000				
AL1614	0.140	0.140	0.151	0.140	0.140	0.000			
AIB0228		0.267	0.267	0.301	0.267	0.267	0.257	0.000	
AL1515	0.256	0.256	0.288	0.256	0.256	0.247	0.009	0.000	
AL1610	0.259	0.259	0.302	0.260	0.259	0.267	0.177	0.179	0.000

Table 3. Pairwise distances between MUSCLE multiply aligned 16S rRNA gene sequences.

DISCUSSION

According to the 16S rRNA gene sequencing, five of the strains in present study belong to the genus of *Paenibacillus*. The paenibacilli are ubiquitous in nature, having been isolated from environments as diverse as soil, clinical specimens, and hot springs (16). Members of the genus are highly diverse, with member species representing psychrophiles, mesophiles, and thermophiles, strict aerobes and facultative anaerobes, and are known for their production of various glycanolytic exoenzymes (28). Since its formation from members of *Bacillus* (2), the genus *Paenibacillus* currently has grown to 200 species and 4 subspecies according to the LPSN (27). Xylanolytic activity is common among the paenibacilli (6) and several investigators have reported the isolation and characterization of glycohydrolase family 8 (23), family 10 (17, 37) and family 11 (34, 38) xylanases from these organisms. Several paenibacilli are psychrotrophic and, hence, cold-active xylanases are found among them (40).

Much like the paenibacilli, the pseudomonads are very commonly encountered in plant- and soil-associated environments, and a number of studies have described xylanolytic activity in, and the isolation and characterization of xylanases from these bacteria (20, 29). The P. fluorescens family 10 xylanase A and family 11 xylanase E have been well studied (13).

The identity of xylanolytic isolate AL1610 as a *Stenotrophomonas sp.* was more surprising. While the stenotrophomonads are typically plant-associated bacteria and therefore expected in the habitats under study, they are generally, as the genus name implies, quite fastidious with a nutritional spectrum mostly limited to mono- and disaccharides (25). Very little literature currently exists on xylanolytic activity in these organisms. Indeed, according to Raj et al. (30), their study on an inducible, thermostable xylanase from *Stenotrophomonas maltophilia* was the first to describe xylanase activity in that species. Malfliet *et al.* (21) also reported S. maltophilia xylanase activity among the consortia present in malting barley.

	Paenibacillus AIB0216B	*Paenibacillus* AIB0216C	*Paenibacillus* OV2120	*Paenibacillus* OV2121	*Paenibacillus* OV2122	*Bacillus* AL1614	*Pseudomonas* AIB0228	*Pseudomonas* AL1515	*Stenotrophomonas* AL1610
Hydrolysis of:									
ONPG	+	+	+	+	–	–	+	+	–
Arginine dihydrolase		–	–	–	–	+	+	+	+
Lysine decarboxylase		–	–	–	–	–	–	–	–
Ornithine decarboxylase		–	–	–	–	–	–	–	–
Citrate utilization	–	–	–	–	+	+	+	+	–
H2S production	–	–	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	–	–	–
Tryptophan deaminase	–	–	–	–	–	–	–	–	–
Indole production	–	–	–	–	–	–	–	–	–
Acetoin production	–	–	–	–	–	–	–	–	+
Gelatinase activity	–	–	–	–	+	+	+	+	+
NO3 reduction		–	–	–	–	–	–	–	–
Fermentation/oxidation:									
D–glucose	+	+	+	+	+	+	+	+	+
D–mannitol	–	–	+	+	–	–	–	+	–
Inositol	–	–	–	–	–	–	–	–	–
D–sorbitol	–	–	–	–	–	–	–	–	–
L–rhamnose	–	–	–	–	–	–	–	+	–
D–sucrose	+	+	+	+	–	w	–	+	–
D–melibiose	+	+	+	+	+	+	+	+	+
Amygdalin	+	+	+	+	–	+	–	+	+
L–arabinose	+	+	+	+	+	+	–	+	w

Table 4. Selected phenotypic characteristics (positive (+), negative (-) or weak (w)) of xylanolytic isolates obtained using API 20E test strips

The stenotrophomonads have been used in agricultural and environmental biotechnology, such as for bioremediation, in spite of the potential of *S. maltophilia* as an opportunistic human pathogen (4). Xylanolytic activity may further expand the biotechnological potential of these organisms. A pairwise distance comparison of the aligned 16S rRNA sequences, obtained for all nine strains, revealed a close relatedness of strains isolated in widely separated parts of the country. These results are striking but not necessarily unexpected, as the sporeforming paenibacilli disperse easily by wind over considerable distances and are thus not subjected to the physical isolation required for diversification (26). The fifth *Paenibacillus*, strain OV2122 was more divergent, displaying on average 67 base substitutions to each of the other paenibacilli over the 987 nucleotide positions tested. The other four strains were more heterogeneous, although the two pseudomonads were fairly similar to each other (9 substitution over the 987 positions). The highest divergence observed was between *Paenibacillus* OV2122 and *Stenotrophomonas* AL1610, 298 base substitutions among the 987 nucleotide positions tested. Four isolates, OV2121, OV2120, AIB0216B and AIB0216C, form a clade with *P. amylolyticus, P. xylanexedens, P. tundae and P. tylopili* (Fig. 1). Both *P. xylanexedens* and *P. tundrae* were originally isolated as xylanolytic paenibacilli from moist tundra soil in Alaska (24). However, related strains have been isolated from other environments, such as barley grain in Finland (31) and catfish guts in Brazil (8). Isolate OV2122 forms a clade with *P. castaneae and P. endophyticus* supporting the EzTaxon assignment of this isolate as *P. castaneae*, a species described from strains originally isolated from a chestnut tree rhizosphere in Spain (39).

Several of the strains yielded amplicons from more than one xylanase primer pair used for screening. While this may be due to less-than-perfect specificity of the primers employed due to the noted diversity of xylanase amino acid sequences (7), more than one xylanase genes may indeed be present in the respective genomes. Several microorganisms, including both pseudomonads and bacilli, are known to produce multiple xylanases (11, 41). Taken together, these results indicate that the paenibacilli (strains AIB0216B, AIB216C, OV2120, OV2121, and OV2122) possess at least family 10 and family 11 xylanases, strains OV2121 and OV2122 possibly also a family 8 xylanase.

bands	primer	lgth (nt)	best BLAST x hit	% (id)aa
AL1610		261	*Paenibacillus sp.* HY8 endo-xylanase	99
OV2122		263	*Paenibacillus amylolyticus* 1,4-beta-xylanase	99
OV2121		204	*Paenibacillus sp.* PAMC 26794 endo-1,4-beta-xylanase	95
AIB0216B_b1	XynFA	157	*Paenibacillus sp.* O199 hypothetical protein	100

Table 5. BLASTX of partial nucleotide sequences obtained from amplicons obtained using xylanase-specific PCR primers.

The two pseudomonads (AIB0228 and AL1515) display quite divergent results, with AL1515 possibly possessing a family 8 xylanase, although in both cases the identity of the PCR amplicons could not be verified by sequencing as belonging to a xylanase gene. Based on the 261-bp PAxynA amplicon sequence, the Stenotrophomonas AL1610 xylanase appears most similar to the family 11 xylanase of *Paenibacillus* HY-8 (14).

We adapted the xylanase screen to a semi-quantitative assay and monitored the emergence and diameter of the blue dye haloes incubated at different temperature, ranging from 5 – 35°C. The effect of temperature was noticeable most of the tested strains displayed most activity at higher temperature. Of the nine tested strains, only *Paenibacillus amylolyticus* OV2121, displayed the most prominent activity at 5°C. We therefore conclude that of the nine xylanolytic strains studied, *Paenibacillus amylolyticus* OV2121 produces the most cold-active xylanase under the conditions employed, while *Pseudomonas kilonensis* AL1515 and *Bacillus subtilis* AL1614 display higher activity at higher temperatures. *Paenibacillus castaneae* OV2122 only displays xylanolytic activity at lower temperatures.

Strains	5°C	15°C	22°C	35°C
AIBO216B	1.3 ± 0.4	3.5 ± 0.5	4.0 ± 0.0	4.0 ± 0.0
AIBO216B	0.7 ± 0.6	3.2 ± 0.3	4.2 ± 0.3	4.3 ± 0.3
AIBO228	0.3 ± 0.6	2.5 ± 0.9	3.8 ± 0.3	3.8 ± 0.3
AL1515	0.0	0.0	3.2 ± 0.3	6.3 ± 0.6
AL1610	0.0	3.5 ± 0.7	3.6 ± 0.1	5.2 ± 0.3
AL1614	0.0	2.0 ± 0.4	3.6 ± 0.8	6.5 ± 1.3
OV2120	0.5 ± 0.5	3.3 ± 0.6	4.3 ± 0.4	4.3 ± 1.0

Table 6. Terminal halo diameters on Az-xylan test media at different incubation

ACKNOWLEDGEMENTS

This work was in part conducted under an ERASMUS internship exchange programme and was funded by the University of Akureyri Research Fund. The authors would also like to acknowledge Robert W. Jackson and other coordinators of the University of Akureyri and Reading University joint Arctic microbiology field trip and laboratory course of 2012 within which environmental sampling and initial culturing of the isolates was performed by students Árný Ingveldur Brynjarsdóttir and Luke Johnson.

REFERENCES

1. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

2. Ash, C., Priest, F. G., Collins, M. D. (1993). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Antonie Van Leeuwenhoek 64:253–260.

3. Beg, Q. K., Kapoor, M., Mahajan, L., Hoondal, G. S. (2001). Microbial xylanases and their industrial applications: A review. Appl. Microbiol. Biotechnol. 56:326–338.

4. Berg, G., Martinez, J. L. (2015). Friends or foes: Can we make a distinction between beneficial and harmful strains of the Stenotrophomonas maltophilia complex? Front Microbiol. 6, 241. doi: 10.3389/fmicb.2015.00241

5. Butterfield, C. T. (1932). The Selection of a Dilution Water for Bacteriological Examinations. J. Bacteriol. 23:355–368.

6. Chakdar, H., Kumar, M., Pandiyan, K., Singh, A., Nanjappan, K., Kashyap, P. L., Srivastava, A. K. (2016). Bacterial xylanases: biology to biotechnology. 3 Biotech 6, 150. doi: 10.1007/s13205-016-0457-z

7. Collins, T., Gerday, C., Feller, G. (2005). Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol. Rev. 29:3–23.

8. de Castro, A. L. M., Vollú, R. E., Peixoto, R. S., Grigorevsi-Lima, A. L., Coelho, R. R. R., Bon, E. P. S., Rosado, A. S., Seldin, L. (2011). Cellulolytic potential of a novel strain of Paenibacillus sp. isolated from the armored catfish Parotocinclus maculicauda gut. Brazilian J. Microbiol. 42:1608–1615. doi: 10.1590/S1517-83822011000040000048

9. Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792–1797.

10. Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution (N Y) 39:783–791.

11. Gilbert, H. J., Sullivan, D., Jenkins, G., Kellett, L. E., Minton, N. P., Hall, J. (1988). Molecular cloning of multiple xylanase genes from Pseudomonas fluorescens subsp. cellulosa. J. Gen. Microbiol. 134:3239–3247. doi: 10.1099/00221287-135-5-1395

12. Guo, B., Chen, X-L., Sun, C-Y., Zhou, B-C., Zhang, Y-Z. (2009). Gene cloning, expression and characterization of a new cold-active and salt-tolerant endo-β-1,4-xylanase from marine Glaciecola mesophila KMM 241. Appl. Microbiol. Biotechnol. 84: 1107. doi: 10.1007/s00253-009-2056-y

13. Hazlewood, G. P., Gilbert, H. J. (1998). Structure and Function Analysis of Pseudomonas Plant Cell Wall Hydrolases. Prog. Nucleic Acid Res. Mol. Biol. 61:211–241. doi: 10.1016/S0079-6603(08)60828-4

14. Heo, S., Kwak, J., Oh, H. W., Park, D. S., Bae, K. S., Shin, D. H., Park, H. Y. (2006). Characterization of an extracellular xylanase in Paenibacillus sp. HY-8 isolated from an herbivorous longicorn beetle. J. Microbiol. Biotechnol. 16:1753–1759. doi: 2596 [pii]

15. Hosoda, A., Sakai, M., Kanazawa, S. (2003). Isolation and characterization of agar-degrading Paenibacillus spp. associated with the rhizosphere of spinach. Biosci. Biotechnol. Biochem. 67:1048–1055. doi: 10.1271/bbb.67.1048

16. Jessen, J. E., Sveinsson, T., Scully, S. M., Orlygsson, J. (2015). Ethanol production by a Paenibacillus species isolated from an Icelandic hot spring—Production yields from complex biomass. Icelandic Agric. Sci. 28:15–24.

17. Kim, M-S., Woo, M-H., Chang, Y-H., Chung, N., Kim, J-S. (2016). Biochemical characterization of a noble xylanase from Paenibacillus sp. EC116. Appl. Biol. Chem. 59: 313–320. doi: 10.1007/s13765-016-0159-6

18. Kim, O-S., Cho, Y-J., Lee, K., Yoon, S-H., Kim, M., Na, H., Park, S-C., Jeon, Y. S., Lee, J-H., Yi, H., Won, S., Chun, J. (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int. J. Syst. Evol. Microbiol. 62:716–21. doi: 10.1099/ijs.0.038075-0

19. Kudou, M., Okazaki, F., Asai-Nakashima, N., Ogino, C., Kondo, A. (2015). Expression of cold-adapted β-1,3-xylanase as a fusion protein with a ProS2 tag and purification using immobilized metal affinity chromatography with a high concentration of ArgHCl. Biotechnol. Lett. 37:89–94. doi: 10.1007/s10529-014-1666-3

20. Liang, W-S., Fang, T-Y., Lin, H-T., Liu, T. C., Lu, W-J., Tzou, W-S., Tang, S-J., Lin, F-P., Liu, S-M., Pan, C-L. (2015). Cloning, expression, and characterization of Pseudomonas vesicularis MA103 β-1,3-xylanase in Escherichia coli Clear Coli BL21(DE3). Fish. Sci. 81:1135–1143. doi: 10.1007/s12562-015-0933-0

21. Malfliet, S., Justé, A., Crauwels, S., Willems, K., De Cooman, L., Lievens, B., Aerts, G. (2013). Assessing the xylanolytic bacterial diversity during the malting process. Food Microbiol 36:406–415. doi: 10.1016/j.fm.2013.06.025

22. Myers, E. W., Miller, W. (1988). Optimal alignments in linear space. Comput. Appl. Biosci. 4:11–17. doi: 10.1093/bioinformatics/4.1.11

23. Na, H. B., Jung, W. K., Jeong, Y. S., Kim, H. J., Kim, S. K., Kim, J., Yun, H. D., Lee, J-K., Kim, H. (2015). Characterization of a GH family 8 β-1,3-1,4-glucanase with distinctive broad substrate specificity from Paenibacillus sp. X4. Biotechnol Lett 37: 643–655. doi: 10.1007/s10529-014-1724-x

24. Nelson, D. M., Glawe, A. J., Labeda, D. P., Cann, I. K. O., Mackie, R. I. (2009). Paenibacillus tundrae sp. nov. and Paenibacillus xylanexedens sp. nov., psychrotolerant, xylan-degrading bacteria from Alaskan tundra. Int. J. Syst. Evol. Microbiol. 59:1708–14. doi: 10.1099/ijs.0.004572-0

25. Palleroni, N. J., Bradbury, J. F. (1993). Stenotrophomonas, a New Bacterial Genus for Xanthomonas maltophilia (Hugh 1980) Swings et. al. 1983. Int. J. Syst. Bacteriol. 43: 606–609.

26. Papke, R. T., Ward, D. M. (2004). The importance of physical isolation to microbial diversification. FEMS Microbiol. Ecol. 48: 293–303.

27. Parte, A. C. (2014). LPSN--list of prokaryotic names with standing in nomenclature. Nucleic. Acids. Res. 42:D613-6. doi: 10.1093/nar/gkt1111 [doi]

28. Priest, F. G. (2010). Genus I. Paenibacillus Ash, Priest and Collins 1994, 852VP (Effective publication: Ash, Priest and Collins 1993, 259) emend. Shida, Takagi, Kadowaki, Nakamura and Komagata 1997a, 297. In: Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer K-H, Whitman W (eds) Bergey's Manual of Systematic Bacteriology, Vol. 3 The Firmicutes. Springer, New York, pp 269–295.

29. Raghothama, S., Simpson, P. J., Szabó, L., Nagy, T., Gilbert, H. J., Williamson, M. P. (2000). Solution Structure of the CBM10 Cellulose Binding Module from Pseudomonas Xylanase A. Biochemistry 39:978–984. doi: 10.1021/bi992163+

30. Raj, A., Kumar, S., Singh, S. K. (2013). A highly thermostable xylanase from Stenotrophomonas maltophilia: Purification and partial characterization. Enzyme Res. doi: 10.1155/2013/429305

31. Rasimus, S., Mikkola, R., Andersson, M. A., Teplova, V. V., Venediktova, N., Ek-Kommonen, C., Salkinoja-Salonen, M. (2012). Psychrotolerant Paenibacillus tundrae Isolates from Barley Grains Produce New Cereulide-Like Depsipeptides (Paenilide and Homopaenilide) That Are Highly Toxic to Mammalian Cells. Appl. Environ. Microbiol. 78:3732–3743. doi: 10.1128/AEM.00049-12

32. Rivas, R., García-Fraile, P., Mateos, P. F., Martínez-Molina, E., Velázquez, E. (2007). Characterization of xylanolytic bacteria present in the bract phyllosphere of the date palm Phoenix dactylifera. Lett. Appl. Microbiol. 44:181–187. doi: 10.1111/j.1472-765X.2006.02050.x

33. Saitou, N., Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.

34. Sermsathanaswadi, J., Pianwanit, S., Pason, P., Waeonukul, R., Tachaapaikoon, C., Ratanakhanokchai, K., Septiningrum, K., Kosugi, A. (2014). The C-terminal region of xylanase domain in Xyn11A from Paenibacillus curdlanolyticus B-6 plays an important role in structural stability. Appl. Microbiol. Biotechnol. 98:8223–8233. doi: 10.1007/s00253-014-5748-x

35. Tamura, K., Nei, M., Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. 101, 11030–11035.

36. Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30:2725–2729. doi: 10.1093/molbev/mst197

37. Valenzuela, S.V., Díaz, P., Javier Pastor, F. I. (2010). Recombinant expression of an alkali stable GH10 Xylanase from Paenibacillus barcinonensis. J Agric Food Chem 58: 4814–4818. doi: 10.1021/jf9045792

38. Valenzuela, S. V., Diaz, P., Pastor, F. I. J. (2014). Xyn11E from Paenibacillus barcinonensis BP-23: A LppX-chaperone-dependent xylanase with potential for upgrading paper pulps. Appl. Microbiol. Biotechnol. 98:5949–5957. doi: 10.1007/s00253-014-5565-2

39. Valverde, A., Peix, A., Rivas, R., Velázquez, E., Salazar, S., Santa-Regina, I., Rodríguez-Barrueco, C., Igual, J. M. (2008). Paenibacillus castaneae sp. nov., isolated from the phyllosphere of Castanea sativa Miller. Int. J. Syst. Evol. Microbiol. 58:2560–2564. doi: 10.1099/ijs.0.65663-0

40. Yadav, A. N., Sachan, S. G., Verma, P., Kaushik, R., Saxena, A. K. (2016). Cold active hydrolytic enzymes production by psychrotrophic Bacilli isolated from three sub-glacial lakes of NW Indian Himalayas. J. Basic Microbiol. 56:294–307. doi: 10.1002/jobm.201500230

41. Yang, R. C., MacKenzie, C. R., Bilous, D., Narang, S. A. (1989). Identification of two distinct Bacillus circulans xylanases by molecular cloning of the genes and expression in Escherichia coli. Appl. Environ. Microbiol. 55:568–572.

CHARACTERIZATION OF ANTIBIOTIC-PRODUCING BACTERIUM ISOLATED FROM ANTHILL SEDIMENT WITH ACTIVITY AGAINST ESKAPE PATHOGENS

CODY A. POSTICH, KEVIN B. KISER*
UNIVERSITY OF NORTH CAROLINA WILMINGTON, NC

MANUSCRIPT RECEIVED 07 JUNE 2018; ACCEPTED 10 NOVEMBER 2018

CORRESPONDING

Kevin B. Kiser
kiserk@uncw.edu

KEYWORDS

- Antibiotic
- ESKAPE
- monolauryl maleate
- mass spectrometry
- *Pseudomonas*

ABSTRACT

Healthcare institutions have seen an increase in infections caused by antibiotic-resistant ESKAPE pathogens. Current antibiotics have become less potent against pathogenic bacteria due to their overuse and misuse. In recent years, scientists have revisited local environments in search of novel antibiotic-producing microbes to address the increasing threat of resistance. One species of bacteria was isolated from anthill sediment in coastal North Carolina. This environment was selected for its abiotic properties, including organic substrates, moisture saturation and aeration. Anthill isolate A2, inhibited various Gram-positive and negative ESKAPE pathogens or their surrogates, including *Staphylococcus aureus, Klebsiella pneumoniae,* and *Acinetobacter calcoaceticus,* in cross-streak tests. 16S rRNA sequencing identified isolate A2 as *Pseudomonas koreensis.* Mass spectrometry and small-molecule analysis performed on ethyl-acetate extracts of culture supernatant were used to evaluate bioactivity and identify the probable structure of one potential antimicrobial compound, monolauryl maleate. Discovery of novel antimicrobial compounds to replace overused antibiotics may help reduce the impact of antibiotic-resistant pathogens.

INTRODUCTION

Antibiotics are becoming increasingly ineffective in treating bacterial infections because of misuse and overuse, particularly in healthcare institutions. Antibiotic-resistant bacteria, known as ESKAPE pathogens, are a rising concern in healthcare settings (2). The ESKAPE pathogens – *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter sp.* – have been designated as global threats because they have developed resistance mechanisms and "escape" death by antibiotics.

Discovering and developing novel antibiotics are one solution to combat antibiotic-resistant infections. Unfortunately, there were

only 16 broad-spectrum antimicrobial agents approved and utilized in the U.S. between 1983–1987, and only 5 novel antimicrobial compounds were approved for use from 2003–2007 (2). Soil microbes are a potential source of new antibiotics. When microbes are in close quarters in terrestrial and aquatic environments, they compete for resources and utilize "chemical weapons" to inhibit growth of other microbes. The renewed search for antibiotic-producing microbes has focused on recruiting students at all levels to explore unique environments across the globe (4). Furthermore, new approaches to identifying previously uncultured microbes have shown promise, as demonstrated by the discovery of teixobactin, an antibiotic with a novel mechanism of action against Gram-positive bacteria, with use of the iChip (11).

In this study, bacteria were cultured from anthill soil in southeastern North Carolina and evaluated for their potential to produce antibiotics. Anthills were chosen for investigation because ant activity was shown to alter soil properties where they build their nests, and these soils were shown to contain an abundance of organic matter and cellulosic polysaccharides that could foster novel microbial communities (7). In fact, some ants that "farm" fungi for food have been shown to have mutualistic relationships with actinomycetes that produce antifungal agents to protect their "crops" against fungal parasites (1). However, little is known about antibacterial activities that may be associated with anthills. Antibiotic activities of anthill isolates were characterized with use of inhibition assays against a variety of Gram-positive and negative bacteria, including ESKAPE pathogens. A cell-free extract from one anthill isolate was analyzed to identify the active compound and to determine whether this finding was novel. One previously unidentified compound, monolauryl maleate, appears to possess activity against the Gram-positive pathogen, *S. aureus*.

MATERIALS AND METHODS

ISOLATION OF ANTIBIOTIC-PRODUCING BACTERIA

Soil was collected from the surface of an anthill (red imported fire ant, *Solenopsis invicta*) located in front of a residential area in Shallotte, North Carolina (33°58'36"N 78°31'30"W). 1:100 dilutions of soil in sterile water were plated on actinomycete isolation agar (AIA; HiMedia Laboratories, West Chester, PA) and incubated at 35°C for 3 days. 100 µL of an overnight tryptic soy broth (TSB; Hardy Diagnostics, Santa Maria CA) culture of *S. epidermidis* was transferred to 8 mL of molten TSB soft agar (TSB with 0.7% agar), vortexed, and poured over the AIA culture. After solidification, the plate was incubated at 35°C for 24 h, and then observed for zones of inhibition (ZOI) in the overlay. Isolates showing ZOI were picked with a sterile loop and streaked for isolation on AIA. *S. epidermidis* was used in initial screens because it has little natural resistance and should display ZOI against a variety of antimicrobials. Additionally, as a BSL-1 microbe, it is safer to work with as students are training in the lab and learning these techniques.

CROSS-STREAK TEST

Each isolate was streaked down the center of Mueller-Hinton agar (MHA; Hardy Diagnostics) and incubated at 35°C for 3 d. Bacteria used in this study are listed in Table 1. Some were acquired from Carolina

Biological Supply Company (Burlington, NC) and strain identity was unknown. Strains with known identity (indicated with ATCC number) were purchased from Fisher Scientific (Hampton, NH). Six ESKAPE pathogens or surrogates (Table 1 and Fig. 2) were streaked perpendicular to the isolate, and the plate was incubated at 35°C for an additional 24 h. Isolate A2 was later retested by cross streak against a panel of 10 Gram-positive and negative bacteria, including some ESKAPE pathogens and surrogates (Table 1 and Fig. 3). The inhibition distance was measured in mm.

IDENTIFICATION OF ANTHILL ISOLATES

Each isolate was Gram-stained and observed at 1000x magnification with a Lecia DM750 compound light microscope. Genomic DNA was extracted from each isolate with the use of the Ultra Clean Microbial DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA), according to manufacturer's specifications. The cell disruption step was performed in a Bead Bug homogenizer (Benchmark Scientific, Edison, NJ) for 3 x 60 seconds at 320 rpm with cooling on ice between cycles. The 16S rRNA sequence was amplified with use of primers Eco8F (5'-AGAGTTTGATCATGGCTCAG-3') and 1509R (5'-GGTTACCTTGTTACGACTT-3') (5). Each 50-µL PCR reaction (GeneMate reagents, BioExpress, Kaysville, UT) contained: 1x PCR buffer, 0.025 U/µL Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer, and 5.0 µL of genomic DNA. PCR was carried out in an Eppendorf (Hauppauge, NY) Master Cycler under the following conditions: 95°C for 5 min; 30 cycles of 95°C for 1 min, 50°C for 30 s, 72°C for 1.5 min; and then 72°C for 5 min. The 1.5-kb PCR product was confirmed by gel electrophoresis, and then purified with use of the E.Z.N.A Cycle Pure kit (Omega Bio-tek, Norcross, GA), according to manufacturer's specifications, and sequenced by Eurofins Genomics (Louisville, KY). Raw sequences were edited with Finch TV software, v1.4 (Geospiza). A contiguous sequence was created from the forward and reverse sequences from the same isolate with Serial Cloner software, v 2.6 (Serial Basics). Sequences were analyzed with BLAST (National Center for Biotechnology Information) with use of the 16S ribosomal RNA sequences (Bacteria and Archaea) database to identify species with similar sequences

Table 1. Test bacteria used in this study.

Gram-negative	Gram-positive
*Acinetobacter calcoaceticus***	*Bacillus cereus*
Aeromonas hydrophila	*Enterococcus faecalis ATCC 29212***
*Escherichia coli***	*Kocuria rhizophila*
*Klebsiella pneumoniae ATCC 13883**	*Staphylococcus aureus ATCC 25923**
Proteus vulgaris	*Staphylococcus capitis ATCC 35661***
*Pseudomonas aeruginosa ATCC 27853**	*Staphylococcus epidermidis ATCC 12228***
Serratia marcescens	

* ESKAPE pathogen
** ESKAPE surrogate

PREPARATION OF CELL-FREE ETHYL-ACETATE EXTRACT

Isolate A2 was cultured in TSB at 25°C for 7 d, and cells were pelleted by centrifugation for 10 min at 2000 rpm in an Eppendorf 5804R centrifuge. The supernatant was passed through a 0.2 µm filter (Corning, Oneonta, NY). The filtrate was transferred to a separatory funnel and combined with 2 parts ethyl acetate (Alfa Aesar, Haverhill, MA). The aqueous layer was removed, extracted again with 2 parts ethyl acetate, and the two organic extracts were combined. The ethyl acetate was evaporated in a rotary evaporator and the dried cell-free extract was dissolved in 100% methanol (Alfa Aesar).

COLUMN CHROMATOGRAPHY

A Strata C18-E (55µm, 70 A) giga tube column (Phenomenex, Torrance, CA) was conditioned with three washes of 100% HPLC-grade methanol to rinse the column, and then was equilibrated with three washes 20% methanol. 23.0 mg of isolate A2 extract was suspended in 5.0 mL of 20% methanol, then was pipetted onto the column. The crude extract was washed with increasing HPLC-grade methanol gradient with pico-pure filtered H_2O at 20%, 40%, 60%, 80%, and 100% MeOH. A final rinse of 100% acetone removed everything else from the column. The column was placed in a Supelco Visiprep vacuum apparatus for eluting these compounds with vacuum pressure at 8 Hg. The fractionated compounds were dried on a speed vacuum, then were resuspended in 1.0 mL of 100% methanol and tested for antibacterial activity by disk diffusion.

DISK DIFFUSION ASSAY

S. aureus and *E. coli* were freshly cultured on tryptic soy agar (TSA; Hardy Diagnostics) at 35°C for 24 h. Test bacteria were suspended in saline solution (0.9% NaCl) to match a 0.5 McFarland turbidity standard. A sterile swab was used to transfer test bacteria to MHA. Dried extracts of isolate A2 were resuspended in 1.0 mL of 100% MeOH, then 30–40 µL of the mixture was pipetted onto sterile filter disks (Whatman #3, GE Healthcare Life Sciences, Marlborough, MA) in 10 µL increments (10). Dried disks were transferred to the MHA plate, which were then incubated at 35°C for 24 h. Diameters of ZOI were measured in mm.

SMALL-MOLECULE ANALYSIS

Small-molecule analysis of culture supernatants from isolate A2 involved preparation of cell-free ethyl-acetate extracts, as described above. Ultrahigh-performance liquid chromatography, low-resolution mass spectrometry, and small-molecule analysis yielded an S-plot, chromatogram of mass over ionization ratios, and UV absorption of a specific secondary metabolite. These results were analyzed with Progenesis QI software (Nonlinear Dynamics, Durham, NC) to compare known compounds to the secondary metabolites with potential antimicrobial capabilities (3).

ULTRAHIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-LOW RESOLUTION MASS SPECTROMETRY (UPLC-LRMS)

C-18 column chromatography elutions 3, 4, 5, and 6 were analyzed by mass spectrometry to search for similar patterns within the fractions that showed inhibition against S. *aureus*. The 60–100% methanol and 100% acetone washes were dried down on a speed vacuum, and then were suspended in 1 mL of 1:1 acetonitrile: pico-pure water. 30 µL of elutions 3, 4, 5, and 6 were run through

RESULTS

ISOLATION OF ANTIBIOTIC- PRODUCING BACTERIA FROM ANTHILL SEDIMENT

Because anthills have unique characteristics compared to the surrounding soil and ants have been shown to have symbiotic relationships with antibiotic-producing microbes, bacteria were cultured from an anthill in southeastern North Carolina colonized by red imported fire ants to search for antibiotic producers. The culture of anthill sediment on AIA was overlayed with *S. epidermidis* and yielded a few small ZOI (Fig. 1). Potential antibiotic-producers were picked and streaked on AIA. In initial cross-streak tests against ESKAPE species or surrogates, one of the anthill isolates, A2, demonstrated inhibition of five of six test bacteria, but not Gram-positive *E. faecalis*, an ESKAPE surrogate for *E. faecium* (Fig. 2). Overall, isolate A2 yielded the strongest inhibitory characteristics against three Gram-negative ESKAPE bacteria, particularly *E. coli* (surrogate for *Enterobacter sp.*), *K. pneumoniae* and *A. calcoaceticus* (surrogate for *A. baumannii*). Moderate inhibition was shown against Gram-negative *P. aeruginosa* and Gram-positive *S. aureus*. Additional cross-streak tests with A2 showed inhibition of variety of Gram-negative and Gram-positive bacteria, including another species of *Staphylococcus* (Fig. 3). Two Gram-negatives, *S. marcescens*

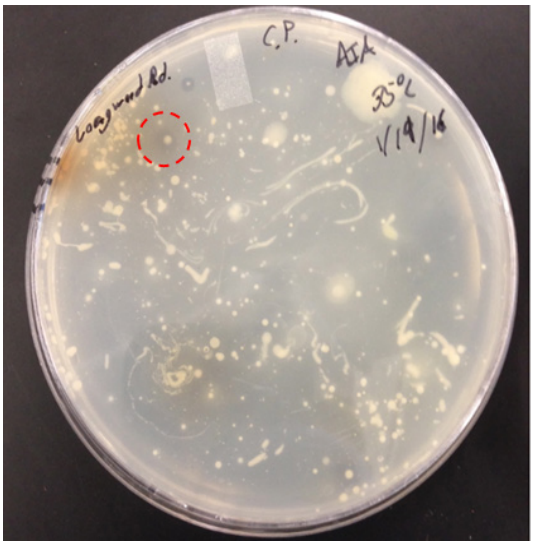


Figure 1. Soft agar overlay of *S. epidermidis* on AIA culture of anthill soil. The red-dashed circle shows the location of the A2 colony showing ZOI.

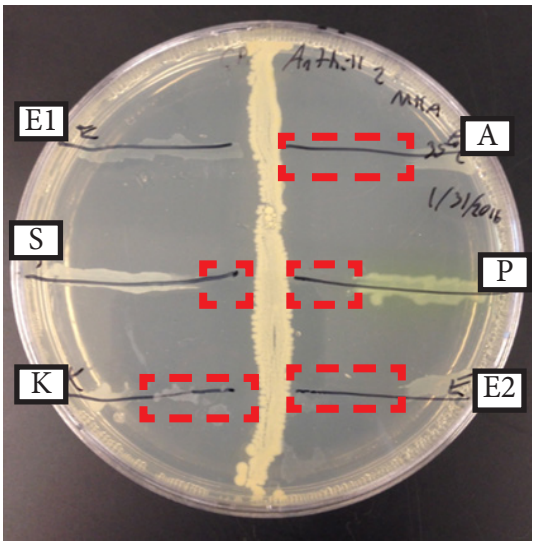


Figure 2. Cross-streak testing with cultured isolate A2 on MHA plates against ESKAPE pathogens and surrogates. Isolate A2 was streaked vertically, and the test bacteria were streaked horizontally. A lack of culture growth from the horizontally streaked test bacteria revealed inhibition (represented by areas boxed by red-dashed lines). E1, *E. faecalis*; S, *S. aureus*; K, *K. pneumoniae*; A, *A. calcoaceticus*; P, *P. aeruginosa*; E2, *E. coli*.

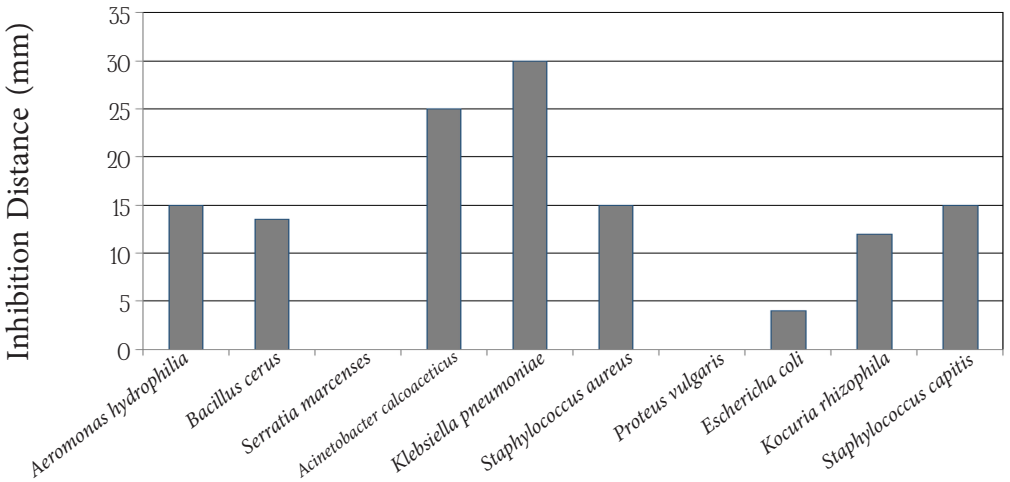


Figure 3. The inhibition distance (mm) displayed by isolate A2 in a cross-streak test against various Gram-positive and negative test bacteria. Bars represent data from a single cross-streak assay. There were no ZOI detected when tested against S. *marcescens* and *P. vulgaris*.

and *P. vulgaris*, were not inhibited.

IDENTIFICATION OF ISOLATE A2

BLAST analysis of the 16S rRNA sequence from the A2 isolate retrieved a sequence with only one mismatch in the 973-base alignment. This species, *P. koreensis*, was previously identified in soils from a Korean farm (8) and a rice paddy (9). Gram staining of A2 revealed a Gram-negative bacillus, as expected for a *Pseudomonas*. Limited knowledge regarding antibacterial activity has been reported for this species, so it is likely the broad-spectrum inhibition seen in cross-streak tests represents a novel finding. To further characterize this activity, an attempt to isolate and identify the antibacterial compound(s) secreted by the A2 isolate was pursued.

ANTIBIOTIC ACTIVITY OF FRACTIONATED CELL-FREE A2 EXTRACTS

Culture supernatants of isolate A2 (putative *P. koreensis*) were extracted with ethyl acetate. These cell-free extracts were tested for antibacterial activity in disk diffusion assays against two of the bacteria that were inhibited in cross-streak tests – Gram-positive S. *aureus* and Gram-negative *E. coli*. The A2 extract produced a 10-mm zone of inhibition against S. *aureus* (Fig. 4, disk A); however, no inhibition was produced against *E. coli* (data not shown). The ability to detect inhibitory activity in cell-free extracts was useful for the preliminary isolation of antibacterial compound(s).

C-18 column chromatography was used to fractionate compounds from 23.0 mg of A2 ethyl-acetate extract suspended in 20% methanol. The largest portion of the extract (9.1 mg) was eluted in the second fraction at 40% methanol, while the smallest portion (0.2 mg) was eluted at 80% methanol (Table 2). Elutions 2-6, were tested by disk diffusion against S. *aureus* to determine which fraction(s) contained the active compound(s). Only 10% of each fractionated sample was loaded onto the sterilized disks (Table 2). Elution 2 produced a small and poorly defined ZOI that could not be measured, while elution 3 and 6 yielded no ZOI (Fig. 4, disks 2, 3 and 6, respectively). Elutions 4 and 5 both produced 10-mm ZOI, similar to that displayed by the original cell-free extract

Table 2. C-18 column chromatography distribution of compounds from the cell-free, ethyl-acetate extract based on polarity with increasing methanol (MeOH) concentrations.

Elutions	[Solvent]	Solvent	Dried Sample Weight (mg)	Amount Loaded onto Disk (mg)
1	20%	MeOH/H_2O	2.0	0.02
2	40%	MeOH/H_2O	9.1	0.91
3	60%	MeOH/H_2O	2.1	0.21
4	80%	MeOH/H_2O	0.2	0.02
5	100%	MeOH/H_2O	0.9	0.09
6	100%	Acetone	0.4	0.09

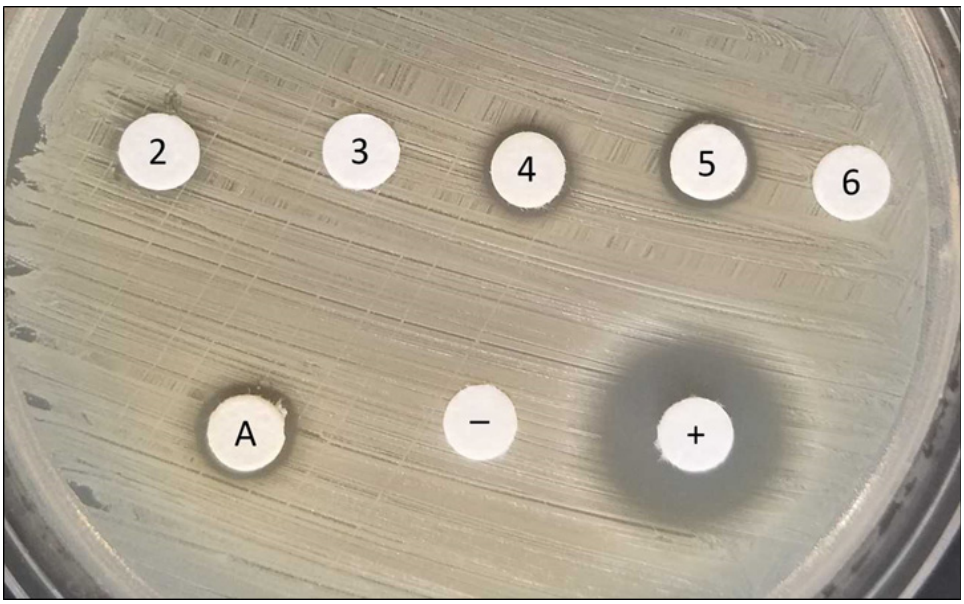


Figure 4. Disk diffusion assay performed with cell-free ethyl-acetate extract of A2 (A) and its C-18 column fractions (2-6) against S. *aureus*. Streptomycin was the positive control (+). Methanol was the negative control (-).

(Fig. 4, disks 4 and 5, respectively).

MASS SPECTROMETRY ANALYSIS OF A2 COLUMN FRACTIONS

C-18 column elutions 3–6 were selected for analysis by mass spectrometry. There were clear differences in number and location of peaks between the four fractions observed on the chromatogram, whereas elutions 4 and 5, which showed antibacterial activity by disk diffusion, had 13 peaks in common (Fig. 5). There was an abundance of one compound at a 276.6 m/z ratio in both active fractions that was absent from the other two non-active fractions (Fig. 5). There was another peak of interest at 294.5 m/z only present in elutions 4 and 5. Although, this may represent an additional active compound, focus was directed at peak 276.6 m/z because of its greater abundance in the extract. Future studies will isolate and assess the antibiotic potential of other peaks.

SMALL-MOLECULE ANALYSIS OF CELL-FREE A2 EXTRACT

Small-molecule analysis was performed on cell-free, ethyl acetate extracts of A2 culture supernatant, which yielded over 1,000 different compounds, as displayed on the S-plot (Fig. 6). The S-plot, where each dot represents a different compound, compared compounds from the A2 cell-free, ethyl-acetate extract and a matched TSB media control. Focusing on the 13 outlier dots in the bottom left quadrant, each represented a compound unique to the A2 culture supernatant. One, if not more, of these 13 compounds may represent the antimicrobial agent produced by isolate A2. The potential active compound identified by mass spectrometry from elutions 4 and 5 was similar to one of the outliers (265.23 m/z) identi-

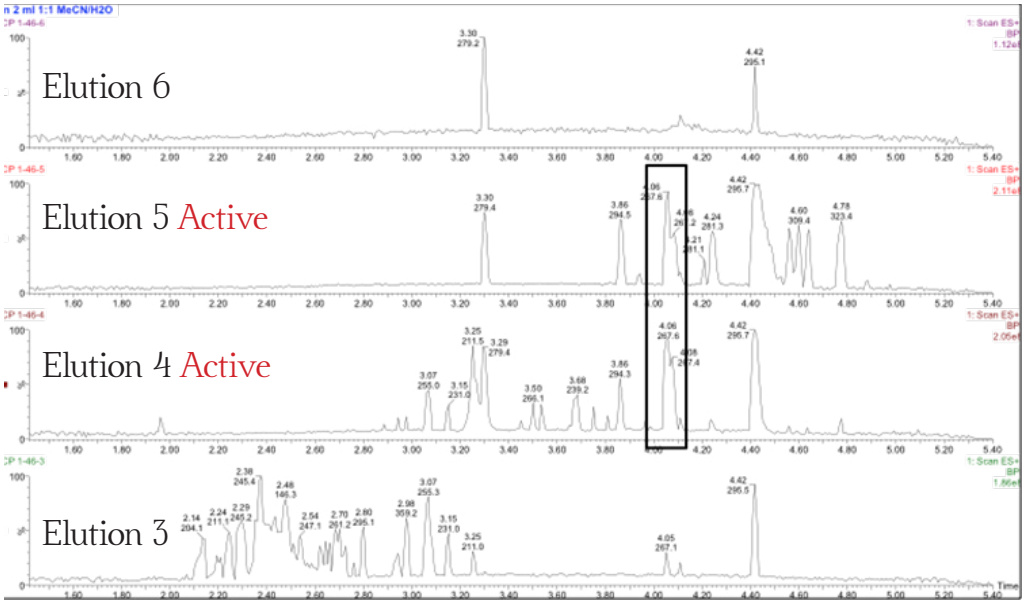


Figure 5. UPLC-LRMS chromatogram results from 60%, 80%, 100% MeOH fractions, and the 100% acetone extraction (elutions 3, 4, 5, and 6, from top to bottom). The boxed area is a selected peak of interest with a mass of 267.6 m/z in the 80% and 100% MeOH fractions.

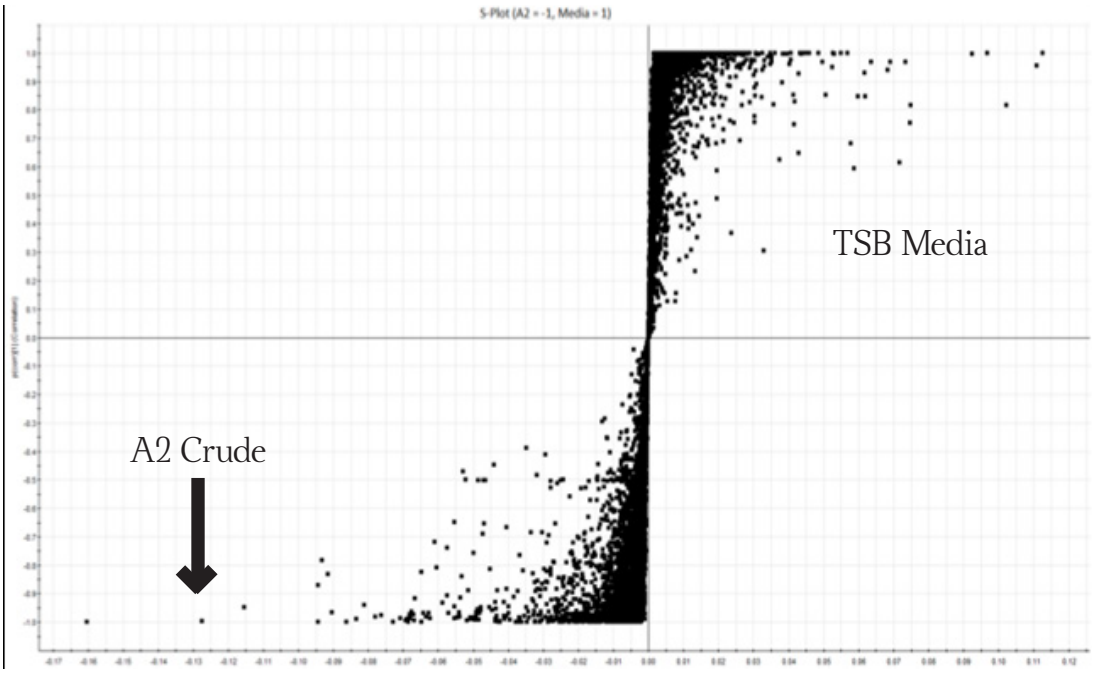


Figure 6. S-plot from small-molecule analysis of triplicate cell-free extracts of A2 culture supernatants (bottom left quadrant) and the TSB media control for comparison (top right quadrant). Arrow indicates compound at 265.23 m/z, which was identified as monolauryl maleate.

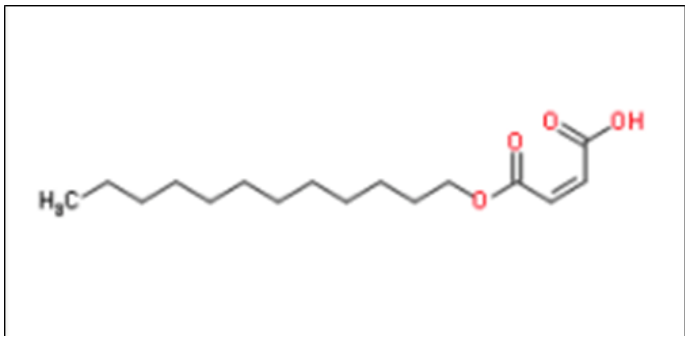


Figure 7. Chemical structure of monolauryl maleate from Progenesis QI software, a compound identified in bioactive C-18 column fractions from A2 cell-free, ethyl acetate extracts.

fied by small-molecule analysis. A cleavage product of this compound was also revealed with a 44 m/z ratio difference, which likely represents a potential carboxylic acid group in the complete compound (data not shown). This finding was consistent with QI Progenesis prediction (97% probability) of the identity of the unknown 266 m/z compound as monolauryl maleate, which has a molecular formula $C_{16}H_{28}O_4$ with a probable carboxylic acid group attached to a 12-carbon chain (Fig. 7).

DISCUSSION

In response to the growing global problem of antibiotic-resistant ESKAPE pathogens in healthcare institutions, soil samples were collected in coastal North Carolina and investigated for the presence of novel antibiotic-producing bacteria. One promising isolate (A2) found in anthill soil inhibited a variety of Gram-positive and Gram-negative bacteria in cross-streak tests (Fig. 3). Broad-spectrum antimicrobial activity is likely beneficial to ants that become colonized by this microbe from sediment manipulation for protection against various pathogenic bacteria, as suggested previously (1). Detection of this antibiotic-producing species on the heads or bodies of imported fire ants could reveal a new mutualistic relationship, similar to ones observed in other ant species. The fact that A2 also inhibited five of six ESKAPE pathogens or their surrogates suggests that anthills may warrant further investigation for bioactive natural products.

BLAST analysis of 16S rRNA gene sequence of A2 showed a match with *P. koreensis*, a newly defined species first discovered in Korean farming soil (8). Genome sequence analysis of another *P. koreensis* isolate from a Korean rice paddy revealed a putative gene cluster for the synthesis of cyclic lipopeptide (CLP), which is a biosurfactant shown to have antagonistic properties (9). *Pseudomonas* species are known to produce a variety of CLPs with antimicrobial activity that likely inhibit colonization of ants by pathogens in the environment (12). One of three *P. koreensis* isolates from a water basin in Mexico demonstrated biosurfactant activity, but inhibition of bacterial growth was limited to two Gram-positive species, *Bacillus subtilis* and *Exiguobacterium aurantiacum* (13). In fact, no broad-spectrum antibacterial activity has been described previously for this species. In this study, not only did the A2 isolate inhibit several different bacterial species, including ESKAPE pathogens, crude and fractionated cell-free extracts from A2 inhibited *S. aureus* (Fig. 3 & 4). Genomic comparison of the two Korean *P. koreensis* strains demonstrated genetic diversity among *Pseudomonas* strains, with 631 putative genes unique to one isolate (9), so it is expected that the North Carolina anthill isolate possesses genetic elements and metabolic capabilities not present in the Korean or Mexican isolates. One of the potentially active compounds, monolauryl maleate, produced by isolate A2 is structurally distinct from CLPs and represents a novel finding in *P. koreensis*. Whether this new compound has similar biosurfactant activity to CLPs might be determined in the future with an in vitro biofilm inhibition assay, or potentially through the development of an ant colonization model. Genomic sequencing of A2 would also reveal differences and help identify a gene cluster involved in antibiotic synthesis.

It was curious that the A2 cell-free extract did not inhibit *E. coli* in a disk diffusion assay, since the A2 isolate did inhibit E. coli in a cell-based, cross-streak assay rather well. This finding indicates that the broad-spectrum activity demonstrated in cross-streaks may represent two or more distinct antibacterial compounds produced by A2 – one absent from cell-free extract that is active against *E. coli* and other Gram-negatives, and another present in cell-free extract that is active against *S. aureus* and other Gram-positives. The compound(s) that inhibit Gram-negative bacteria may not have be soluble in ethyl acetate or may have remained with the cell pellet during collection of the culture supernatant. In hindsight, *K. pneumoniae*, which displayed the greatest inhibition in cross streaks, should have been first Gram-negative tested. Unfortunately, the extraction yielded only enough material to complete a preliminary round of disc-diffusion assays against one Gram-positive and one Gram-negative species. It will be important to revisit the extraction technique to

explore other options for isolating bioactive metabolites and increasing yield, including modifying culture conditions and performing extractions on lysed cells in the pellet. Furthermore, these extracts should also be tested for antibacterial activity against other Gram-negative ESKAPE pathogens, *K. pneumoniae* and *A. calcoaceticus*, which were also inhibited in the cross-streak assay.

A potential novel antimicrobial compound, monolauryl maleate, was identified by small-molecule analysis and low-resolution mass spectrometry of a cell-free extract of isolate A2. Mass spectrometry, UV light absorbance, and small-molecule profiling of isolate A2 provided evidence of a potential antimicrobial compound in the S-plot with a 266 m/z ratio that was characterized through QI Progenesis software. There was a 97% match with the compound, monolauryl maleate (Fig 7). This compound consists of a carbon chain connected to a carboxylic acid group, as confirmed by a 44 m/z fractionation with low-resolution mass spectrometry. No findings had been previously reported regarding the properties of monolauryl maleate as an antibiotic agent. Monolauryl maleate is not available commercially for testing purposes at this time. Synthesis of monolauryl maleate may be possible and could be valuable for future comparative studies. This is one of thirteen potential antimicrobial compounds residing within the active elutions 4 and 5 (Fig. 5). HPLC purification and NMR analysis (3) on the compounds of interest in these two fractions, including peak 294.5 m/z, will be required to ultimately confirm the identity of the active compound(s) and to characterize antimicrobial activity against the ESKAPE pathogens that were inhibited in the cell-based assays. Once identified, the potential of any active A2 compound as a therapeutic agent can be explored.

In 2005, there were 8,987 cases of methicillin-resistant *S. aureus*, or MRSA, infections in hospital and communal locations across the United States, and there were a total of 988 deaths from complications of these infections (6). The constant battle against antibiotic-resistant infections in the healthcare community must be addressed, in part, through the discovery of novel antibiotics. One method includes studying environmental bacteria and their natural products from unique habitats around the world (4, 11). In this study, antimicrobial activity against *S. aureus*, and other ESKAPE pathogens, was discovered in a *Pseudomonas* species isolated from an anthill. The preliminary identification of monolauryl maleate may represent the initial step in the discovery of the next novel antimicrobial agent to address the increasing prevalence of antibiotic-resistant infections.

ACKNOWLEDGMENTS

This study was supported by funds from UNCW Center for the Support of Undergraduate Research and Fellowship (CSURF), Experiencing Transformative Education through Applied Learning (ETEAL), and Department of Biology and Marine Biology. C. Postich was also supported, in part, by the Paul E. Hosier Undergraduate Fellowship. Thomas Coombs, Jeffery Wright, and Jeremy Morgan of UNCW Department of Chemistry and Biochemistry provided additional lab resources and encouragement. Allison Stewart and Wendy Strangman of MARBIONC assisted with small-molecule analysis and mass spectrometry analyses. This research was performed in compliance with federal regulations and institutional policies relating to the handling of infectious agents.

REFERENCES

1. Barke J, Seipke RF, Grüschow S, Heavens D, Drou N, Bibb MJ, Goss RJM, Yu DW, Hutchings MI. (2010) A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus*. *BMC Biology* 8:109. doi:10.1186/1741-7007-8-109

2. Boucher H, Talbot G, Bradley J, Edwards J, Gilbert D, Rice L, Scheld M, Spellberg B, Bartlett J. (2009) Bad bugs, no drugs: No ESKAPE! An update from the Infectious Diseases Society of America. *Clinical Infectious Diseases*. 48:1-12.

3. Covington B, McLean J, Bachmann B. (2017) Comparative mass spectroscopy-based metabolomics strategies for the investigation of microbial secondary metabolites. *Nat Prod Rep*. 34(1):6-24. doi: 10.1039/c6np00048g.

4. Davis E, Sloan T, Aurelius K, Barbour A, Bodey E, Clark B, Dennis C, Drown R, Fleming M, Humbert A, Glasgo E, Kerns T, Lingro K, McMillin M, Meyer A, Pope B, Stalevicz A, Steffen B, Steindl A, Williams C, Wimberley C, Zenas R, Butela K, Wildschutte H. (2017) Antibiotic discovery throughout the Small World Initiative: A molecular strategy to identify biosynthetic gene clusters involved in antagonistic activity. *Microbiologyopen* 6(3). doi: 10.1002/mbo3.435

5. Erwin PM, Olson JB, Thacker RW. (2011) Phylogenetic diversity, host-specificity and community profiling of sponge-associated bacteria in the Northern Gulf of Mexico. *PLoS ONE* 6(11): e26806. https://doi.org/10.1371/journal.pone.0026806

6. Klevens RM., Morrison MA, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Craig AS, Zell ER, Fosheim GE, McDougal LK, Carey RB, Fridkin SK. (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. *JAMA*.298 (15): 1763-1771. DOI: 10.1001/jama.298.15.1763

7. Kristiansen SM, Amelung W. (2001) Abandoned anthills of *Formica polyctena* and soil heterogeneity in a temperate deciduous forest: morphology and organic matter composition. *European Journal of Soil Science*. 52(3): 355-363. https://doi.org/10.1046/j.1365-2389.2001.00390.x

8. Kwon SW, Kim JS, Park IC, Yoon SH, Park DH, Lim CK, Go SJ. (2003) *Pseudomonas koreensis* sp. nov., *Pseudomonas umsongensis* sp. nov. and *Pseudomonas jinjuensis* sp. nov., novel species from farm soils in Korea. *International Journal of Systematic and Evolutionary Microbiology* 53:21–27. DOI 10.1099/ijs.0.02326-0

9. Lin H, Hu S, Liu R, Chen P, Ge C, Zhu B, Guo L. (2016) Genome Sequence of *Pseudomonas koreensis* CRs05-R5, an antagonistic bacterium isolated from rice paddy field. *Microbia*l. https://doi.org/10.3389/fmicb.2016.01756

10. Matuschek E, Brown J, Kahlmeter G. (2014) Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *European Society of Clinical Microbiology and Infectious Disease*. 20:255-266.

11. Piddock LJ. (2015) Teixobactin, the first of a new class of antibiotics discovered by iChip technology? *J Antimicrob Chemother*. 70(10):2679-80. doi: 10.1093/jac/dkv175.

12. Raaijmakers JM, de Bruijn I, de Kock MJD. (2006) Cyclic lipopeptide production by plant-associated *Pseudomonas spp.*: diversity, activity, biosynthesis, and regulation. *Mol. Plant. Microbe Interact*. 19:699–710. doi: 10.1094/MPMI-19-0699

13. Toribio J, Escalante AE, Caballero-Mellado J, González-González A, Zavala S, Souza V, Soberón-Cháveza G. (2011) Characterization of a novel biosurfactant producing *Pseudomonas koreensis* lineage that is endemic to Cuatro Ciénegas Basin. *Systematic and Applied Microbiology* 34:531– 535. doi:10.1016/j.syapm.2011.01.007

MOLECULAR PATHOGENESIS OF *BACILLUS* SPP., WITH EMPHASIS ON THE DAIRY INDUSTRY

ALYSSA A. GRUTSCH, PIERRE S. NIMMER, RACHEL H. PITTSLEY, KATHERINE G. KORNILOW, & JOHN L. MCKILLIP

DEPARTMENT OF BIOLOGY & BIOTECHNOLOGY CERTIFICATE PROGRAM, BALL STATE UNIVERSITY, MUNCIE, IN

MANUSCRIPT RECEIVED 07 JUNE 2018; ACCEPTED 10 NOVEMBER 2018

CORRESPONDING

Katherine G. Kornilow
kgkornilow@bsu.edu

KEYWORDS

• *Bacillus* spp.
• enterotoxins
• food pathogen

ABSTRACT

The bacterial species *Bacillus cereus* accounts for 1.4–12% of foodborne illness outbreaks worldwide, a statistic that is certainly an underestimate. This bacterial genus is capable of contaminating a wide range of food products, including rice, chicken, vegetables, spices, and dairy products. *B. cereus* endospores are partially resistant to pasteurization, dehydration, gamma radiation, and other physical stresses used in food processing, and their adhesive characteristics promote biofilm-forming capability on a variety of substrates in dairy operations. *B. cereus* and other closely-related species produce several types of exotoxins, including at least four hemolysins, three phospholipases, a heat/acid stable emetic toxin called cereulide, and three well-studied heat-labile enterotoxins that all cause gastroenteritis following ingestion. While a great deal of information on virulence gene presence and expression is known in *B. cereus*, very little has been done to explore the virulence potential of thermoduric spore-formers that may be found in ultrahigh temperature (UHT) pasteurized milk, and their ability to produce biofilms. Biofilm production is understood to be under similar regulation as toxins and other extracellular virulence determinants. This chapter describes the current status of knowledge with *Bacillus* spp. relevant to the dairy industry, virulence potential, and biofilm production from the perspective of food safety.

BACILLUS – GENERAL INFORMATION

Bacillus spp. bacteria show a wide range of characteristics that allow them to live in most natural environments (Griffiths, 2010). *Bacillus* comprise a large group of ubiquitous Gram-positive, rod-shaped, aerobic-to-facultatively anaerobic endospore-forming saprophytes (Weber & Rutala, 1988). Although the majority of *Bacillus* spp. are nonpathogenic, a few *(Bacillus cereus, Bacillus anthracis, and Bacillus thuringiensis)* opportunistically infect animal hosts (mammals and insects) (Vilian *et al.*, 2006). *Bacillus* microscopic morphology may be individual or as long chains in primary isolates from soil or water samples (Weber & Rutala, 1988). The size

of an individual rod can range from 0.5 x 1.2 um to 2.5 x 10 um. Spores produced by *Bacillus* spp. are resistant to heat (including to some extent, pasteurization conditions), cold, ionizing radiation, dehydration, and many disinfectants (Griffiths, 2010). The endospores are oval or cylinder shaped and are found centrally, sub-terminally or terminally. Over 30 species of *Bacillus* spp. are recognized, and are divided into two groups based mostly on the 16S rRNA/DNA sequences: the *Bacillus subtilis* group and the *Bacillus cereus* group. *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis* and *Bacillus pumilus* are mesophilic, have ellipse shaped spores, and are the most common members of the *B. subtilis* group. *B cereus, B. anthracis, B. thuringiensis, Bacillus weihenstephanensis, and Bacillus mycoides* do not ferment mannitol, produce lecithinase, and comprise the *B. cereus* group.

The colony morphology of *Bacillus* spp. is diverse across species. *Bacillus* spp. grow on nutrient agar or peptone media and exhibit ideal growth at a pH 7; however some *Bacillus* spp. grow at a pH of 9, while other species can endure pH 2. *Bacillus* spp. grow best within a temperature range of 30°–45°C, but thermophilic variants grow optimally at 65°C. All *Bacillus* spp. metabolize organic substrates such as amino acids, organic acids and sugars by aerobic respiration, anaerobic respiration, or fermentation, depending on species and environment. The enzymatic processes and metabolic characteristics are typically the criteria for *Bacillus* species differentiation.

*Bacillaceae f*amily members demonstrate a wide range of characteristics, including the ability to produce a battery of enzymes, antibiotics, and other secondary metabolites (Schallmey *et al*., 2004). For example, *Bacillus* spp. have unique abilities to synthesize and/or secrete many substances which are beneficial and show great success in agriculture and industry. Many *Bacillus* species exhibit antibacterial and antifungal activity against phytopathogens through secretory products (Yu *et al*., 2002), a logical evolutionary strategy since *Bacillus* spp. are soilborne or are found in epiphytes (plant that grows non-parasitically on another plant) and/or endophytes (living within a plant host)(Fravel, 2005). Many antimicrobial compounds are well recognized in the biotechnology and biopharmaceutical industries for their surfactant properties are derived from *B. subtilis* (Jacques, 2007). A surfactant lowers surface tension between two liquids or a solid and a liquid (Singhal, 2007). Surfactants are used for foam creation and stabilization in food processing, household products (paint, detergent, fabric softener), solubilization of agrochemicals, oil recovery, crude oil drilling lubricants, and bioremediation of water insoluble pollutants.

Phenotypically, the genus is difficult to delineate into species, but using genotypic methods, determination of relatedness has been revisited in recent years (Sneath, 1986). The mole % G + C content of the DNA is a well-regarded metric by which organisms may be compared genetically. The *Bacillus* genus is diverse and has a G + C content from 33–69% (Winn *et al*., 2006). Sequencing of16S rRNA genes and DNA–DNA hybridization methods have been used to assign species names (Goto *et al*., 2000). 16S rDNA has a hypervariant region (HV region) on the 5' end. This HV region is highly specific to each *Bacillus* spp. and is a good genotyping target. Overall, much emphasis has been placed in recent years on defining criteria for species determination within the genus *Bacillus*, although no single accepted system or approach has been established yet.

Other molecular techniques are also used to identify bacterial species including *Bacillus* spp. The following molecular techniques are used to confirm identify of *Bacillus* and other bacterial species: (1) polymerase chain reaction (PCR)(Adzitey *et al*., 2013); (2) pulsed field gel electrophoresis (PFGE); (3) random amplified polymorphism deoxyribonucleic acid (RAPD); and (4) matrix assisted laser desorption/ionization time of flight (MALDI-TOF)(Murray, 2012). PCR is a DNA replication process that amplifies small portions of DNA (amplicons) exponentially with the help of oligonucleotide primers and DNA polymerase. PCR has many different variations, including real time PCR (qPCR). qPCR is a powerful approach wherein template bacterial DNA is amplified and quantified at the same time using a standard curve-based comparison of type strain standards. PFGE is an agarose gel electrophoresis method that separates large pieces of genomic DNA. This separation of DNA is done by applying an electrical current that periodically changes between three different directions, providing a means to accurately resolve small differences in genomic sequences for bacterial community analyses. RAPD is a PCR based method that uses arbitrary primers to randomly amplify segments of target DNA, essentially acting as a DNA fingerprinting system for bacterial species. MALDI-TOF is a simple and rapid technique. Bacterial colonies are removed from the plate, mixed with a UV absorbing matrix (saturated solution of α-cyano-4- hydroxy-cinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and dried on a target plate. The target plates are exposed to laser pulses that develop an energy transfer from the matrix to the nonvolatile analyte molecules. The analyte is removed in the form of gas. The molecules are enhanced in a flight tube to the mass spectrometer. MALDI-TOF is accurate, rapid, and after initial purchase, inexpensive. These characteristics perhaps explain why MALDI-TOF is being used in hospitals for quick identification of bacterial infections.

BACILLUS IN CLINICAL SETTINGS

Through biofilm production, *B. cereus* has been implicated in contaminating intravenous catheters (Hernaiz *et al*., 2003) resulting in *B. cereus*-mediated sepsis (Kuroki *et al*., 2009; Ozkocaman *et al*., 2006). The formation of biofilms also allows the release of planktonic bacteria that produce additional biofilms increasing the severity of the infection (Costerton *et al*., 1999).

In addition to catheter contamination, *B. cereus* and its endospores have been shown to contaminate air filtration and ventilation equipment (Bryce *et al*., 1993), fiber optic bronchoscopy equipment (Goldstein & Abrutyn, 1985; Richardson *et al*., 1986), linens (Barrie *et al*., 1994), gloves (York, 1990), specimen collection tubes and balloons used in manual ventilation (VanDerZwet *et al*., 2000), alcohol-based hand wash solutions (Hsueh *et al*., 1999), plaster-impregnated gauze (Rutala *et al*., 1986), and many antiseptics such as chlorhexidine and povodone iodine (Dubuoix *et al*., 2005). The most common types of infections *B. cereus* causes, other than foodborne illness, include fulminant bacteremia, central nervous system (CNS) involvement (meningitis and brain abscesses), pneumonia, gas gangrene-like cutaneous infections and endophthalmitis.

B. CEREUS-MEDIATED ENDOPHTHALMITIS

B. cereus is not only capable of causing food-associated toxicoinfections, but can cause endophthalmitis as well (Davey & Tauber, 1987; Hermandy *et al.*, 1990; Ullman *et al.*, 1987). *B. cereus* is not the only pathogen capable of causing endophthalmitis, but is considered one of the most aggressive pathogens causing this condition. Because there is a limited immune response when a pathogen enters the eye, a wide spectrum of pathogens can enter and elicit a wide array of effects. Symptoms can range from a relatively painless anterior chamber inflammation (Aaberg *et al.*, 1998), to an explosive ocular and periorbital infection caused by *B. cereus* (Schemmer & Drebe, 1987). Specific toxin production by a particular microorganism is theorized to account for the difference in symptoms. *B. cereus* induced endophthalmitis is characterized by a corneal ring abscess followed by increased pain, chemosis, proptosis, retinal hemorrhage, and perivasculitis (Callegan *et al.*, 1999). Fever, leukocytosis, and general malaise often appear as the systemic manifestations of this condition (Martinez *et al.*, 2007).

B. cereus induced endophthalmitis can be divided into two categories: exogenous and endogenous. An exogenous source is due to blunt trauma that penetrates the eye, which may occur due to occupation (for example, metal workers), in an agricultural setting (David *et al.*, 1994) or infection resulting from unsterile instruments during cataract surgery. In one example in Rome, an ophthalmologist had four of his cataract patients lose vision in their treated eye one day after their cataract surgery (Simini, 1998). *B. cereus* is ranked second behind *Staphylococcus aureus* which is responsible for about 70% of post-cataract surgery endophthalmitis (Han *et al.*, 1996). The three main risk factors surgeons need to be aware of to reduce posttraumatic endophthalmitis are the presence of an intraocular foreign body, delay in closure of the globe, and the location/extent of the laceration of the globe.

Endogenous sources represent about 2–8% of all endophthalmitis cases (Romero *et al.*, 1999) and are due to bacteria entering the posterior segment of the eye. The most common pathogen to enter the posterior segment of the eye is *Candida albicans* but other common pathogens include S. *aureus, B. cereus, Escherichia coli, Neisseria meningitides* and *Klebsiella spp.* *B. cereus* can accomplish this route of entry through blood transfusion, contaminated needles/illicit drug injection paraphernalia (Grossniklaus *et al.*, 1985), or by iatrogenic administration of medications such as B vitamins or insulin (Motoi *et al.*, 1997).

Moyer *et al* (2009) demonstrated that *B. cereus* is capable of disrupting tight junctions between endothelial cells and the basement membrane of retinal capillaries and retinal pericytes as early as 4h post-infection. Such changes are hypothesized to be responsible for causing the loss of retinal structure and function (Kopel et al., 2008; Moyer *et al.*, 2009). The exact toxins from *B. cereus* responsible for causing this breakdown of the blood retinal barrier are unknown but are theorized to consist of the following molecules that may be working individually or in concert to achieve this effect: the Hbl enterotoxin, the Nhe enterotoxin, a crude exotoxin (CET) derived from cell-free *B. cereus* culture filtrates, phosphatidylcholine-preferring phospholipase C (PC-PLC), collagenase, cereolysin O (Shany *et al.*, 1974), or cereolysin AB (Scott *et al.*, 1996). However, only the Hbl enterotoxin protein has been identified for its role in endophthalmitis (Callegan *et al.*, 1999a).

Hbl enterotoxin has been shown to cause irreversible tissue damage to the photoreceptors of the retina in less than 12–24h causing blindness in the infected eye (Beecher *et al*., 1995; Davey & Tauber, 1987).

B. cereus is capable of disrupting the blood retinal barrier as early as 4h in retinal tissues, 6h post-infection in aqueous humor, and in all other ocular tissues 12h post-infection (Callegan *et al*., 1999b). *B. cereus* has been shown to be a more rapid and virulent endophthalmitis pathogen compared to S. *aureus* and *Enterococcus faecalis*. Additionally, *B. cereus* seems to exhibit an almost immediate inflammatory response despite low numbers of the organism present at the early stages of infection.

Limited research exists addressing the exact role the immune system plays in endophthalmitis, but the eye is known to be an immunoprivileged site as was first described by Medawar in 1948 (Cunha-Vaz, 1997). The eye restricts both the adaptive and innate immune systems in such a way to balance the challenge of pathogen infection against inflammation-induced vision loss (Streilien, 2003).

In most instances of *B. cereus* induced endophthalmitis, vision loss occurs regardless of the type of therapeutic or surgical intervention utilized because the severity of the disease has progressed to such a condition, that too many toxins have been released by *B. cereus* and many bacteria will have migrated in the eye out of the reach of antibiotics (Callegan *et al*., 2006). Thus within a 12–18h time frame, massive tissue destruction occurs to the retina and surrounding ocular tissues resulting in antibiotics no longer being maximally effective (Callegan *et al*., 2002). In addition, the inflammatory response inside the eye is so aggressive that even if the antibiotics control *B. cereus*, the inflammation produced causes damage to surrounding ocular structures thus making it difficult to manage ocular infections.

BACILLUS IN FOOD

Foodborne illness from a variety of microorganisms affects on average 76 million individuals in the U.S. each year resulting in some 5,000 deaths (Mead *et al*., 1999). Worldwide statistics on *Bacillus cereus* foodborne illness are underestimated due to a variety of factors, including emetic symptoms similar to *Staphylococcus aureus* intoxication and diarrheal symptoms similar to those elicited by *Clostridium perfringens* type A. Most affected individuals do not seek medical attention due to the short duration of signs and symptoms. *B. cereus* seems to account for between 1.4–12% of foodborne illness outbreaks worldwide (Stenfors *et al*., 2008).

Bacillus spp. are capable of contaminating a wide range of food products, including rice, chicken, vegetables, spices, and dairy products. Contamination in the dairy industry may occur when *B. cereus* spores come in contact with the udders of cows (Andersson et al. 1995), if the spores colonize feed or bedding, or if the spores survive pasteurization (Claus & Berkley, 1986; Sneath, 1986). This is a serious problem in the food industry because *B. cereus* endospores are in many instances partially resistant to the

heat of pasteurization, dehydration, gamma radiation, and other physical stresses. This resistance is due to the ultrastructure of the endospore of course, but also in part to the hydrophobic nature of the spores that allows them to adhere strongly to surfaces and develop biofilm-like properties (Mattson *et al.*, 2000; Ronner *et al.*, 1990). For example, an irradiation dose of 1.25–4 kGy needs to be administered to reduce spores by 90% (De Lara et al., 2002). Also, pasteurization may result in the activation and germination of spores (Hanson *et al.*, 2005). In addition, *B. cereus* endospores germinate in response to particular nutrients such as glycine or in response to physical stress such as temperature (spore germination can occur over 5–50°C in cooked rice) (Granum, 1994) and high pressures (i.e. 500 MPa). Thus foods need to be cooked at least at a temperature of 100°C (212°F) or above to kill most of the endospores (Griffiths & Shraft, 2002).

Thermoduric sporeformers have many important reasons to be the subject of great interest within the dairy industry (Burgess *et al.*, 2010). Thermophilic bacilli produce heat-resistant (80–100°C) and highly heat-resistant (>106°C) endospores in UHT treated products, which can lay dormant for years. Heat, chemicals, and pH levels can activate a spore for germination and outgrowth. This is particularly important in the dairy industry because heat is used as a preservation mechanism. *B. subtilis* has a low activation temperature of 65–70°C. Once the spores are activated, germination is elicited by nutrients that bind to germination receptors. A nutrient mixture of asparagine, glucose, fructose and K+ (AGFK) triggers *B. subtilis* spore germination (Setlow, 2003).

Many people consider *B. anthracis*, *B. thuringensis*, and *B. cereus* to be the same species (Helgason *et al.*, 2000). *B. anthracis* is found in the soil and infects primarily herbivorous animals, causing human disease (Winn *et al.*, 2006; Kolsto *et al.*, 2009). This disease may be contracted by local infections of skin lesions, through the gastrointestinal (GI) route, or by inhalation. Respiratory and GI-acquired routes are highly lethal forms of anthrax. *B. anthracis* virulence mechanisms easily allows for the spread of the bacteria to the lymph nodes. Once in the lymph nodes, the bacteria disseminate via the bloodstream and internal organs. *B. anthracis* spores are highly resistant to adverse environmental conditions and it is difficult to be certain that the organism has been fully eradicated from endemic areas (Winn *et al.*, 2006). The endospores are maintained in soil and stay dormant indefinitely. The virulence determinants produced by *B. anthracis* are composed of three proteins: a protective antigen (PA), an edema factor (EF), and the lethal factor (LF). Virulent strains are also typically capsule-producers. Toxin expression and production is enhanced by elevated CO2 and growth temperatures of 35–37° C. *B. anthracis* strains harbor two large plasmids, pXO1 and pXO2 (Kolsto *et al.*, 2009). These plasmids are needed for full virulence. pXO1 contains the coding for the PA (pag), EF (cya), and LF (lef). pXO2 contains a five-gene operon for the biosynthesis of a polyglutamate capsule. This capsule is important for the ability to escape the host immune system, by protecting the vegetative cells from phagocytosis.

B. thuringensis classification has been accomplished by H serotyping, which utilizes bacterial flagellar antigens (Sneath, 1986). This species has unique insecticidal properties demonstrating activity against several insect orders, as well as nematodes, mites and protozoa. *B. thuringensis* produces protoxins during sporulation (Aronson *et al.*, 1986). These toxins are either parasporal inclusions or found on the spore surface. *B.*

thuringensis produces parasporal crystals during sporulation, which are inclusions of insecticidal toxins. The midgut of the larvae have proteases that convert protoxins to toxins, activating the toxin to bind to receptors on columnar midgut cells. This binding event results in pore formation of the midgut epithelium, and susceptible insects die from this extensive damage and pH changes as midgut contents mix with the hemocoel cavity. Three common subspecies variants have been recognized and well characterized over the last 40 years: (1) *B. thuringiensis subsp. kurstaki*; (2) *B. thuringiensis subsp. israelensis*; and (3) *B. thuringiensis subsp. japanensis*. Each produces crystalline endotoxin specific for a unique order of insect for selectively toxic biological control. Interestingly, each is also extremely genetically similar to the type strain pathogen in this family, *B. cereus*.

B. cereus and other *Bacillus* spp. are a major cause of foodborne illness globally and a major cause of endophthalmitis (Weber & Rutala, 1988; Stenfors *et al*., 2008; Moyer *et al*., 2008). *B. cereus* has an optimum growth temperature of 30°–40°C, although psychotrophic members can grow in temperatures as low as 4°C. *B. cereus* can grow in a pH of 5.0–8.8 with optimal pH of 6.0–7.0. Food poisoning due to *B. cereus* is underreported because it is short-term and self-limiting. In 2005, *Bacillus* spp. were responsible for 1.4% of foodborne illness in Europe. In the Netherlands 12% of foodborne illness was caused by *B. cereus* between 1993–1998. In 2006, an average of 63,400 (0.4%) people were domestically affected with *B. cereus food* poisoning (Scallan *et al*., 2011). Reports of *B. cereus* induced food poisoning has increased in industrialized countries, however reporting and testing is variable. In the US passive surveillance is usually performed due to low hospitalization of *B. cereus* food poisoning.

Foods frequently contaminated by *B. cereus* include milk, dairy products, dry foods, rice, egg products and legumes. Two types of food-related illnesses are caused by *B. cereus*: (1) Type 1: short-incubation "emetic" and (2) Type 2: long incubation "diarrheal." Type 1 has an incubation time of 2 hours and lasts approximately 9 hours. Type 2 has an incubation time of 9 hours and lasts 24 hours. Type 1 is mostly associated with contaminated rice and type 2 is associated to contaminated meat or vegetables.

The main virulence factor for type 1 food poisoning caused by *B. cereus* is cereulide (Cueppens et al., 2011). Cereulide is a small molecular weight heat stable exotoxin that can withstand treatment at 121°C for 2 hours at a pH of 7.0. This stability means the toxin can withstand frying, roasting, and microwave exposure, eliciting a foodborne emetic intoxication in susceptible individuals. The main causes of type 2 foodborne illness are hemolysin B (Hbl) and non-hemolytic enterotoxin (Nhe), both comprised of three components encoded by separate operons (Fig. 1) – typical AB toxin architecture. Hbl is made of the cytolytic subunits HblC and HblD, and the protein B binding domains. The Hbl operon also has a fourth member, the *hblB* gene. However, *hblB* is not transcribed and is likely a pseudogene. Nhe is made of the cytolytic protein NheA, and the protein B binding sections NheB and NheC. In recent research, 7.5% of reported emetic symptoms have been linked to Hbl and Nhe. These toxins are a product of aerobic, spore forming *B. cereus*.

Aerobic spore formers in food are ubiquitous. This ubiquity makes it impossible to prevent aerobic spore formers from being present in many fresh foods. Spore counts in raw milk vary throughout the year, but are highest in winter when dairy cows are primarily indoors. Pasteurization is effective

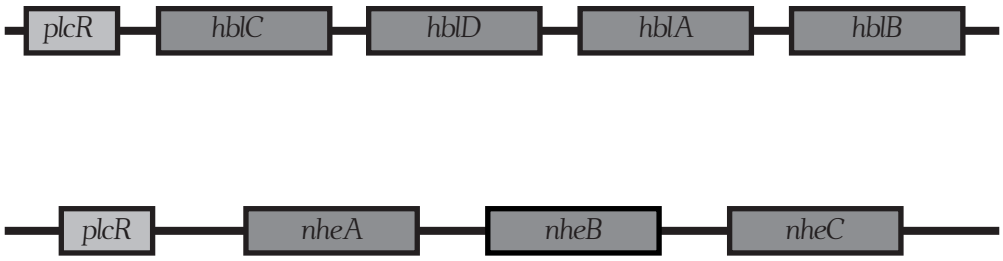


Figure 1. *Bacillus* spp. HBL and NHE operons. The *plcR* region is the regulator gene as described in the text [24]."

in inactivating vegetative cells in raw milk, but fails to kill many spores. The spores have no competition from vegetative cells, so they proliferate rapidly if the product is mishandled or improperly stored. The sporulated *Bacillus*, upon germination, can adhere to pipelines and equipment, causing biofilm formation. These spores and vegetative cells in equipment and raw milk may be tolerant to sterilization. Biofilm extrapolymeric substances (EPS) offer a significant survival strategy to established populations of bacteria. These counteractive techniques include ultra-high temperature (UHT) processing, previously known to inactivate all living material, however spores are now known to survive UHT-processing.

UHT-processing is achieved by treating fluid milk at 135–150°C for 1–8 seconds. The milk flows continuously during this process and is packaged into pre-sterilized containers (aseptic packaging). The UHT process is designed to kill almost all organisms including spores. The concern is that some spores still survive and there is no competition for these spores, giving them an ideal environment to proliferate. The growing concern for psychrotolerant spore formers is that they show potential to induce foodborne illness and produce spoilage defects caused by enzymatic activity. These concerns are due to a combination of the following reasons: (1) longer refrigeration storage pre-pasteurization; (2) higher temperatures used for pasteurization; (3) prolonged shelf life; and (4) pasteurization activates the germination of spores. A combination of these "advantages" are beneficial for *B. cereus* endospores to form from vegetative cells or vegetative cells to form endospores.

Production length of milk treatment has been reduced to 6–8 hours to help reduce thermophile growth (Burgess *et al*., 2010). Once a production cycle is complete, a cleaning-in-place (CIP) method is performed on the equipment. CIP consists of the following steps: (1) a warm water rinse; (2) a 1.5% caustic wash at 75°C for 30 minutes; (3) a water rinse; (4) a 0.5% nitric acid wash at 70°C for 20 minutes; and (5) a second water rinse. These steps have helped with growth within the equipment, but not within the milk itself. Table 1 indicates the time and temperature requirements laid out by the Food and Drug administration for pasteurization regimes, including UHT pasteurization.

BACILLUS SPP. BIOFILMS

Adherence of microbial biofilms to dairy production surfaces makes sanitization more difficult, and increases cost via labor and chemical usage along with lost production time. FDA involvement and subsequent product recalls can also occur causing further financial problems for dairies. Araújo et al. (2009) have proposed a basic mechanism for biofilm adhesion based on six general stages. First, the biofilm surface must be primed for adhesion with the existence of food deposits. The biofilm-producing microorganism must then come into contact with the primed surface. Positive and negative biochemical forces including van der Waals forces and other electrostatic forces then allow the biofilm to make a non-permanent attachment to the surface when microorganism are between 20 and 50 nm away. Irreversible adhesion results within 1.5 nm when extracellular polysaccharide production, ionic bonds, and hydrophobic forces occur. The fourth stage is described by the multiplication of bacterial cells and an increase in secreted polysaccharides and the fifth stage involves strong metabolism in the biofilm. Lastly, microorganisms begin to be released from the biofilm during the sixth stage, shedding bacteria to generate new biofilms elsewhere.

Several authors have identified a variety of mesophilic *Bacillus* subspecies capable of surviving ultra-high temperature pasteurization via endospore formation (Araújo *et al.* 2009; Lindsay *et al.* 2002; Scheldeman et al. 2006; Sutyak *et al.* 2008). Using bacterial cultures sampled from dairies, 16s rRNA, and PCR amplification some of the most prevalent and potentially problematic species, in regards to biofilm production, have been characterized. These species include *B. cereus*, *B. amyloliquefaciens*, and several others.

The level of virulence activity in *B. cereus* cells is due to a number of different environmental factors, including temperature, pH, oxygen tension, glucose concentrations, and specific antimicrobial chemical compounds (Glatz and Goepfeort, 1976; Sutherland and Limond, 1993). Biofilm production is understood to be under similar regulation as toxins and other extracellular virulence determinants, which suggests that subinhibitory stress may have great influence on overall potential for *Bacillus spp.* to become problematic in dairy microbiology settings.

QUORUM SENSING

Quorum sensing is a regulatory system where the bacterium (*B. cereus*) recognizes an extracellular signal caused by an autoinducer (AI) to sense the density of *B. cereus* in the immediate environment. Quorum sensing is used to govern cell density, and the corresponding regulation of relevant gene expression that would enhance survival during the log-to-stationary phase transition in dense cultures, or in a natural environment such as soil, food, or within a host [28]. Quorum sensing mechanisms control many processes in the bacterial cell, including sporulation, biofilm production, and virulence factor secretion [27]. Quorum sensing involves direct or indirect activation of a related receptor protein by the AI (Graumann, 2012). This activation results in up- or down-regulation of specific genes. All quorum sensing routines are dependent on three principles: (1) the bacterial species produces AIs; (2) AIs are detected by membrane or cytoplasm receptors; and (3) AIs produce a positive feedback loop (Rutherford *et al.*, 2012).

Gram-positive bacteria use small, post-translationally modified peptides as same species AIs, called Autoinducing Peptides (AIPs) (Graumann, 2012). AIPs are expressed as large, precursor peptides and processed into smaller, cyclic, thiolactone-containing peptides that are transported across the membrane. This transportation can happen in two ways: (1) two-component signaling (Fig. 2) or (2) AIP-binding transcription factor signaling (Fig. 3). In the two-component signaling method, once the AIPs are transported outside of the cell they are too hydrophilic to cross the membrane without help. The AIPs remain in the extracellular matrix. The bacteria sense the AIP as it binds to the receptor protein (histidine kinase) located in the neighboring bacterial cell surface. This binding induces phosphorylation of the kinase. The phosphoryl group is then transferred to an aspartate residue of the response regulator. Then this binds to the promoter region of target genes, which activates or represses transcription.

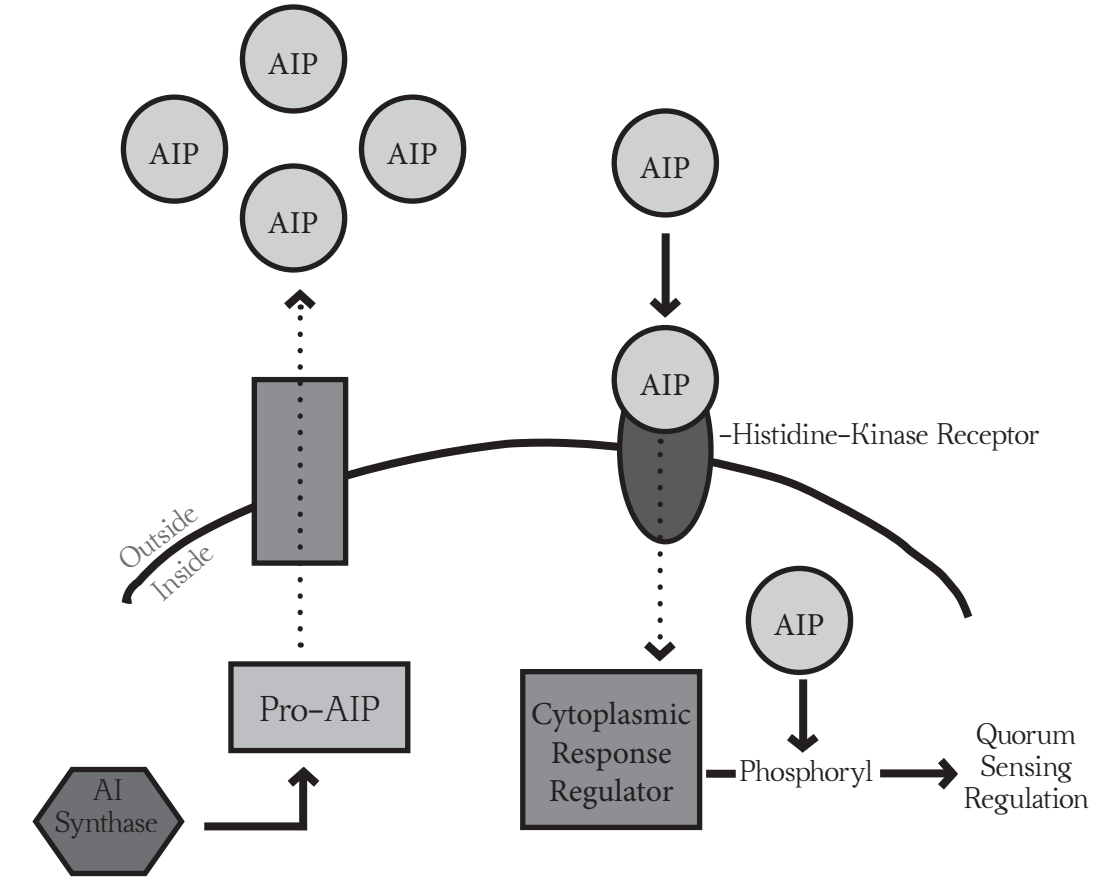


Figure 2. Two-component quorum sensing of Gram-positive bacteria. AI synthase is used to process and transport the pro-AIP out of the cell. Once the concentration of AIP outside the cell is high, AIP binds to histidine kinase receptors. This binding activates the kinase activity of the receptor, inducing autophosphorylation. The phosphoryl group binds to the response regulator and activates transcription of the quorum sensing system genes [27].

QUORUM SENSING & *BACILLUS* SPP. PATHOGENESIS

Quorum sensing in *B. cereus* is dependent on a protein PlcR. PlcR is a pleiotropic regulator of most virulence factors specific to the B. cereus group (Nhe and Hbl) (Rutherford *et al.*, 2012). The activity of PlcR depends on binding to the AIP that is produced from the PapR protein. PapR is a small signaling peptide that acts as a quorum sensing effector (Slamti & Lereclus, 2005) (Fig. 3). PapR is 48 amino acids long and is encoded by an open reading frame located downstream from plcR. PapR is secreted from the cell forming a PapR pro-AIP. PapR pro-AIP is processed by neutral protease B (NprB) to form the active AIP. The AIP is transported back into the cell by an oligopeptide permease system (Opp). AIP then binds to the transcription factor PlcR, activating the protein. This PlcR-AIP complex regulates the production of virulence factors and a positive feedback loop for *papR*. It has been shown that PlcR expression is positively regulated by CodY expression (Frenzel *et al.*, 2012).

CodY is a global transcriptional regulator that facilitates advantageous changes in response to variations of available nutrients in Gram-positive bacteria (Sonenshein, 2005). CodY is a GTP and isoleucine binding protein that also initiates endosporulation. The binding of GTP and isoleucine act as co-repressors of the transcription of many genes.

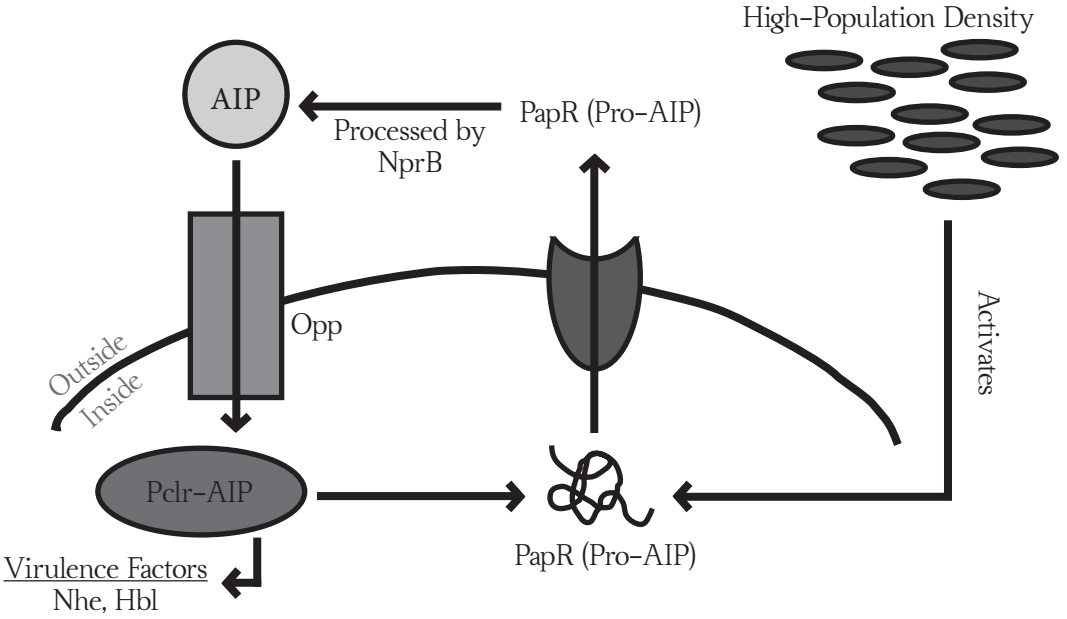


Figure 3. AIP binding PlcR (transcription factor) quorum sensing signaling in *B. cereus*. A high population density outside the cell activates PapR (pro-AIP) and then the PapR (pro-AIP) is secreted outside of the cell. PapR (pro-AIP) is processed by the protease NprB to become a heptapeptide AIP. AIP is transported back into the cell using an Opp. Once AIP is inside the cell, it binds and activates PlcR. This PlcR-AIP complex regulates virulence factors and also produces a positive feedback loop for PapR secretion [27].

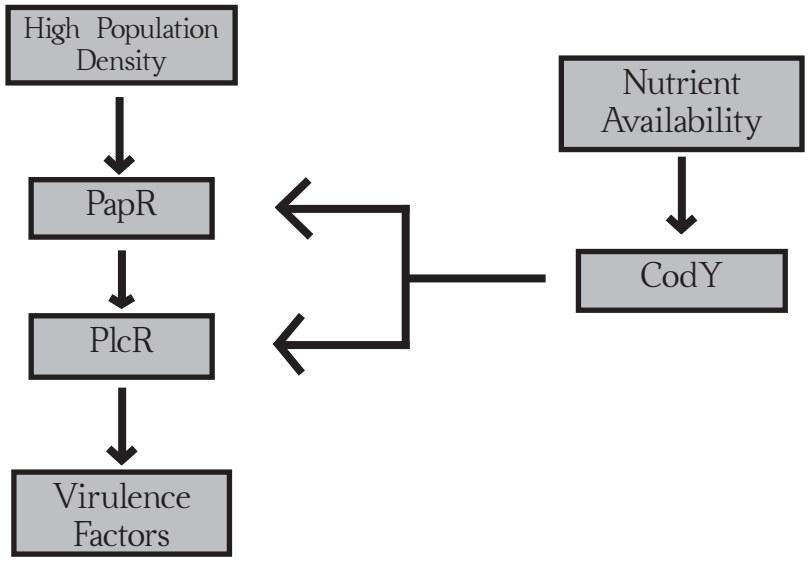


Figure 4. CodY regulates PapR and PlcR expression in *B. cereus*. Nutrient availability for the cell regulates the expression of CodY [27, 30].

Endosporulation happens when there is a response to bacterial starvation by limited levels of carbon, nitrogen or phosphorous. Endosporulation is a defense mechanism of Gram-positive bacteria, like a turtle hiding in its shell, to protect its genome. Nucleotide synthesis is dependent on carbon, nitrogen, and phosphorus. This spore keeps the genome dormant until the environment is favorable enough to replicate. CodY is a transcriptional activator of the plcR gene [30] (Fig. 4). In a Δ*codY B. cereus* strain, PlcR expression was strong in exponential, late exponential, and stationary phases of growth. In contrast, a wildtype *B. cereus* strain showed expression of PlcR in only the stationary phase of growth. CodY was first found in *B. subtilis* to control expression of more than 100 stationary phase genes. Thus, it is generally accepted that (like *plcR*), *codY* is widely conserved among Bacillaceae family members.

B. amyloliquefaciens belongs to the *B. subtilis* group (Priest *et al.*, 1987). Members of this group exhibit similar behaviors physiologically, although *B. amyloliquefaciens* is not a subspecies of *B. subtilis* due to the difference in □-amylase production. *B. amyloliquefaciens* has been found to share less than 5% homology at the DNA level with *B. subtilis*. *B. subtilis* has been shown to express CodY (Serror & Sonenshein, 2002; Ratnayake-Lecamwasam *et al.*, 2001). Phelps and McKillip (2002), using DNA PCR, found that hblC, hblD, hblA, nheA, and nheB genes or gene homologues were present in a different strain of *B. amyloliquefaciens* obtained from a Louisiana creamery, although expression of these genes was not measured. Thus, the potential for this species to harbor and express these or other virulence factors (via global effectors CodY and/or PlcR) is a realistic possibility, despite this species being placed (at least currently) in the *B. subtilis* group rather than the *B. cereus* group.

SUMMATION & FUTURE WORK

The debate over proper identification and understanding of *Bacillus* virulence has been ongoing for over 50 years (Rasko et al., 2005). Recent public awareness of potential bioterrorism using the anthrax toxin produced by *B. anthracis* has lead government agencies to fund multiple studies aimed at rapidly differentiating *B. anthracis* from other closely related *Bacillus* species such as *B. cereus* and *B. thuringiensis*, since *B. anthracis* produces the anthrax toxin encoded by two plasmid-based operons, pXO1 and pXO2. The anthrax toxin primarily kills herbivore mammals, but can also kill humans. Not to be underestimated, *B. cereus* can cause severe food poisoning through its production of emetic and diarrheal toxins. While heavily used as an insecticidal agent in crops with its Cry crystalline toxins, *B. thuringiensis* has also recently been demonstrated to cause food poisoning symptoms in humans similar to *B. cereus*. Ironically, species like *Bacillus coagulans*, which has been found to harbor the *nheA* gene, are readily used as probiotics in human health.

Bacillus spp. were originally differentiated into species at a time when biologists did not possess the molecular tools to delve deeper than biochemical tests and phenotypical observations. While this strategy worked well for other genera, 16S rRNA analysis of differences among *B. cereus*, *B. thuringiensis*, and *B. anthracis* have shown these species to have a nucleotide sequence difference of less than 1%. Recent advances in molecular biology have allowed scientists to scrutinize the genetic properties of these three "species". After exhaustive studies using DNA-DNA hybridization, 16S and 23S rRNA comparative analyses, multilocus sequence typing (MLST), fluorescent amplified fragment length polymorphism analysis, rep-PCR, and small nucleotide polymorphism (SNP) analyses, scientists have been unable to reliably differentiate these three *Bacillus* species.

While many methods have been pursued, most results have suggested that *B. cereus*, *B. thuringiensis*, and *B. anthracis* should be considered the same species due to highly conserved nucleoidal genetic sequences. Due to the easily identifiable symptoms of *B. anthracis* and *B. cereus*, there is recent concern among biologists that the "*B. anthracis*" species may in fact be an oversampled subset of *B. cereus*. Other scientists speculate that *B. anthracis* may have only recently evolved to the point to be considered distinct from *B. cereus*. Unfortunately, recent literature is contradictory when discussing how similar two separate *Bacillus* genomes need to be in order to be considered the same species. There are claims that *B. thuringiensis*, *B. cereus*, and *B. anthracis* should be considered one species on the basis of genetic evidence. Alternatively, other scientists claim that current taxonomy has not divided *Bacillus* strains enough, suggesting that more species or subspecies than currently listed in literature exist. No commonly accepted definition that separates these species on genetic evidence has been found.

There are three nhe genes that are encoded on the nheABC operon, and have been shown to remain conserved as a cluster during genetic recombination. It can reasonably be assumed that the presence of the most proximal subunit of nhe indicates the presence of the other two genes. In the literature, all genes encoding the Nhe and Hbl enterotoxins have been readily located downstream in both *B. cereus* and *B.*

thuringiensis (Phelps & McKillip, 2002).

The presence of the nheABC operon does not necessarily indicate a virulent strain, but has a very high likelihood of expressing these genes in a host environment or in food under permissive conditions. Thus, future work to determine the pathogenicity of nheA positive samples could include the use of a Tecra VIA to detect enterotoxin proteins. Without this step, the virulence of nheA positive samples cannot be definitively determined. A large degree of genetic variation exists in nhe sequences among Bacillus spp., giving rise to false negative results in PCR-based detection assays. Strains negative for nheA in real-time PCR have been found to produce the enterotoxin Nhe as determined using a Tecra VIA kit.

The nheABC operon is mobile among Bacillus spp. through horizontal gene transfer (HGT). Indeed, HGT has been observed among *Bacillus* spp. and can serve as a mechanism explaining the incidence of non-*B. cereus* samples positive for nheA. While no data has been found to suggest that this gene transfer mechanism uses an integron, the anthrax-like operon pXO16 found in *B. thuringiensis* is part of a conjugative plasmid. It is reasonable conjecture that other *Bacillus* species may also harbor conjugative plasmids that aid in HGT.

Within *Bacillus*, most virulence factors are encoded on plasmids (55), which have been demonstrated to readily transfer between differing species. Indeed, a recent study indicated that the virulence genes associated with *B. cereus* infection undergo frequent rearrangement both within the bacterial nucleoid and between species. Thus, a better method than traditional biochemical tests to detect pathogenic *Bacillus* strains is to screen for virulence operons present in plasmids or in nucleoidal DNA.

Bacillus genomes that have been sequenced display a high level of genetic synteny in their gene order. Two genes that encode for bacterial ribosomes, 16S and 23S rDNA, contain genetic sequences that are less than 1% different when compared between B. *cereus, B. thuringiensis*, and *B. anthracis* (12). A dissimilarity of 3% between 16S or 23S rDNA sequences is the minimal "cut off" between two strains to be considered as distinct species. Additionally, the *gyrB* gene sequence shared among these species is very homologous. Because these genes are shared among different species within the *Bacillus* genus, they cannot be used to differentiate species. However, 16S and 23S rRNA can be used to differentiate between different strains of *B. anthracis*.

Interestingly, there are a number of mechanisms that facilitate the movement of genes between different members of the *Bacillus* genus. One such mechanism is through the natural action of bacteriophage. After lysing its host cell, the bacteriophage will insert its genes into *Bacillus* genomes. While normally either lytic or lysogenic, it is possible for prophage to undergo random mutation, which renders it unable to enter the lysogenic cycle. In this way, genes from one species of bacteria can be transferred to *Bacillus spp.* As previously mentioned, *Bacillus* operons may be on conjugative plasmids. Additionally, *Bacillus* spp. are naturally competent, allowing these microbes to naturally take up random DNA in their vicinity.

The virulence genes for Nhe are present in more strains of Bacillus than is currently accepted within the scientific community. This research identified several "species" of *Bacillus* that were not previously known to harbor the Nhe enterotoxin operon. Given

that a debate is currently underway about the very identity of *B. cereus* and other strains, it is improper for food safety experts to screen food products only for *B. cereus*. Phenotypic-based classification techniques have failed to accurately differentiate *Bacillus* species. Additionally, no molecular-based approach can accurately differentiate *Bacillus*. The bottom line is the determination of species within *Bacillus* does not even matter when concerned with food safety. Molecular techniques should instead screen for virulence determinants in microbes instead of identifying said microbes. Since endospore formation enables *Bacillus spp.* to be ubiquitous in the environment and on food, all foods should be examined in this way. This is the only true way to determine whether food products are safe for human consumption.

REFERENCES

1. Aaberg, T.M., Flynn, H.W., Schiffman, J., & Newton, J. 1998. Nosocomial acute-onset postoperative endophthalmitis survey: a 10 year review of incidence and outcomes. *Ophthalmology* 105:1004-1010.

2. Adminstration, U.S.F.a.D., Grade A "Pasteurized Milk Ordinance", P.H.S. *Department of Health and Human Services, Food and Drug Adminstration, Editor*. 2001: Washington, D.C.

3. Adzitey, F., Huda, N., & Ali, G. R. R. 2013. Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. *3 Biotech*: p. 97–107.

4. Andersson, A., Ronner, U., & Granum, P.E. 1995. What problems does the food industry have with the spore-forming pathogens *Bacillus cereus* and Clostridium perfringens? *Int. J. Food Microbiol.* 28:145-155.

5. Aronson, A. I., Beckman, W., & Dunn, P. 1986. Bacillus thuringiensis and related insect pathogens. *Microbiological Reviews*, 50(1), 1–24.

6. Barrie, D., Hoffman, P. N., Wilson, J. A., & Kramer, J. M. 1994. Contamination of hospital linen by *Bacillus cereus*. *Epidemiology and Infection*, 113(2), 297–306.

7. Beecher, D. J., Pulido, J. S., Barney, N. P., & Wong, A. C. 1995. Extracellular virulence factors in Bacillus cereus endophthalmitis: methods and implication of involvement of hemolysin BL. *Infection and Immunity*, 63(2), 632–639.

8. Bryce, E., Smith, J., Tweeddale, M., Andruschak, B., & Maxwell, M. 1993. Dissemination of Bacillus cereus in an Intensive Care Unit. *Infection Control and Hospital Epidemiology*, 14(8), 459-462.

9. Burgess, S. A., Lindsay, D., & Flint, S. H. 2010. *Thermophilic bacilli* and their importance in dairy processing. *International Journal of Food Microbiology*, 144(2), 215-225.

10. Callegan, M. C., Booth, M. C., Jett, B. D., & Gilmore, M. S. 1999. Pathogenesis of gram-positive bacterial endophthalmitis. *Infection and Immunity*, 67(7), 3348-3356.

11. Callegan, M. C., Engelbert, M., Parke, D. W., Jett, B. D., & Gilmore, M. S. 2002. Bacterial Endophthalmitis: Epidemiology, Therapeutics, and Bacterium-Host Interactions. *Clinical Microbiology Reviews*, 15(1), 111–24

12. Callegan, M. C., Jett, B. D., Hancock, L. E., & Gilmore, M. S. 1999. Role of Hemolysin BL in the Pathogenesis of Extraintestinal *Bacillus cereus* Infection Assessed in an Endophthalmitis Model. *Infection and Immunity*, 67(7), 3357–3366.

13. Callegan, M. C., Novosad, B. D., Ramirez, R., Ghelardi, E., & Senesi, S. 2006. Role of swarming migration in the pathogenesis of *Bacillus endophthalmitis*. *Investigative Ophthalmology & Visual Science*, 47(10), 4461-4467.

14. Ceuppens, S., Rajkovic, A., Heyndrickx, M., Tsilia, V., Van De Wiele, T., Boon, N., & Uyttendaele, M. 2011. Regulation of toxin production by *Bacillus cereus* and its food safety implications. *Critical Reviews in Microbiology*, 37(3), 188-213.

15. Chapman, K. W., & Boor, K. J. 2001. Acceptance of 2% ultra-pasteurized milk by consumers, 6 to 11 years old. *Journal of Dairy Science*, 84(4), 951-954.

16. Claus, D. & Berkley, R.C.W. 1986. Genus *Bacillus* Cohn 1872, 174. In P.H.A Sneath, N.S. Mair, M.E. Sharpe, & J.G. Holt (Ed.), *Bergey's Manual of Systematic Bacteriology*.(pp. 1105-1137) Williams and Wilkins, Baltimore.

17. Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science*, 284(5418), 1318-1322.

18. Cunha-Vaz, J. G. 1997. The blood-ocular barriers: past, present, and future. *Documenta Ophthalmologica*, 93(1-2), 149-157.

19. Davey Jr, R. T., & Tauber, W. B. 1987. Posttraumatic endophthalmitis: the emerging role of *Bacillus cereus* infection. *Clinical Infectious Diseases*, 9(1), 110-123.

20. David, D. B., Kirkby, G. R., & Noble, B. A. 1994. *Bacillus cereus* endophthalmitis. *The British Journal of ophthalmology*, 78(7), 577.

21. De Lara, J., Fernández, P. S., Periago, P. M., & Palop, A. 2002. Irradiation of spores of *Bacillus cereus* and Bacillus subtilis with electron beams. *Innovative Food Science & Emerging Technologies*, 3(4), 379-384.

22. Dubouix, A., Bonnet, E., Alvarez, M., Bensafi, H., Archambaud, M., Chaminade, B., ... & Marty, N. 2005. *Bacillus cereus* infections in traumatology–orthopaedics department: retrospective investigation and improvement of healthcare practices. *Journal*

of Infection, 50(1), 22–30.

23. Priest, F. G., Goodfellow, M., Shute, L. A., & Berkeley, R. C. W. 1987. *Bacillus amyloliquefaciens* sp. nov., nom. rev. *International Journal of Systematic and Evolutionary Microbiology*, 37(1), 69–71.

24. Fravel, D. R. 2005. Commercialization and implementation of biocontrol 1. Annu. Rev. *Phytopathol.*, 43, 337–359.

25. Frenzel, E., *et al.*, 2012. CodY orchestrates the expression of virulence determinants in emetic *Bacillus cereus* by impacting key regulatory circuits. *Molecular Microbiology*, 85(1), 67–88.

26. Goldstein, B., & Abrutyn, E. 1985. Pseudo-outbreak of *Bacillus* species: related to fibreoptic bronchoscopy. *Journal of Hospital Infection*, 6(2), 194–200.

27. Goto, K., Omura, T., Hara, Y., & Sadaie, Y. 2000. Application of the partial 16S rDNA sequence as an index for rapid identification of species in the genus *Bacillus*. *The Journal of General and Applied Microbiology*, 46(1), 1–8.

28. Gracias, K. S., & McKillip, J. L. 2011. Triplex PCR-based detection of enterotoxigenic *Bacillus cereus* ATCC 14579 in nonfat dry milk. *Journal of Basic Microbiology*, 51(2), 147–152.

29. Granum, P. E. 1994. *Bacillus cereus* and its toxins. *Journal of Applied Microbiology*, 76(S23).

30. Graumann, P. (Ed.). 2012. *Bacillus*: cellular and molecular biology. *Horizon Scientific Press*.

31. Griffiths, M. W. 2010. *Bacillus cereus* and Other *Bacillus spp. Pathogens and Toxins in Foods*,1–19.

32. Griffiths, M. W., & Schraft, H. 2002. *Bacillus cereus* food poisoning. *Foodborne Diseases*, 2, 261–270.

33. Grossnikiaus, H., Bruner, W. E., Frank, K. E., & Purnell, E. W. 1985. *Bacillus cereus* panophthalmitis appearing as acute glaucoma in a drug addict. *American Journal of Ophthalmology*, 100(2), 334–335.

34. Yu, G. Y., Sinclair, J. B., Hartman, G. L., & Bertagnolli, B. L. 2002. Production of iturin A by *Bacillus amyloliquefaciens* suppressing Rhizoctonia solani. *Soil Biology and Biochemistry*, 34(7), 955–963.

35. Han, D. P., *et al.*, 1996. Spectrum and susceptibilities of microbiologic isolates in the Endophthalmitis Vitrectomy Study. *American Journal of Ophthalmology*, 122(1), 1–17.

36. Hanson, M. L., Wendorff, W. L., & Houck, K. B. 2005. Effect of heat treatment of milk on activation of *Bacillus* spores. *Journal of Food Protection*, 68(7), 1484–1486.

37. Helgason, E., *et al.*, 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—One Species on the Basis of Genetic Evidence. *Applied and Environmental Microbiology*, 66(6), 2627–2630.

38. Hemady, R., Zaltas, M., Paton, B., Foster, C. S., & Baker, A. S. 1990. *Bacillus*-induced endophthalmitis: new series of 10 cases and review of the literature. *The British Journal of Ophthalmology*, 74(1), 26–29.

39. Hernaiz, C., Picardo, A., Alos, J. I., & Gomez Garces, J. L. 2003. Nosocomial bacteremia and catheter infection by *Bacillus cereus* in an immunocompetent patient. *Clinical Microbiology and Infection*, 9(9), 973–975.

40. Hsueh, P.-R., *et al.*, 1999. Nosocomial Pseudoepidemic Caused by *Bacillus cereus* Traced to Contaminated Ethyl Alcohol from a Liquor Factory. *Journal of Clinical Microbiology*, 37(7), 2280–2284.

41. Ongena, M., & Jacques, P. 2008. *Bacillus lipopeptides*: versatile weapons for plant disease biocontrol. *Trends in Microbiology*, 16(3), 115–125.

42. Kolstø, A. B., Tourasse, N. J., & Økstad, O. A. 2009. What sets *Bacillus anthracis* apart from other *Bacillus* species? *Annual Review of Microbiology*, 63, 451–476.

43. Kopel, A.C., Carvounis, P.E., & Holz, E.R. 2008. *Bacillus cereus* endophthalmitis following invitreous bevacizumab injection. *Ophthalmic Surg. Lasers Imaging* 39:153–154.

44. Kuroki, R., *et al.*, 2009. Nosocomial bacteremia caused by biofilm-forming *Bacillus cereus* and *Bacillus thuringiensis*. *Internal Medicine*, 48(10), 791–796.

45. Martinez, M. F., Haines, T., Waller, M., Tingey, D., & Gomez, W. 2007. Probable occupational endophthalmitis from *Bacillus cereus*. *Archives of Environmental & Occupational Health*, 62(3), 157–160.

46. Mattson, M. P., Culmsee, C., Yu, Z., & Camandola, S. 2000. Roles of nuclear factor B in neuronal survival and plasticity. *Journal of Neurochemistry*, 74(2), 443–456.

47. Mead, P. S., *et al.*, 1999. Food-related illness and death in the United States. *Emerging Infectious Diseases*, 5(5), 607–625.

48. Motoi, N., *et al.*, 1997. Necrotizing *Bacillus cereus* infection of the meninges without inflammatory

reaction in a patient with acute myelogenous leukemia: a case report. *Acta Neuropathologica*, 93(3), 301–305.

49. Moyer, A. L., *et al.*, 2008. *Bacillus cereus* induces permeability of an in vitro blood–retina barrier. *Infection and Immunity*, 76(4), 1358–1367.

50. Moyer, A. L., Ramadan, R. T., Novosad, B. D., Astley, R., & Callegan, M. C. 2009. Bacillus cereus–induced permeability of the blood–ocular barrier during experimental endophthalmitis. *Investigative Ophthalmology & Visual Science*, 50(8), 3783–3793.

51. Murray, P. R. 2012. What Is New in Clinical Microbiology—Microbial Identification by MALDI-TOF Mass Spectrometry: A Paper from the 2011 William Beaumont Hospital Symposium on Molecular Pathology. *The Journal of Molecular Diagnostics* : JMD, 14(5), 419–423.

52. Ozkocaman, V., *et al.*, 2006. *Bacillus spp.* among hospitalized patients with haematological malignancies: clinical features, epidemics and outcomes. *Journal of Hospital Infection*, 64(2), 169–176.

53. Phelps, R. J., & McKillip, J. L. 2002. Enterotoxin production in natural isolates of *Bacillaceae* outside the *Bacillus cereus* group. *Applied and Environmental Microbiology*, 68(6), 3147–3151.

54. Pomerantsev, A. P., Pomerantseva, O. M., & Leppla, S. H. 2004. A Spontaneous Translational Fusion of Bacillus cereus PlcR and PapR Activates Transcription of PlcR-Dependent Genes in Bacillus anthracis via Binding with a Specific Palindromic Sequence. *Infection and Immunity*, 72(10), 5814–5823.

55. Rasko, D. A., Altherr, M. R., Han, C. S., & Ravel, J. 2005. Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiology Reviews*, 29(2), 303–329.

56. Ratnayake-Lecamwasam, M., Serror, P., Wong, K. W., & Sonenshein, A. L. 2001. Bacillus subtilis CodY represses early-stationary-phase genes by sensing GTP levels. *Genes & Development*, 15(9), 1093–1103.

57. Richardson, A. J., Rothburn, M. M., & Roberts, C. 1986. Pseudo-outbreak of *Bacillus* species: related to fibreoptic bronchoscopy. *Journal of Hospital Infection*, 7(2), 208–210.

58. Romero, C. F., Rai, M. K., Lowder, C. Y., & Adal, K. A. 1999. Endogenous endophthalmitis: case report and brief review. *American Family Physician*, 60, 510–523.

59. Rönner, U., Husmark, U., & Henriksson, A. 1990. Adhesion of *Bacillus* spores in relation to hydrophobicity. *Journal of Applied Microbiology*, 69(4), 550–556.

60. Rutherford, S. T., & Bassler, B. L. 2012. Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harbor Perspectives in Medicine*, 2(11), a012427.

61. Scallan, E., *et al.*, 2011. Foodborne illness acquired in the United States—major pathogens. *Emerging Infectious Diseases*, 17(1), 7.

62. Schallmey, M., Singh, A., & Ward, O. P. 2004. Developments in the use of *Bacillus* species for industrial production. *Canadian Journal of Microbiology*, 50(1), 1–17.

63. Scheldeman, P., Herman, L., Foster, S., & Heyndrickx, M. 2006. *Bacillus sporothermodurans* and other highly heat-resistant spore formers in milk. *Journal of Applied Microbiology*, 101(3), 542–555.

64. Schemmer, G. B., & Driebe, W. T. 1987. Posttraumatic *Bacillus cereus* endophthalmitis. *Archives of Ophthalmology*, 105(3), 342–344.

65. Scott, I. U., et al., 1996. Endophthalmitis associated with microbial keratitis. *Ophthalmology*, 103(11), 1864–1870.

66. Vilain, S., Luo, Y., Hildreth, M. B., & Brözel, V. S. 2006. Analysis of the life cycle of the soil saprophyte *Bacillus cereus* in liquid soil extract and in soil. *Applied and Environmental Microbiology*, 72(7), 4970–4977.

67. Serror, P., & Sonenshein, A. L. 1996. CodY is required for nutritional repression of *Bacillus subtilis* genetic competence. *Journal of Bacteriology*, 178(20), 5910–5915.

68. Setlow, P. 2003. Spore germination. *Current Opinion in Microbiology*, 6(6), 550–556.

69. Shany, S., Bernheimer, A. W., Grushoff, P. S., & Kim, K. S. 1974. Evidence for membrane cholesterol as the common binding site for cereolysin, streptolysin O and saponin. *Molecular and Cellular Biochemistry*, 3(3), 179–186.

70. Shaligram, N. S., & Singhal, R. S. 2010. Surfactin- A Review on Biosynthesis, Fermentation, Purification and Applications. *Food Technology and Biotechnology*, 48(2), 119–134.

71. Simini, B. (1998). Outbreak of *Bacillus cereus* endophthalmitis in Rome.

72. Slamti, L., & Lereclus, D. 2005. Specificity and polymorphism of the PlcR–PapR quorum–sensing system in the *Bacillus cereus* group. *Journal of Bacteriology*, 187(3), 1182–1187.

73. Sneath, P., Bergey's Manual of Systematic Bacteriology. Vol. 2. 1986, Baltimore, MD: Wiliam and Wilkins Co.in Liquid Soil Extract and in Soil. *Applied and Environmental Microbiology*, 2006. 72: p. 4970–4977.

74. Sonenshein, A. L. 2005. CodY, a global regulator of stationary phase and virulence in Gram–positive bacteria. *Current Opinion in Microbiology*, 8(2), 203–207.

75. Stenfors Arnesen, L. P., Fagerlund, A., & Granum, P. E. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology Reviews*, 32(4), 579–606.

76. Stenfors Arnesen, L. P., Fagerlund, A., & Granum, P. E. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiology* Reviews, 32(4), 579–606.

77. Streilein, J. W. 2003. Ocular immune privilege: therapeutic opportunities from an experiment of nature. Nature reviews. *Immunology*, 3(11), 879.

78. Ullman, S., Pflugfelder, S. C., Hughes, R., & Forster, R. K. 1987. *Bacillus cereus* panophthalmitis manifesting as an orbital cellulitis. *American Journal of Ophthalmology*, 103(1), 105–106.

79. Van Der Zwet., *et al*., 2000. Outbreak of *Bacillus cereus* infections in a neonatal intensive care unit traced to balloons used in manual ventilation. *Journal of Clinical Microbiology*, 38(11), 4131–4136.

80. Weber, D., & Rutala, W. 1988. *Bacillus* Species. *Infection Control & Hospital Epidemiology*, 9(8), 368–373.

81. Wilson, G. S. 1943. The pasteurization of milk. *British Medical Journal*, 1(4286), 261.

82. Winn, W. C. 2006. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology*. Lippincott williams & wilkins.

83. York, M. K. 1990. *Bacillus* species pseudobacteremia traced to contaminated gloves used in collection of blood from patients with acquired immunodeficiency syndrome. *Journal of Clinical Microbiology*, 28(9), 2114–2116.

UNDERGRADUATE PERSPECTIVE: THE BENEFITS OF PERFORMING UNDERGRADUATE RESEARCH

ELLEN WAGNER
UNIVERSITY OF WISCONSIN - MADISON

As is common for the majority of incoming biology majors, I began my undergraduate experience expecting to eventually find myself in medical school. Now, almost five years after starting my undergraduate education, I am a first year graduate student pursuing a PhD in Cellular and Molecular Biology and could not be further from medical school. How did my career trajectory change? What were the key events during my four years as an undergraduate student that triggered the switch from medical doctor to basic biology researcher?

When it came time to search for the university I would call home for the four years of undergrad, one of my priorities was the opportunity to work in a research lab. Everyone I had spoken with at universities I was considering said research is a great resume builder for medical school. My only previous laboratory experience was in high school science labs. I had never seen a real research lab, nor did I understand what the research process truly was. The only perspective I had of research at this time was knowing pharmaceuticals are developed from research and go through clinical trials to be approved for use. There I was, 17 years old and putting top priority on something I did not understand.

“I BEGAN EQUATING THE RESEARCH WITH SOLVING PUZZLES (ONE OF MY FAVORITE HOBBIES). IN ORDER TO MAKE DISCOVERIES IN RESEARCH (SOLVE THE PUZZLE), YOU NEED TO BE ABLE TO THINK CRITICALLY ABOUT ALL OF THE DATA YOU HAVE ACQUIRED (THE PUZZLE PIECES) AND COMPILE IT INTO THE BIGGER PICTURE.”

Flashforward to my sophomore year of undergrad, when I took my first microbiology class. The major assignment for the lab section of the class was identifying an unknown bacterium using the skills we had learned throughout the semester. At first, this assignment seemed overwhelming. I kept thinking to myself, “How I am supposed to identify an unknown bacterium?” However, as I began to plan out the tests I wanted to perform on my bacterium, I discovered this would actually be fun. I began equating the research with solving puzzles (one of my favorite hobbies). In order to make discoveries in research (solve the puzzle), you need to be able to think critically about all of the data you have acquired (the puzzle pieces) and compile it into the bigger picture. This class taught me how to approach scientific questions, and I wanted to seek out a research lab to learn more about this side of science. This assignment was the turning point in my path of deciding between medicine and research.

The process for joining a research lab as an undergraduate differs between universities and between labs at the same university. Getting involved with research as an undergraduate was relatively easy for me. I simply looked up what type of research was occurring in the biology labs using the biology department website and emailed professors whose work sounded interesting to me. However, certain labs and universities have a more intensive application process. The professors at Ball State University want

to be involved with the students and help them learn what they need to succeed, so they are very willing to talk to students about their research and joining their labs. That is one of the greatest benefits I enjoyed attending a smaller undergraduate university. Moreover, the labs are not filled with post-doctoral fellows or PhD students, so as an undergrad I was working directly with the professor. This helped me gain confidence in my abilities because I knew I was getting the best training possible. However, I do not believe this is solely a small university trait. Since I began graduate school at the University of Wisconsin-Madison, I have also noticed that the professors at this top R01 research university care about giving both the undergraduate and graduate students the best education and training possible.

I had an unusual undergraduate education in that I received training in two very different research labs. The first lab centered on studying the virulence and biofilm potential of Bacillus amyloliquefaciens isolated from ultra-high temperature pasteurized milk. The second lab studied the mechanism and purpose of pseudouridine RNA modifications in Candida albicans. My time in the two labs partially overlapped, which is not something I would necessarily encourage a busy undergraduate student to do. I really enjoyed having the opportunity to work on two completely different projects, but it took time away from other parts of my life in order to equally commit time to both projects. While it is important for undergraduates to learn research techniques, the most important aspect of research an undergraduate student can learn is how to critically think about scientific questions.

For any undergraduate student interested in pursuing a career in any type of research, my advice is to find a lab working on something interesting. I think it is very important for undergraduates to find a stimulating project to work on because this will ultimately determine if they want to continue pursuing research. If their first research experience is working on something they do not find intriguing, then they will not be motivated to commit time to working on the project. It's important for undergraduates beginning in a lab to feel inspired by the science, so that they develop the desire to pursue answers for unknown questions. I encourage undergraduates to get involved in research as early as possible, so they have time to develop this curiosity, but I do not believe joining a research lab as soon as you start at your undergraduate institute is necessary. If you are truly interested in science and research, your curiosity to pursue questions will develop naturally and extra time in the lab will not provide benefit for that aspect of learning. The main benefit of joining early as an undergraduate is that it presents students with more opportunities to participate in summer research programs at other universities. I began reaching out to professors during my sophomore year of undergrad and did not begin working in the lab until the summer before my junior year. I believe my two years working in the lab was the perfect amount of time for me to discover that I enjoyed doing research and wanted to pursue graduate school. I was a competitive applicant when applying for graduate school, and I ultimately ended up at my first-choice school. Two years was enough time for me to develop a strong foundation of skills that I need to succeed as a graduate student.

I encourage students to not be discouraged in their abilities when they are meeting

new people at conferences, interviews, jobs, and graduate school who have experiences different from theirs. I have always struggled with impostor syndrome, the belief that your achievements occur by random chance and at any moment "truly" successful people will realize that you are not good enough to be where you are with your career. Impostor syndrome is very prevalent in academia and extremely common in graduate students. During the first week of orientation for graduate school, I finally had the chance to meet the other students in my cohort. This was when my impostor syndrome really set in. To my surprise, I quickly learned that all but three of the students in my cohort had had at least one year off of school where they worked in a research lab. Most of my peers had either attended R01 universities for undergraduate or worked at an R01 institute as a technician. They all talked about their incredibly interesting and diverse research, and it made me feel like I had not experienced or learned enough about research yet to be in the same place along our paths for our careers. However, something that I have had to continually remind myself is that everyone experiences a learning curve when beginning graduate school. We were all starting over in our research, so while it is possible that I may not know all of the techniques my classmates know, they still have to learn new techniques for their thesis projects, as do I.

> "MY ABILITY TO PROBLEM SOLVE IN RESEARCH IS NOT PERFECT, AND I WILL CONTINUALLY WORK TO DEVELOP IT THROUGHOUT GRADUATE SCHOOL, BUT I WOULD BE LACKING IN THAT SKILL IF I HAD NOT HAD PRIOR RESEARCH EXPERIENCE."

If I had not performed research as an undergraduate, I do not believe I would be prepared for graduate school. It provided me the exposure I needed to understand how research labs work, and it taught me the fundamental skill of thinking critically when approaching a research question. My ability to problem solve in research is not perfect, and I will continually work to develop it throughout graduate school, but I would be lacking in that skill if I had not had prior research experience. I am very grateful for the opportunity to perform research as an undergraduate student. It taught me many valuable skills and helped me determine what type of career I want.

Ultimately, I advise anyone entering a STEM major to look into joining a research lab, even if they are not necessarily interested in a research career. Conducting research supplements and solidifies undergraduate education because students learn to apply the theories they are taught in class. Performing research as an undergraduate is beneficial because it teaches many critical thinking skills that can be used in any work environment. Research also helps to diversify students' experiences, making them more competitive when entering the workforce. When an undergraduate student conducts research for a significant period of time, it shows that they have experience with problem solving, big picture thinking, and collaborating with peers. I encourage students to branch out from their comfort zone, pursue something new (like research), and use that experience to help them develop new skills and discover what they enjoy related to their field.

EDITORIAL BOARD MEMBERS

FINE FOCUS

CONCENTRATED ON STUDENT OPPORTUNITY

2018 FINE FOCUS TEAM

CALL FOR MANUSCRIPT

SUBMISSIONS: We are an international journal dedicated to showcasing undergraduate research in all fields of microbiology. Fine Focus is managed entirely by undergraduate students from production to print, but utilizes an external Editorial Board of experts for double-blinded peer review of manuscripts

MISSION: We publish original research by undergraduate students in microbiology. This includes works in all microbiological specialties and microbiology education.

ABOUT US: Fine Focus is a peer-reviewed academic journal with a mission to publish findings of international undergraduate microbiology research in both print and electronic platforms.
As an international journal, Fine Focus accepts article submissions from undergraduates, and works with more than 50 Editorial Board members from around the world. We use a double-blind review system to ensure fair and objective review.

WHY WE ARE UNIQUE: We are the first international undergraduate research journal specifically in microbiology. Fine Focus allows interested students the opportunity to see their research efforts through to fruition via publication while learning about the scientific peer review process at the same time. By offering an opportunity for undergraduate students to publish early in their training, they become the authors of, and authorities on, their own education and professional development.

DONATE: We are a non-profit journal and we rely on donations that able us to continue our work with undergraduates and gettheir word heard in the field of microbiology. To donate, please visit *finefocus.otg/donate*

WANT TO GET INVOVLED?: Undergraduate students can submit microbiology research by going to *finefocussubmissions.org*. Microbiology professionals can get involved with Fine Focus as editors or reviewers. Let us know if you woud like Fine Focus in your college or university library.

Fine Focus at a glance:

- Free – no page charges or subscription costs
- Partnering with American Society for Microbiology & University of Detroit –Mercy
- Published articles searchable and indexed via EBSCO Host & Cardinal Scholar