UC Merced
UC Merced Electronic Theses and Dissertations

Title
Biopesticides as Unintentional Reservoirs and Vectors of Antibiotic Resistance Genes and Mobilizable Elements

Permalink
https://escholarship.org/uc/item/6zp600r2

Author
KAZE, MO

Publication Date
2020

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA, MERCED

Biopesticides as Unintentional Reservoirs and Vectors of Antibiotic Resistance Genes and Mobilizable Elements

A dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Quantitative and Systems Biology

by

Mo Kaze

Committee in charge:

Professor Miriam Barlow, Chair
Professor Gordon Bennett
Professor Susannah Tringe

2020

Copyright (or ©)
Mo Kaze, 2020
All rights reserved

The Dissertation of Mo Kaze is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Professor Gordon Bennett

Professor Susannah G. Tringe

Chair, Professor Miriam Barlow

University of California, Merced

2020

Table of Contents

Section 1
Abstract page 1

Section 2
Introduction page 1

Section 3
Chapter 1 page 1

Chapter 2 page 28

Chapter 3 page 45

Section 4
Conclusion page 1

List of Figures

Chapter 1

Antibiotic resistance content of mined biopesticide genomes page 11

Co-occurrence network of biopesticide resistomes of homologous ARG page 15

Antibiotic resistance content of commercial biopesticide genomes page 16

Photographs of replicate plates of Minimum Inhibitory Concentrations assays page 19

Photographs of replicate plates of disk diffusion assays page 19

Co-occurrence network of commercial biopesticide resistomes page 20

Chapter 2

Hi-C results of the biopesticides indicate location of antibiotic resistance genes page 26

Representative plates showing resistant phenotypes page 38

Alteration to resistance phenotypes before and after co-culturing page 39

Antibiotic resistance genes exchanged during co-culture of *K. pneumoniae* and *Bacillus*- based biopesticides page 40

Chapter 3

Phage genomic content identified in biopesticide strains page 54

Bacteriophage genomes and genes identified in plasmids page 55

VIBRANT phage analysis of mined and commercial biopesticide genomes page 56

Comparison of orthology databases for phage and prophage associated genes page 57

VIBRANT identified auxiliary metabolic gene categories for commercial page 58

biopesticides

VIBRANT identified auxiliary metabolic gene categories for mined biopesticides page 59

Integrative and mobilizable elements identified in commercial biopesticides page 60

Antibiotic resistance gene pattern found in forty-four *B. thuringiensis* genomes page 61

List of Tables

Chapter 1

Table 1 Minimum Inhibitory Concentrations of eight clinically relevant antibiotics tested on *Bacillus*-based biopesticide products page 18

Table 2 Disk diffusion assay of five clinically relevant antibiotics tested on *Bacillus* based biopesticide products page 18

Table 1 (Supplemental) List of biopesticides compiled from EPA and CADPR records page 22

Chapter 2

Table 1 Minimum Inhibitory Concentration Results page 37

Mo Kaze

mokrobial@gmail.com www.mokrobial.com Twitter: @MoKrobial LinkedIn: MoKaze

Research Interests

My current research as a DOE Fellow, supervised by Dr. Susannah Tringe at JGI, focuses on engineered transitional systems, such as aqueducts and networks of irrigation canals. These terrestrial aquatic interfaces cover large regions and are not currently being routinely measured for carbon flux and the microbial communities of these waterways and their potential contribution of greenhouse gases to the atmosphere have yet to be investigated. These engineered interfaces may be acting as "hotspots" with impacts disproportionate to their geographic area. Human-made waterway sediments likely contain microorganismal communities that impact carbon gas flux. The research project I developed involves mapping the biogeographical distribution of organisms and defining their functional potential across the water distribution system. My field green house gas sampling and metagenomic analysis will provide input data for predictive models of landscape features not currently reflected in climate models

My doctoral research involves investigating genetic movement across environmental gradients with a focus on biopesticides. These commercial products' genomic content contain antibiotic resistance genes and viral sequences which have clinical and environmental consequences. My publications regarding biopesticides indicate these widely-sed microbial products are behaving as unexpected reservoirs and vectors for antibiotic resistance.

Education

University of California, Merced 8/2020	PhD Quantitative Systems Biology
California State University, East Bay, Hayward, CA 2017	MS Molecular Microbiology
Mills College, Oakland, CA 2013	BA Molecular Biology

Skills

- Clinical and environmental microbiology
- Library prep, Next gen sequencing and analysis (illumina, Nanopore platforms), Hi-C
- Confocal, Super res, fluorescent, light microscopy and image analysis
- Immunohistochemistry, immunohistology, FISH, laser-based microdissection
- Python, Obj C, R
- Experimental design, sample collection and processing
- Computational and statistical analysis
- Viral resuspension, concentration, and extraction
- Protein purification and isolation
- NMR and LCMS analysis, GHG analysis, biogeochemical analysis
- Scientific communication, grant writing

Publications

- Lauren Elisabeth Brooks, Mo Kaze, and Mark Sistrom. "A Curated, Comprehensive Database of Plasmid Sequences." *Microbiol Resour Announc* 8.1 (2019): e01325-18.
- Lauren Elisabeth Brooks, Mo Kaze, and Mark Sistrom. "Where the plasmids roam: large-scale sequence analysis reveals plasmids with large host ranges." *Microbial genomics* 5.1 (2019).

Currently under review:

- Mo Kaze, Lauren Elisabeth Brooks, and Mark Sistrom. "*This Gras Isn't Greener: Biopesticides as Reservoirs and Vectors of Antibiotic Resistance*." (2020)

Awaiting submission

- Mo Kaze, Lauren Elisabeth Brooks, and Mark Sistrom. "*Assessing the diversity of antibiotic resistant bacteria in waste water using a whole genome approach*."
- Mo Kaze, Lauren Elisabeth Brooks, and Mark Sistrom. "*Welcome to Thunderdome: The Resistome and Virome of the Plasmidome*."
- Mo Kaze, Lauren Elisabeth Brooks, and Mark Sistrom. "*Mixing with a Bad Crowd: ARG exchange between Klebsiella pneumoniae and Bacillus based-Biopesticides Leads to Carbapenem Resistant Phenotypes.*"

In process

- Mo Kaze, "*The Phage, Prophage, AMG and IME Content of Biopesticides.*"

Doctoral thesis

- Mo Kaze, "*Biopesticides as Unintentional Reservoirs and Vectors of Antibiotic Resistance Genes and Mobilizable Elements*."

Awards

UCM QSB Dissertation Award	2020
Department of Energy Science Graduate Fellowship	2019
Best Poster Microbiology Student Symposium	2019
UCM Summer Research Fellowship	2019
DOE Joint Genome Institute Distinguished Scholar	2018
Computational Biology Research Fellowship	2018
UCM Summer Research Fellowship	2018

Conference Presentations

- American Society for Virology 2020: *The Viral Content of Widely-used Biopesticides* (1st author) **talk**, poster **- postponed**
- American Society for Microbiology 2020: *Microbial Community Structure and Methane Production in a Large-scale Engineered Irrigation System* (1st author), poster **- postponed**

- American Society for Microbiology 2020: Title: *This Gras Isn't Greener: Biopesticides as Reservoirs and Vectors of Antibiotic Resistance* (1st author), **talk**, poster **- postponed**
- Microbiology Society Annual Meeting 2020: *Biopesticides as Unintentional Reservoirs and Vectors of Antibiotic Resistance genes* (1st author), **talk - postponed**
- Bay Area Ecology and Evolution of Infection Disease Meeting 2020: Title: *Poisoning the Apples? Investigating the Viral Content of Widely-used Biopesticides* (1st author), **talk**
- American Society for Microbiology 2019: Title: *Factors Influencing Microbial Community Composition of Historic and Restored Wetlands* (1st author), **talk**, poster
- Microbiology Student Symposium 2019: Title: *This GRAS Isn't Greener: Biopesticides as Unintentional Reservoirs of Resistance* (1st author), **talk**, poster
- Northern California Botanists Symposium 2019: Title: *Endophyte community shiKs in response to drought in monkey flowers (Erythranthe laciniata) grown in native soil*, poster
- Northern California Computational Biology 2018: *Genomic Mining Reveals Reservoir of Resistance Biopesticides Used in California* (1st author), poster
- American Society for Microbiology 2018: Title: *Genomic Mining Reveals Reservoir of Antibiotic Resistance Genes in Widely Used Biopesticide* (1st author), poster
- American Society for Microbiology 2018: Title: *Harboring a Fugitive: Identifying Unexplored Vectors and Carriers of Antibiotic Resistance Plasmids*, poster
- American Society for Cell Biology 2016: UCSF and UC Berkeley poster presentation Title: *Whole-slide Imaging Approach to Assessing Allogenic Transplantation of hESC-CMs using Histological Analysis and Immunofluorescence* (1st author), poster
- American Society for Cell Biology 2016: Title: *Characterization of the Microbiome of a Geothermal Pool Proximal Species, Pisolithus tinctorius* (1st author), poster

Professional Affiliations

American Society for Microbiology
American Society for Virology
Pride In STEM
Graduate Women in Science
Women in Bio
Microbiology Society

Teaching Experience

TEACHING ASSISTANT | **UC MERCED - MERCED, CA** | **2017-2018**

Courses: Molecular Basis of Health & Disease
Microbiology Lab

ADJUNCT INSTRUCTOR	**MERRITT COLLEGE - OAKLAND, CA**	**2016 -2017**
Course:	Advanced Microscopy	
TEACHING ASSISTANT	**CSU EAST BAY - HAYWARD, CA**	**2015 - 2017**
Courses:	Microbiology Lab Intro to Biology Lab Intro to Microbiology Lab	
TEACHING ASSISTANT	**MILLS COLLEGE - OAKLAND, CA**	**2011-2012**
Courses:	Immunology Microbiology Nursing: Genetics Nursing: Microbiology	

Relevant Coursework

GRADUATE

- Computational Biology and Genomics [UC Berkeley]
- Microbial Physiology [UC Berkeley]
- Microbial Ecology [UC Berkeley]
- Microbial Genetics (audit) [UC Berkeley]
- Microbial Diversity and Evolution (audit) [UC Berkeley]
- Phylogenetics [UCM]
- Dynamics of Soil Organic Matter [UCM]
- Molecular Cell Biology I & Molecular Cell Biology II [CSUEB]
- Advanced Molecular Techniques [CSUEB]
- PCR & Fragment Analysis [CSUEB]
- Functional Genomics [CSUEB]
- Microbial Symbioses [CSUEB]
- Protein Chemistry [CSUEB]
- Environmental Microbiology [CSUEB]
- Parasitology [CSUEB]

POST-BACCALAUREATE

- Advanced Microscopy
- Live Cell Imaging
- Cell Culturing
- Immunohistochemistry

UNDERGRADUATE

- Genetics
- Immunology
- Microbiology
- Protein Chemistry & Enzymology
- Molecular Cell Biology

Service and Outreach

PANELIST - MILLS COLLEGE WOMEN AND STEM	**2016**
PANELIST - UC MERCED LGBT IN STEM	**2017**
UC MERCED GRADUATE DEAN'S ADVISORY COUNCIL ON DIVERSITY	**2018 -2020**

Research Experience

CLINICAL & ENVIRONMENTAL MICROBIOLOGY - UNIVERSITY OF CALIFORNIA, MERCED, CA DISSERTATION RESEARCH

Thesis project: Investigating Widely Used Microbial Products as Reservoirs and Vectors of Antibiotic Resistance

ENVIRONMENTAL MICROBIOLOGY - DEPARTMENT OF ENERGY / JGI; BERKELEY, CA - 2019- PRESENT

Project: Microbial Community Structure and Methane Production in California's Canal System
Responsibilities: experimental design, sample collection and processing, computational and statistical analysis, orthologous cluster analysis, scientific communication

ENVIRONMENTAL MICROBIOLOGY - JOINT GENOME INSTITUTE, DOE; WALNUT CREEK, CA - 2018-PRESENT

Project: Investigating Factors Influencing Microbial Community Composition in Bay Area Historic and Restored Wetlands
Responsibilities: computational analysis of large datasets, statistical analysis, orthologous cluster analysis, figure design, scientific communication

EXOBIOLOGY, NASA; CA, MT, WY - 2016 - 2017

Project: Archaea isolation and identification
Responsibilities: Research design, literature review, sample collection and environmental analysis at Norris geothermal pools in Yellowstone National Park, archaeal and bacterial cell culturing, DNA extraction, PCR, next-gen sequencing, biochemical analysis, LCMS and analysis, Super Res microscopy

MASTERS THESIS, CAL STATE EAST BAY, HAYWARD, CA; COMPLETED MAY 2017

Project: Characterizing the microbiome of a thermophilic fungus
Responsibilities: AHL extraction and GCMS analysis, documentation, microscopy and image analysis, protocol design, run undergraduate research team with journal club and personalized assignments, create supportive network for women and LGBT STEM students

MICROSCOPIST, UC BERKELEY CANCER RESEARCH LAB; BERKELEY, CA – 2015-2017

Projects: Histological Analysis and Immunofluorescence of Allogenic Transplantation of hESC-CMs, Imaging Toxicity Effects on Gut Tissue, Imaging Retinal Wound Tissue

HIV & AGING, UCSF; SAN FRANCISCO, CA - 2012-2013

Literature review, PPT presentations on topics and findings, study design, IRB applications and review, data collection & analysis

BIOPHOTONICS, MILLS COLLEGE; OAKLAND, CA 2011-2012

Project: bioengineering far red fluorescing cellular tags for HIV infection visualization
Responsibilities: Literature review, contamination troubleshooting, organizing samples, PCR, DNA sequencing & analysis, cell culturing, primer design, transformation, protein purification, spectrophotometry, documentation

Professional Experience

IMAGING ASSISTANT, UC BERKELEY CANCER RESEARCH LAB; BERKELEY, CA – 2015-2017

Maintenance, fluorescent microscopy, sample preparation, technical support utilizing confocal and slide scanning, support staff for conferences and educational courses

IOS LEAD TEST ENGINEER, VOXER INC; SAN FRANCISCO, CA – 2013 - 2014

Automation development, documentation, issue tracking, managing offsite team, managing internal & external beta projects, presenting performance & progress data, business account liaison, metric analysis

SW ENGINEER, APPLE INC; CUPERTINO, CA – 2006-2011

Projects: iPhone, iOS, iTunes, iPhoto

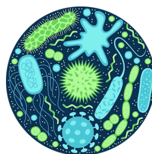
Academic References

Dr. Susannah Tringe
DOE Advisor
Joint Genome Institute
Lawrence Berkeley National Lab
1 Cyclotron Road
Berkeley, CA 94720 USA
stringe@lbl.gov

Dr. Carol Lauzon
MS Advisor
California State University, East Bay
25800 Carlos Bee Blvd
Hayward, CA 94542 USA
carol.lauzon@csueastbay.edu

Dr. Mark Sistrom
PhD Advisor
University of California, Merced
5200 North Lake Road
Merced, CA 95343 USA
msistrom@ucmerced.edu

Dr. Lauren E. Brooks
Utah Valley University
800 West University Parkway
Orem, UT 84058 USA
lauren.brooks@uvu.edu



Biopesticides as Unintentional Reservoirs and Vectors of Antibiotic Resistance Genes and Mobilizable Elements
Mo Kaze
A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy
UNIVERSITY OF CALIFORNIA, MERCED 2020
Professor Miriam Barlow, Chair

Antibiotic resistant infections are, globally, the cause of nearly 700,000 deaths every year. It has been estimated that by the year 2050, 10 million deaths could occur annually. Multi-drug resistant infections are extensive and pervasive and are most often connected with hospital-acquired infections. However, there has been a steady and alarming increase in community-acquired multi-drug resistant infections. The use of microorganisms for pest control, frost prevention, and rhizosphere enhancements in agriculture has steadily increased over the last 20 years. In California alone, the amount of biopesticide active ingredients distributed annually is close to 8 million pounds. Considered safe for consumption, non-toxic, and nonpathogenic, microbial products offer welcome alternatives to traditional chemically synthesized pesticides known to cause damage to human health and the environment. The addition of bacterial products, such as biopesticides, may be contributing reservoirs of clinically relevant antibiotic resistance genes (ARG) and creating increased opportunities for exchange in the environment. These bacterial products may be acting as unintentional vectors of antibiotic resistance and as sources of viral and mobilizable genetic elements. This work uses a combination of classic microbiology techniques and current sequencing technologies and bioinformatics approaches to answer the questions: are commercial bacteria-based biopesticides acting as reservoirs of ARG, can they act as vectors of ARG, and what is the viral and mobilizable genetic element content of these products? This work demonstrates that yes, biopesticides are current reservoirs for diverse clinically relevant ARG and have antibiotic resistant phenotypes, they can easily exchange important ARG with well-characterized pathogen carbapenem resistant *Klebsiella pneumoniae*, and they contain a range of bacteriophage genomes and genes and multiple integrative and mobilizable elements.

Antibiotic resistant infections are, globally, the cause of nearly 700,000 deaths every year. It has been estimated that by the year 2050, 10 million deaths could occur annually. Potentially 2.4 million deaths could occur in high-income countries in the next thirty-five years.[1] Antibiotic resistant diseases can lead to massively increased health care costs and wide-spread deaths can lead to losses in food production and distribution, trade, and employment. When combined with increases in poverty and inequitable wealth distribution, economic disaster may also be a result of the rise in global antibiotic resistant infections.[2] Multi-drug resistant infections are extensive and pervasive and are most often connected with hospital-acquired infections. However, there has been a steady and alarming increase in community-acquired multi-drug resistant infections.[3]

Many of the origins of this complicated issue have been identified, such as: lack of new antibiotics [4], agricultural and livestock practices, inappropriate and overuse of antibiotics, and nosocomial infections. Examples of agricultural and livestock practices include overuse of veterinary prophylactic antibiotics in livestock [5–7], use of reclaimed water for irrigation [8,9], and application of animal manure as fertilizer.[10] Antibiotic resistant bacteria generated by agricultural and livestock practices can exchange antibiotic resistance genes (ARG) with soil microbes and generate novel pathogenicity; environmental microbes can then act as reservoirs or vectors of ARG.[11,12] However, there are many other practices that could be contributing to the generation, spread, and maintenance of antibiotic resistance genes.[13] It is crucial to investigate ARG and "their spread from environment to clinic."[14] There is an essential need to look comprehensively at genetic movement and contact in the environment and research additional large-scale agriculture practices and their contribution and impact on the antibiotic resistant bacteria crisis. [15] One industrial agricultural practice that has yet to be investigated for its potential role as a reservoir or vector of antibiotic resistance genes is the use of biopesticides.

The use of microorganisms for pest control, frost prevention, and rhizosphere enhancements in agriculture has steadily increased over the last 20 years.[16–18] In California alone, the amount of biopesticide active ingredients distributed annually is close to 8 million pounds[19]. Considered safe for consumption, non-toxic, and nonpathogenic,[20–23] microbial products offer welcome alternatives to traditional chemically synthesized pesticides known to cause damage to human health and the environment.[24–26] The addition of bacterial products, such as biopesticides, may be contributing reservoirs of clinically relevant antibiotic resistance genes and creating increased opportunities for exchange in the environment. These bacterial products may be acting as unintentional vectors of antibiotic resistance. It is crucial to identify clinically relevant antibiotic resistance genes and define the resistomes of bacterial biopesticides used in large scale applications in highly modified agricultural environments in order to prevent unintentional contributions to the spread of antibiotic resistance genes and expansion of antibiotic resistant genes reservoirs.

Biopesticides are the most commonly used microbial product in industrial agriculture and *Bacillus*-based species dominate the global market.[27] Many

general species of biopesticides are considered non-pathogenic for a variety of reasons; *Bacillus subtilis* strain GB03 does not produce toxins [28] and *Pseudomonas syringae* strain ESC-10 is incapable of growth at human body temperature.[16] However, not all commercial biopesticide strains in use are as innocuous, some strains such as *Agrobacterium radiobacter* are considered a low virulent, opportunistic, nosocomial pathogen[29], while strains of *Bacillus pumilis* produce a toxin responsible for food poisoning and lesions.[30,31]

Bacillus thuringiensis is the most widely used biopesticide in industrial agriculture.[32] In California close to 400,000 pounds of *B. thuringiensis* alone were used in 2017[19]; it is considered the safest biopesticide and has been in use for more than 80 years.[21,33,34] *B. thuringiensis* is a Gram positive, aerobic, soil-dwelling bacteria [35], identified by the presence of its characteristic plasmids containing *cry* and *cyt* genes. These two toxin genes and their variations confer the unique insecticidal properties of *B. thuringiensis.* [36] It is a member of BacillusACT, a group sharing a common genome with differing extrachromosomal, independently replicating DNA. The presence of ARG in *B. thuringiensis* is not unexpected especially given its pathogenic siblings *Bacillus anthracis* and *Bacillus cereus* – well characterized toxin producers. *B. thuringiensis* has a special capability regarding plasmid acceptance and maintenance and has previously been shown to host as many as 17 plasmids.[37]

Bacteria acquire new traits and capabilities by horizontal gene transfer, an element of which is plasmid transfer and uptake.[38] Plasmid exchange between bacterial kin remains an essential evolutionary stratagem. It is not unexpected for bacteria to contain bacteriocin genes that offer competitive advantages against rival species, especially as conjugative plasmid transfer can confer these new abilities.[39] Genes carrying instructions for antibiotic resistance, pathogenicity, and virulence can be transferred via horizontal gene transfer, either through viral or plasmid exchange.[40] There are many methods for conjugative plasmid exchange between Gram positive pathogens[41] and are not confined to Gram positive relatives. Exchange between Gram negative and Gram positive species has been previously been demonstrated.[42] Very broad host ranges have recently been identified allowing for previously unknown plasmid promiscuity.[43] Both Gram positive and negative biopesticides examined for this work have the capability of conjugative plasmid exchange. Antibiotic resistance, pathogenicity, and virulence genes can be transferred on plasmids and taken up phylogenetically distant species in the environment.[44] Plasmids can be taken up by common soil microbes[45] and confer resistance, such as: NDM-1 a carbapenem resistance plasmid, MCR-1 a colistin resistance plasmid, and pZB18 a vancomycin resistance plasmid.[46] Plasmids bearing antibiotic resistance genes and novel pathogenicity should be considered part of the resistomes when investigating ARG of bacterial biopesticides. The widespread use and application of bacterial biopesticides to crops in high concentrations raises the possibility of unintentional contributions to the movement and generation of antibiotic resistance genes (ARG) in the environment.

Previous work performed by Dr. Lauren E. Brooks investigating multi-drug resistant *Staphylococcus aureus* in wastewater treatment samples identified the presence of *B. thuringiensis.* This project generated twelve whole genome sequences of *B. thuringiensis*. I used bioinformatic analyses to determine if ARG are present in published *B. thuringiensis* genomes. I generated a database of all 44 complete, whole genome sequences from NCBI and split the nucleotide sequences by location (plasmids and chromosomes). To determine if ARG were present in *B. thuringiensis*, I queried these sequences for antibiotic resistance genes against the Comprehensive Antibiotic Resistance Database (CARD). Following removal of duplicates, 295 occurrences of 15 unique genes encoding antibiotic resistance were identified. Of the eight ARG located on plasmid sequences, six were located on one single plasmid, encoding resistance to both tetracycline and vancomycin.

This initial work inspired my thesis research and the hypotheses I wished to explore: Are there clinically relevant antibiotic resistance genes present in commercially available bacterial biopesticides? Are the bioinformatically identified antibiotic resistance genes expressed and do they demonstrate resistance to clinically relevant antibiotics? Is there genetic exchange between widely available *Bacillus*-based bacterial biopesticide products and a bioinformatically informed pathogen? What is the role of plasmids in relation to antibiotic resistance genes occurring in commercially available *Bacillus*-based bacterial biopesticides? This research begins the foundational work of determining the role of microorganismal biopesticides as reservoirs and potential vectors of antibiotic resistance genes.

My research analyzed publicly available genomes sequences for widely used agricultural bacterial biopesticide products in order to identify the presence of antibiotic resistance genes. Commercially available *Bacillus*-based biopesticides were the basis of antibiotic resistance experiments and whole genome sequencing. My research to address the four hypotheses will be presented in the following analyses. I used classic microbiology techniques and bioinformatic analyses to determine the role of biopesticides as reservoirs and vectors of ARG. I used bioinformatic analyses to determine if ARG are present in twenty-nine publicly available, published whole genomes for different species of bacterial biopesticides. I compiled a database of these genome sequences from NCBI. I queried all sequences for antibiotic resistance genes using CARD. I performed quantitative analysis and network analyses in order to present a comprehensive view of the resistome landscape of biopesticides.

I generated pure cultures for four readily available biopesticide products: two *B. thuringiensis* kurstaki, one *B. amyloliquefaciens* product, and one *B. subtilis* product. I extracted DNA from each of the *Bacillus*-based biopesticides, generated libraries and sequenced the strains using next generation methods on illumina MiSeq and illumina iSeq platforms prior to experimentation. I tested for antibiotic resistance using classic Kirby-Bauer assays and minimum inhibitory concentrations of multiple clinically relevant antibiotics. Bioinformatics results indicated a strong candidate for ARG exchange in *K. pneumoniae*. I determined the permissivity of the commercial *Bacillus*-based biopesticides to antibiotic

resistance genes by co-culturing them with a carbapenemase positive strain of *K. pneumoniae*. The co-cultures were isolated and the minimum inhibitory concentrations for two carbapenems were measured. I determined the occurrence of transfer of multiple genes between the biopesticide products when cultured together. The co-cultured *Bacillus*-based biopesticides were cultured, DNA extracted, libraries generated and were sequenced on the illumina iSeq platform. Genomes were assessed for quality, assembled, annotated and queried against CARD. Multiple sequence alignment determined the differences between the "before" and "after" *Bacillus*-based biopesticides. The genomes were also analyzed for partial gene transfer from *K. pneumoniae.*

In order to investigate the role of mobile genetic elements I looked at the ARG content of plasmids. I performed a type of chromosome conformation capture, in vivo proximity-ligation method, a Hi-C library preparation protocol in order to assuredly identify the location of the ARG present in the *Bacillus*-based biopesticides. Hi-C has been used previously to demonstrate the location of ARG and clearly identify where they occur in a microbiome; either on plasmids or within the bacterial chromosome.[14] Hi-C sequences were demultiplexed, assembled and visualized. Our curated database of plasmids[47] was used to determine the presence of ARG in plasmids and make the case for including plasmids when defining the resistome of pathogens. This database was also quantified for the presence of antibiotic resistance genes as well as viral sequences, prophages, and virus-associated proteins. This analysis provides a current census of clinically relevant ARG present in the plasmidome and quantified its virome, i.e. viral genetic elements. Incorporating plasmid sequences when investigating ARG content of various bacterial species is an efficient and cost-effective method and allows for a more extensive topographical view of a resistome.

Bibliography

1. World Health Organization. No Time to Wait: Securing the future from drug resistant infections. *Rep to Secr United Nations*. 2019.
2. World Bank Group. Drug-resistant infections: A Threat to Our Economic Future. *World Bank Rep*. 2017;(September):1-132. doi:10.1007/s11947-009-0181-3
3. David van Duin and DP. Multidrug Resistant Bacteria in the Community: Trends and Lessons Learned. *Infect Dis Clin North Am*. 2017. Doi:10.1016/j.idc.2016.02.004
4. Theuretzbacher U, Outterson K, Engel A, Karlén A. The global preclinical antibacterial pipeline. *Nat Rev Microbiol 2019*. 2019:1-11. doi:10.1038/s41579-019-0288-0
5. Schwarz S, Feßler AT, Loncaric I, et al. Antimicrobial Resistance among Staphylococci of Animal Origin. *Microbiol Spectr*. 2018;6(4) doi:10.1128/microbiolspec.ARBA-0010-2017
6. Jechalke S, Heuer H, Siemens J, Amelung W, Smalla K. Fate and effects of veterinary antibiotics in soil. *Trends Microbiol*. 2014;22(9):536-545. doi:10.1016/j.tim.2014.05.005
7. Munk P, Knudsen BE, Lukjacenko O, et al. Abundance and diversity of the fecal resistome in slaughter pigs and broilers in nine European countries. *bioRxiv*. 2017;(February):194647. doi:10.1101/194647
8. Deng S, Yan X, Zhu Q, Liao C. The utilization of reclaimed water: Possible risks arising from waterborne contaminants. *Environ Pollut*. 2019;254:113020. doi:10.1016/j.envpol.2019.113020
9. Intriago JC, López-Gálvez F, Allende A, et al. Agricultural reuse of municipal wastewater through an integral water reclamation management. *J Environ Manage*. 2018;213:135-141. doi:10.1016/j.jenvman.2018.02.011
10. Han XM, Hu HW, Chen QL, et al. Antibiotic resistance genes and associated bacterial communities in agricultural soils amended with different sources of animal manures. *Soil Biol Biochem*. 2018;126(March):91-102. doi:10.1016/j.soilbio.2018.08.018
11. Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MOA, Dantas G. The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens. *Science (80)*. 2012;337(August):1107-1111. doi:10.1126/science.1220761
12. Naik GA, Bhat LN, Chopade BA, Lynch JM. Transfer of broad-host-range antibiotic resistance plasmids in soil microcosms. *Curr Microbiol*. 1994;28(4):209-215. doi:10.1007/BF01575963
13. Smalla K, Cook K, Djordjevic SP, Klümper U, Gillings M. Environmental dimensions of antibiotic resistance: assessment of basic science gaps. *FEMS Microbiol Ecol*. 2018;94(12):1-6. doi:10.1093/femsec/fiy195
14. Stalder T, Press MO, Sullivan S, Liachko I, Top EM. Linking the resistome and plasmidome to the microbiome. *ISME J*. 2019;13(10):2437-2446. doi:10.1038/s41396-019-0446-4

15. Berendonk TU, Manaia CM, Merlin C, et al. Tackling antibiotic resistance: The environmental framework. *Nat Rev Microbiol*. 2015;13(5):310-317. doi:10.1038/nrmicro3439
16. EPA. Pseudomonas syringae strain ESC-10 (006441) Pseudomonas syringae strain ESC-11 (006451) Fact Sheet. 2008;10(006441):10-11.
17. Parnell JJ, Berka R, Young HA, et al. From the lab to the farm: An industrial perspective of plant beneficial microorganisms. *Front Plant Sci*. 2016;7(AUG2016):1-12. doi:10.3389/fpls.2016.01110
18. Akinrinlola RJ, Yuen GY, Drijber RA, Adesemoye AO. Evaluation of Bacillus Strains for Plant Growth Promotion and Predictability of Efficacy by In Vitro Physiological Traits. *Int J Microbiol*. 2018;2018. doi:10.1155/2018/5686874
19. CADPR. Summary of pesticide use report data - 2017. 2017.
20. EFSA. Risks for public health related to the presence of Bacillus cereus and other Bacillus spp. including Bacillus thuringiensis in foodstuffs. *EFSA J*. 2016;14(7). doi:10.2903/j.efsa.2016.4524
21. Yul J, Choi JAEY, Li MS, Jin BR a E, Je YHO. BT as a specific, safe, and effective tool for insect pest control. *J Microbiol Biotechnol*. 2007;17(4):547-559. 22. Federici BA, Siegel JP. Safety of Bacillus thuringiensis and Bt Crops Used in Insect Control. 2008. http://files/285/Federici, Siegel - 2008 - Safety of Bacillus thuringiensis and Bt Crops Used in Insect Control.pdf.
23. Siegel JP. The Mammalian Safety of Bacillus thuringiensis- Based Insecticides. *J Invertebr Pathol*. 2001;77(1):13-21. doi:10.1006/jipa.2000.5000
24. Nicolopoulou-Stamati P, Maipas S, Kotampasi C, Stamatis P, Hens L. Chemical Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture. *Front Public Heal*. 2016;4(July):1-8. doi:10.3389/fpubh.2016.00148
25. Wilson C, Tisdell C. Why farmers continue to use pesticides despite environmental, health and sustainability costs. *Ecol Econ*. 2001;39(3):449-462. doi:10.1016/S0921-8009(01)00238-5
26. Hernández AF, Parrón T, Tsatsakis AM, Requena M, Alarcón R, López-Guarnido O. Toxic effects of pesticide mixtures at a molecular level: Their relevance to human health. *Toxicology*. 2013;307:136-145. doi:10.1016/j.tox.2012.06.009 27.
27. Thakore Y. The biopesticide market for global agricultural use. *Ind Biotechnol*. 2006.
28. McSpadden Gardener BB, Driks A. Overview of the nature and application of biocontrol microbes: Bacillus spp. *Phytopathology*. 2004;94(11):1244. doi:10.1094/PHYTO.2004.94.11.1244
29. Aujoulat F, Jumas-Bilak E, Masnou A, et al. Multilocus sequence-based analysis delineates a clonal population of Agrobacterium (Rhizobium) radiobacter (Agrobacterium tumefaciens) of human origin. *J Bacteriol*. 2011;193(10):2608-2618. doi:10.1128/JB.00107-11

30. Tena D, Martinez-Torres J a., Perez-Pomata MT, Saez-Nieto JA, Rubio V, Bisquert J. Cutaneous Infection Due to Bacillus pumilus: Report of 3 Cases. *Clin Infect Dis*. 2007;44(4):e40-e42. doi:10.1086/511077
31. From C, Hormazabal V, Granum PE. Food poisoning associated with pumilacidinproducing Bacillus pumilus in rice. *Int J Food Microbiol*. 2007. doi:10.1016/j.ijfoodmicro.2006.11.005
32. Raymond B, Federici BA, Raymond B. In defence of Bacillus thuringiensis, the safest and most successful microbial insecticide available to humanity — a response to EFSA. 2017;(February):1-8. doi:10.1093/femsec/fix084
33. Alejandra Bravo et al. Bacillus thuringensis: A story of a successful bioinsecticide. *Insect Biochem Mol Biol*. 2013;41(7):423-431. doi:10.1016/j.ibmb.2011.02.006.
34. Bishop AH, Johnson C, Perani M. The safety of Bacillus thuringiensis to mammals investigated by oral and subcutaneous dosage. 1999.
35. Gibbons F. Firmicutes Gibbons and Murray. 2001;5.
36. Lereclus D, Agaisse H, Grandvalet C, Salamitou S, Gominet M. Regulation of toxin and virulence gene transcription in Bacillus thuringiensis. *Int J Med Microbiol*. 2000;290(4-5):295-299. doi:10.1016/S1438-4221(00)80024-7
37. Reyes-Ramírez A, Ibarra JE, Reyes-Ramirez A, et al. Plasmid Patterns of Bacillus thuringiensis Type Strains. *Appl Environ Microbiol*. 2008;74(1):125-129. doi:10.1128/AEM.02133-07
38. Ochman, Howard, Lawrence, Jeffrey G., Groisman, Eduardo A. Lateral gene transfer and the nature of bacterial innovation. *Nature*. 2000;405(6784):299-304.
39. Dimitriu T, Marchant L, Buckling A, Raymond B. Bacteria from natural populations transfer plasmids mostly towards their kin. *Proc R Soc B Biol Sci*. 2019;286(1905):20191110. doi:10.1098/rspb.2019.1110
40. Frost LS, Leplae R, Summers AO, Toussaint A. Mobile genetic elements: The agents of open source evolution. *Nat Rev Microbiol*. 2005;3(9):722-732. doi:10.1038/nrmicro1235
41. Grohmann E, Muth G, Espinosa M. Conjugative plasmid transfer in gram-positive bacteria. *Microbiol Mol Biol Rev*. 2003;67(2):277-301. doi:10.1128/MMBR.67.2.277
42. Courvalin P. Transfer of Antibiotic Resistance Genes between Gram-Positive and Gram-Negative Bacteria. *Antimicrob Agents Chemother*. 1994;38(7):1447-1451.
43. Brooks LE, Kaze M, Sistrom M. Where the plasmids roam: large-scale sequence analysis reveals plasmids with large host ranges. 2019:1-10. doi:10.1099/mgen.0.000244
44. Bush K, Courvalin P, Dantas G, et al. Tackling antibiotic resistance. *Nat Rev Microbiol*. 2011;9(12):894-896. doi:10.1038/nrmicro2693
45. Miller, RV, Bacterial Gene Swapping in Nature. *Sci Am*. 1998;278(1):66-71.
46. Wellington EMH, Boxall ABA, Cross P, et al. The role of the natural environment in the emergence of antibiotic resistance in Gram-negative

bacteria. *Lancet Infect Dis*. 2013;13(2):155-165. doi:10.1016/S1473-3099(12)70317-1

47. Brooks L, Kaze M, Sistrom M. A Curated, Comprehensive Database of Plasmid Sequences. *Microbiol Resour Announc*. 2018;85(2):1-11.

This GRAS Isn't Greener: Biopesticides as Unintentional Reservoirs of Antibiotic Resistance

Introduction

The increasing prevalence of antibiotic resistant bacterial infections is one of the most pressing public health crises of the current era. Without significant efforts to curb antibiotic resistant infections, it is predicted that 10 million deaths per annum will result by 2050 – more than cancer and diabetes combined.[1-2] There has been a great amount of research into the spread of antibiotic resistance via common agricultural practices. These have been implicated in the spread and generation of antibiotic resistant bacteria,[1–4] especially as up to 80% of antibiotics produced are used in agricultural applications.[5] Understandably, most research on antibiotic resistance focuses on species and mechanisms in clinical settings. However, the widespread use and presence of antibiotics and the genes which encode resistance to them in the broader environment plays a critical role in the evolutionary mechanisms occurring in nonclinical bacterial species and microorganismal communities. These microorganisms, which are often overlooked, can subsequently impact the prevalence of resistant infections presented at the clinic.

The use of microorganisms for pest control, frost prevention, and rhizosphere enhancements in agriculture has steadily increased over the last 20 years.[6–8] In California alone, the quantity of live bacteria distributed as biopesticides annually is close to 8 million pounds.[9] Considered safe for consumption, non-toxic, non-pathogenic and effective,[10–13] microbial products offer a welcome alternative to traditional, chemically synthesized pesticides known to cause damage to human health and the environment.[14,15] Microbes used as biopesticides are classified "Generally recognized as safe" (GRAS) by the FDA[16] for a variety of reasons (e.g., *Bacillus subtilis* strain GB03 does not produce toxins,[17] and *Pseudomonas syringae* strain ESC-10 is incapable of growth at human body temperature).[6] Not all commercially used biopesticides are as innocuous - *Agrobacterium radiobacter* is considered a low virulence, opportunistic, nosocomial pathogen[18], while strains of *Bacillus pumilis* produce a toxin responsible for food poisoning and lesions.[19,20]

Bacillus thuringiensis (Bt) is the most widely used biopesticide in industrial agriculture.[23] In California close to 400,000 pounds of Bt were used in 2017;[9] out of commercial *Bacillus*-based biopesticides Bt is considered the safest biopesticide and has been in use for more than 80 years.[21] Bt is a Gram positive, aerobic, soil-dwelling bacteria, distinguished from its pathogen siblings, *Bacillus anthracis* and *Bacillus cereus* (*Bacillus*-ACT), by the presence of characteristic plasmids containing *cry* and *cyt* genes.[25,26] These two toxin genes and their variations confer the unique insecticidal properties of Bt.[27] Bt has a special capability regarding plasmid acceptance and maintenance and has previously been shown to host as many as 17 plasmids.[22] The widespread use and application of bacterial biopesticides to crops in high concentrations raises the

possibility of unintentional contributions to the movement and generation of Antibiotic Resistance Genes (ARG) in the environment. Plasmid exchange between bacteria has been an essential evolutionary stratagem.[29] Genes carrying instructions for antibiotic resistance, pathogenicity, and virulence can be transferred via horizontal gene transfer, either through viral infection or plasmid exchange.[23] There are many methods for conjugative plasmid exchange between Gram positive pathogens[24] and exchange between Gram negative and Gram positive species has previously been demonstrated.[25] Very broad host ranges have recently been identified allowing for previously unknown plasmid promiscuity.[26] Plasmids bearing ARG and novel pathogenicity should be considered when determining the safety of bacterial biopesticides.

Despite extensive research of Bt and other microbial products as methods of pest control,[27] these commonly occurring soil bacteria have yet to be thoroughly investigated as potential reservoirs and vectors of ARG in the environment. There is an urgent need to understand the role biopesticides play in the transmission of ARG and generation of novel pathogenicity. The addition of biopesticides to the environment creates increased opportunities for horizontal exchange of ARG; these bacteria may act as unintentional reservoirs of antibiotic resistance. It is crucial to identify clinically relevant ARG and define the resistomes of bacterial biopesticides used in large scale applications to prevent unintentional contributions to the spread of antibiotic resistance genes and expansion of ARG reservoirs.

This study represents the first effort to assess the potential role of commercial bacterial biopesticides as reservoirs of ARG and to connect antibiotic resistance genotypes to resistance phenotypes. Using a combination of bioinformatic and microbiological techniques, we determine the resistomes, classification of all ARG in a community of organisms,[28] of all publicly available bacterial biopesticide genomes used in industrial agriculture and determine which of these is encoded on extrachromosomal plasmids. We analyze commercially available commercial *Bacillus*-based biopesticides via isolation, antibiotic susceptibility testing and whole genomic sequencing. This work demonstrates that currently used bacterial biopesticides contain clinically relevant antibiotic resistance genes, bear resistance to multiple drug classes, and are potential vectors of unintended transmission of antibiotic resistance as they are introduced to the environment in large quantities.

Results

Genomic mining to determine biopesticide resistance profiles

Analysis of 31 biopesticide species with publicly available genomes identified 94 unique clinically relevant ARG (Supplementary Table 2). All strains contained at least one (*Bacillus amyloliquefaciens* strain Y2) and up to 107 (Bt-aizawai) (mean = 15.5, S.D. = 21.5 clinically relevant ARG. (Figure 1a). This corresponds to ARG encoding resistance to between one (*Bacillus amyloliquefaciens* strain Y2) and 47 (*Pantoea vagans* C1-1) drug classes (mean

= 38.3 S.D. = 43.0) (Figure 1b . Twenty-seven (80%) biopesticide strains contain resistance to four or more of the 29 drug classes, and more than half contain resistance to ten or more drug classes. Bt strains contained genes conferring resistance from nine to twelve drug classes, and all contained resistance genes for tetracyclines, streptogramins, phenicols, oxazolidinones, penams, macrolides, lincosamides, cephalosporins, and carbapenems. *P. vagans* and the *Pseudomonas* sequences contained the highest number of resistance drug classes with 19, 18, and 17 classes respectively. (Figure 1c) Three drug classes occurred in more than 24 biopesticide strains: macrolides occurred in 26 strains (84%), tetracyclines in 25 strains (81%) and phenicols in 24 strains (77%). ARG conferring resistance to carbapenems, cephalosporins, lincosamides, oxazolidinones, penams and streptogramins occurred in approximately half (47%) of all biopesticide strains. Resistance genes for triclosan were found *in Achromobacter piechaudii*, *P. vagans*, *Pseudomonas fluorescens*, and *Pseudomonas vranovensis* strains. Visualizing the data by drug class (Figure 1d) we observed twenty-six antibiotic resistance gene families, with 68% of observed gene families encoding antibiotic resistance enzymes, and 18% encoding efflux pump structures. Beta-lactamase gene families comprise one third of those detected, 15% belonged to the resistance-nodulation-cell division (RND) antibiotic efflux pump gene family, and 14% belonged to the glycopeptide resistance family.

In widely used members of the *Bacillus*-based biopesticides, we observed that the most frequently occurring ARG (42%), originally identified in *Bacillus*-ACT, occur in Bt and *B. cereus* biopesticide strains. They included two beta lactamases from *B. cereus*, BcI and BcII, and two from *B. anthracis,* Bla1, Bla2, along with a fosfomycin thiol transferase gene FosB. These five ARG make up 65% of the 318 individual genes in Bt strains and confer resistance using antibiotic inactivation mechanisms. Clindamycin resistance gene, lsaB is present in 13% of the analyzed Bt strains, and vanRM, a vancomycin resistance gene, is present in 14%.

Examining biopesticide species outside of the *Bacillus*-based group, we observed thirty-seven genes (18% of those identified) which occurred once in their genomes. Fifty-eight percent of ARG occur three times or fewer in the non-Bt biopesticide strains. The efflux cytoplasmic membrane protein gene ceoB appeared multiple times in the *A. piechaudii* and *Burkholderia ambifaria* strains, however, no strains contained the additional genes required for a complete ceoAB-OpcM efflux pump complex. The ARG cfr(B), clbA, and clcD, all members of the Cfr 23S ribosomal RNA methyltransferase gene family which together confer resistance to multiple antibiotic classes (oxazolidinones, streptogramins, macrolides, lincosamides, phenicols, and, pleuromutilins) were identified in *B. amyloliquefaciens* strains and in *B. pumilis* strains. Vancomycin resistance conferring gene vanZF occurred 2-4 times in strains: *B. cereus, Paenibacillus popillae*, Bt-aizawai, and Bt-morrisoni. *Pseudomonas aeruginosa* efflux pump system component MuxB was found multiple times in biopesticide sequences for *A. piechaudii*, *B. ambifaria, Chromobacterium subtsugae, P.*

fluorescens, and *P. vranovensis*. One MuxA gene was identified in *C. subtsugae*, however, neither essential component genes MuxC nor OpmB were found in any of the sequences. All three genes required for multidrug efflux pump MexAB-OprM are found twice in *P. vranovensis*. The MexB gene was identified by itself in *A. piechaudii* strains and *P. vagans*. MexB and OprM genes, without MexA, were found *in P. fluorescens*.

Gene co-occurrence networks identified shared resistomes between the 88 CARD-defined “important pathogens” and the mined biopesticides. *Bacillus*-based biopesticides form a highly homologous cluster (Fig2a). All Bt-strains and *B. cereus* connected to *B. anthracis* via three genes, BcI, BcII, and FosB. Two strains, Btmorrisoni and Bt-japonensis, have a larger network of connections to pathogens. The ANT(4’-Ib) aminoglycoside resistance gene connected Bt-morrisoni to pathogens *Staphylococcus aureus, Enterococcus faecium* and *Enterococcus faecalis*. Btjapanonesis connected to pathogens *S. aureus, E. faecium* and *E. faecalis* via vancomycin resistance genes vanSA and vanRA as well as *Klebsiella pneumoniae* via vanRA. *K. pneumoniae* connected to *P. vagans* via efflux pump genes acrB and oqxB. *P. vagans* connected to *Citrobacter freundii, Enterobacter hormaechei, Escherichia coli, Klebsiella oxytoca, K. pneumoniae, Salmonella enterica, Shigella dysenteriae, Shigella flexneri,* and *Shigella sonnei* via a suite of seven efflux pump genes. In the center of the network, biopesticide nodes for all four *B. amyloliquefaciens* strains connected to *Clostridia difficile* via RND efflux gene clcD, and to *E. faecium* and *E. faecalis* via multidrug resistance gene cfrB. *B. subtilis* GB03 and *B. velezensis* also connected to *C. difficile, E. faecium* and *E. faecalis* via clcD and cfrB genes. *B. subtilis* MIB 600 connected to *E. faecium* via multi-drug resistance gene ErmB (Fig 2a). Pathogens, *P. aeruginosa* and *P. fluorescens* connected to the *A. piechaudii* strains via efflux pump gene mexI. *C. subtsugae* connected to these pathogens via efflux pump gene MuxC. Biopesticide *P. fluorescens* A506 and *P. vranovensis* connected to both *Pseudomonas* pathogens via a suite of eight efflux pump genes. (figure 2b) *B. ambifaria* connected to *B. pseudomallei* via the beta-lactamase gene Omp38, RND efflux genes amrA, and amrB. (Fig 2c) Biopesticide *S. galbus* connected to *M. tuberculosis* by the RND efflux gene mtrA (Fig 2d).

Antibiotic resistance of Commercial *Bacillus*-based Biopesticides

Bacterial strains were isolated from four commercial Bacillus-based biopesticide products (cpb), *B. thuringiensis* kurstaki strain SA12 (cbp1-Bt -kurstaki, cbp2-Bt -kurstaki)*, B. subtilis* strain QST 713 (cbp3-BsubQST) and *B. amyloliquefaciens* strain D747 (cbp4-BamyD747). Whole genome sequencing of these biopesticide products revealed twenty-eight unique ARG across the strains, containing resistance to 21 unique drug classes (Figure 3a). There were no ARG identified in *B. amyloliquefaciens* D747. Four genes were identified in *B. subtilis* QST 713: three multidrug resistance conferring RNA methyltransferase

genes (cfr(B), clbA, clcD) and the tetracycline efflux protein, tet(L). All other genes were identified in both Bt-kurstaki isolates, and these twenty-four ARG confer resistance to 20 out of the 21 drug classes (oxazolidinone resistance was identified only in *B. subtilis*). 161 unique genes belonging to the TEM and SHV beta lactamase resistance gene families were identified in cbp1-Bt -kurstaki. (Figure 3b) One hundred ninety-one genes (89%) belonged to beta-lactamase resistance families: KPC, Class A *B. anthracis*, *B. cereus*, OKP, LEN and SHV and TEM (Figure 3a). Annotation using RAST provided a different set of ARG than CARD. Annotation yielded 52 unique genes, and CARD yielded 31 unique genes (Supplementary Table 2). There were three genes that were identified by both RAST and CARD: beta-lactamase encoding OXA-1 and efflux transport protein acrA in cbp1Bt -kurstaki, and fosfomycin resistance gene FosB in cbp2-Bt-kurstaki (Figure 3c). The largest groups of CARD-identified genes, TEM and SHV, were not present in the RAST annotations of the four products.

Antibiotic susceptibility of these isolates was tested using twelve clinically relevant antibiotic drugs from ten drug classes identified by sequencing. They included two beta-lactam classes: carbapenems and cephalosporins (Table 2). Minimum inhibitory concentration (MIC) assays identified antibiotic resistance in three of the four biopesticide products. *B. amyloliquefaciens* strain D747 (cbp4-BamyD747) indicated resistance with a MIC of 4 µg/mL of quinupristin/dalfoprisitin (QDA), a streptogramin antibiotic (Figure 4d) and to tetracycline (TE). *B. amyloliquefaciens* strain D747 (cbp4BamyD747) was susceptible to the carbapenems, erythromycin and cephalosporin antibiotics (Figure 4f). One Bt-kurstaki product, cbp2-Bt -kurstaki, was entirely resistant to ceftazidime (CAZ), a third-generation cephalosporin antibiotic (256 µg/mL) (Figure 4e) and cephazolin (KZ) a first-generation cephalosporin (256 µg/mL). Cbp2-Bt -kurstaki was susceptible to clindamycin (CD) a lincosamide antibiotic, erythromycin (E) a macrolide antibiotic, and imipenem (IMI) a group 2 carbapenem. The other Bt-kurstaki product, cpb1-Bt -kurstaki, demonstrated resistance to CAZ (48 µg/mL), ETP (4 µg/mL) and complete resistance to KZ (256 µg/mL). It was susceptible to clindamycin, erythromycin (Figure 4g), and imipenem. *B. subtilis* QST 713, cbp3-BsubQST, demonstrated intermediate resistance to QDA (Fig 4b) and TE (Figure 4c) with no growth occurring past the MIC of 3 µg/mL. It was susceptible to clindamycin, erythromycin, ertapenem, imipenem, and cephazolin (Figure 4a). The four commercial *Bacillus*-based products were also tested against five antibiotics - clindamycin (DA2), doxycycline (DO30), linezolid (LZD30), sulfonamide/trimoxazole (STX 25), and vancomycin (VA30) in a disk diffusion assay (Table 3). *B. amyloliquefaciens* D747, cbp4-BamyD747 and *B. subtilis* QST 713, cbp3-BsubQST, were susceptible to all five. Cbp2-Bt -kurstaki was resistant to sulfonamide/trimoxazole (Figure 5a) and susceptible to the other four antibiotics. Cbp1-Bt -kurstaki demonstrated resistance to clindamycin (Fig 5b) and susceptibility to the other four antibiotics.

We constructed a gene homology co-occurrence network to visualize ARG shared between pathogens and the *Bacillus*-based biopesticides (Figure

6a). Cbp1-Bt kurstaki had the largest number of connections, linking to thirty-eight pathogens via multiple genes. The majority (97%) of the pathogens in this network were Gram negative species. Cbp1-Bt -kurstaki and cbp2-Bt-kurstaki contained 5 genes shared by *B. anthracis*: Bla1, Bla2, BcI, BcII, and FosB. *B. subtilis* QST 713 connected to three pathogens - *Clostridium difficile* via two multi-drug resistance conferring methyltransferase genes, cfrB and clcD, and *E. faecium* and *E. faecalis* via cfrB. Mined biopesticide strains queried against a curated plasmid database resulted in matches from the four genera of genomes represented: *Agrobacterium*, *Bacillus*, *Burkholderia*, and *Pseudomonas. Bacillus* genera were 99.7% of matches with nine plasmid species: *ambifaria, amyloliquefaciens, anthracis, bombysepticus, cereus, fluorescens, mycoides, radiobacter,* and *thuringiensis*. Of these species, there were five serovar/strain-specific Bt plasmids: aizawai (90%), berliner (1%), galleriae (3%), israelensis (5%), japonensis (1%). There were 8 unique CARD-identified ARG present in the sequences from mined biopesticide plasmids: vanRA, vanSA, ANT(4')-Ib, tet(45), tet(L), vanYF, and mphM. Four ARG were not identified in the chromosome-only genomes: vanSA, vanYF, ANT(4')-Ib, and mphM. The plasmid network connected Bt-aizawai strains to five pathogens: *S. epidermidis, S. aureus, E. faecium, E. faecalis*, and *K. pneumoniae.* The *K pneumoniae*-associated ARG were identified in *B. bombysepticus, B. cereus, and* Bt plasmids. *B. anthracis* sequences shared *B. mycoides* and Bt plasmids with Bt-gallierae and Bt-aizawai biopesticide strains. (Fig 6b)

Discussion

Using a combination of bioinformatic and classic microbiology approaches, this study represents a multi-method, comprehensive investigation of the current reservoir of antibiotic resistance genes present in widely used bacteria-based biopesticide products. Through this work, we explore how the presence of suspected ARG translates to observable resistance phenotypes in these bacteria, identify homologous ARG in pathogenic species, and describe ARG located on plasmids in bacteria isolated from commercial biopesticide products. This work builds on past work to identify ARG in biopesticide strains, [29] such as Patel et al.'s identification of vancomycin resistance clusters in biopesticide *P. popillae.*[30] Our work confirms these findings and identifies additional vancomycin gene clusters in *P. popillae*. Systems for selection of Bt strains for pest control without creating resistance to broad spectrum antibiotics have been proposed as early as 1997[31], indicating prior concern regarding application of biopesticides acting as ARG reservoirs.

A large number of homologous ARG, those found in the mined biopesticide genomes, are present in a wide range of clinically relevant pathogens – demonstrating the potential for biopesticides to act as vectors of clinically relevant antibiotic resistance. *B. ambifaria*, while no longer approved for biopesticide use in the United States, due to isolation from infections found in cystic fibrosis patients,[32] is still in use in other countries. We observed *B.*

ambifaria strains with all genes required for ceoAB-OpcM resistance-nodulation-cell division efflux pumps. This complex confers resistance to multiple drugs *in B. cenocepacia.*[33] We also identified five potential multidrug efflux pump complexes that are each missing a single gene required for completion. In *P. vagans*, multiple individual genes were missing from suites of genes required to form multidrug efflux pumps. Incomplete efflux complexes do not confer resistance, nor an obvious evolutionary advantage to bacteria which harbor them. However, many efflux related genes are prevalent on plasmids, [34,35] and are decoupled from evolutionary processes affecting hosts of these mobile genetic elements. Biopesticides are often used in multi-strain consortia[17] which presents the opportunity for individual strains carrying these genes, especially on plasmids, to exchange and complete functional multi-component systems. Engineered consortia may disseminate these complete systems to other bacteria in the environment through horizontal gene transfer. The number of drug classes identified in mined biopesticide strains is cause for concern, especially as the identified drug classes and gene families include the potential for resistance to "drugs of last resort" (e.g. antibiotics such as vancomycin and linezolid). [36,37] Glycopeptide resistance gene cluster and Cfr 23S rRNA gene families encode for resistance to antibiotics such as vancomycin and linezolid respectively. Gene families Erm 23S rRNA and ABC-F ATP binding cassette confer resistance to lincosamides. Resistance to clindamycin, a lincosamide, was observed in the antibiotic susceptibility tests.

The CARD-defined resistome pathogens connect to the mined biopesticides via multiple ARG. Clustering centered mainly around Gram negative biopesticides with Gram negative pathogens, and Gram positive biopesticides and Gram positive pathogens. Closely related species clusters are also evident (Fig 2b,2c,2d). The cluster of Gram negative pathogens (*C. freundi, E. coli, E. hormachei, K. pneumoniae, K. oxytoca, S. enterica, S. dystenteriae, S. flexneri, S. sonnei*) and Gram negative biopesticide *P. vagans* share multiple ARG and belong to the same order.[38] Gram positive pathogens *B. anthracis, C. difficile, E. faecium, S. aureus*, connected to Gram positive *Bacillus*-based biopesticides and are all Firmicutes. Previous work has outlined the challenges of exchange of ARG between taxonomically distant bacteria,[39] however, Gram negative pathogens *E. faecalis, and K. pneumoniae* shared several ARG with Gram positive *Bacillus*-based strains and *P. vagans* respectively providing evidence of ARG across phyla. The sequenced *Bacillus*-based biopesticides showed similar results. The cbp1-Bt-kurstaki resistome contains thirty-eight unique pathogens with 89% (34) Gram negative species. These results add evidence to previous work defining the role of horizontal gene transfer across phyla in evolutionary processes.[40–42] This also points to the lack of physical barriers to exchange of ARG between biopesticides applied in large amounts to and environmental bacteria.

Use of a curated plasmid database provided expanded resistome information and efficiently identified which genes are located on plasmids. Plasmid-based ARG are potentially transmissible between biopesticide strains

and this is demonstrated by *B. thuringiensis*-aizawai. A single Bt-aizawai strain appeared in the CARD-identified resistome network connected to one pathogen (*B. anthracis*) by three genes, FosB, Bla1, and BcII. (Fig 2a) However, Bt-aizawai strains were the main result in the plasmid based co-occurrence network (Fig 6b). Plasmids bearing shared ARG connected Btaizawai to five additional pathogens: *S. epidermidis, S. aureus, E. faecium, E. faecalis*, and *K. pneumoniae. K pneumoniae*-associated ARG were contained in *B. cereus* and *B. thuringiensis* plasmid sequences identified in multiple strains of Bt-aizawai. The presence of *B. anthracis* and *B. cereus* plasmids in the genomes of the mined biopesticides is likely due to their shared common genome which only differs in extrachromosomal, independently replicating plasmid DNA.[43] However, Bt strains containing *B. anthracis* plasmids could potentially come in contact with additional genes or plasmids once added in large quantities in the environment allowing complete sets of toxin genes to occur.

Cultures isolated from the commercial products contained ARG for multiple antibiotic drug classes, a large percentage of which belong to beta-lactamase gene families. These commercially available products also demonstrated resistant phenotypes to several clinically relevant antibiotics. There were discrepancies between the ARG present and resistance phenotypes. Clindamycin resistance gene clcD was present in the sequenced commercial Bt strains, yet only one of the Bt-kurstaki products demonstrated clindamycin resistance and this was observed only in the disk diffusion assay. There were members of multiple vancomycin resistance gene families identified in the mined genomes and none identified in the strains isolated from commercial products; nor did any display resistance to vancomycin in laboratory tests. Both sequenced Bt products contained genes conferring resistance to diaminopyrimidine, yet only the cbp2-Bt -kurstaki product was resistant in the MIC assay. This data shows that biopesticide strains harboring ARG may not necessarily express them, and these ARG may not result in resistant phenotypes. Resultantly, these products can act as latent carriers and as potential vectors of resistance to human pathogens which may not be determined by susceptibility testing of biopesticide products.

Interaction between biopesticide strains and the human microbiome facilitates the horizontal transfer of ARG from biopesticides to human commensal and opportunistically pathogenic bacteria. Both the quantity of biopesticides, and land area treated with them is considerable and increasing. In California 8 million pounds and more than 8 million acres were treated with biopesticides in 2017.[44] Bernstein et al. investigated the nasal and oral colonization of farm workers by Bt-kurstaki and determined that workers directly exposed to biopesticides had a significantly higher likelihood of colonization than those not directly exposed, and 68% of directly exposed workers had positive colonization assay results.[45] Evidence that biopesticide strains can actively colonize exposed individuals and carry clinically relevant resistance genes on mobile genetic elements as shown in this study presents a significant possibility that biopesticides can transfer ARG to the human microbiome. Furthermore,

farm worker populations most likely to be directly exposed to biopesticides face well-documented disparities in access to healthcare, [46,47] and may be more severely impacted by infection by opportunistic pathogens that have acquired antibiotic resistance from biopesticides than the general population.

Despite vociferous support for Bt as the "safest and most successful microbial insecticide available to humanity,"[48] it is essential to examine all facets of common agricultural practices that have contributed to the generation, spread, and maintenance of antibiotic resistance in the environment.[2,3] Massive input of bacterial ARG reservoirs to the environment has the capacity to alter the broader environmental resistome in ways that are complex and difficult to predict.[49,50] Increasing the concentration of biopesticide strains in agricultural systems increases the likelihood of multidirectional exchange. Antibiotic contamination of soil has been demonstrated to drive bacteria to evolve resistance to chemically synthesized pharmaceuticals arriving in soils from hospital effluent, to antibiotics and resistant bacteria input by manure application, and via irrigation using reclaimed municipal wastewater.[4,49,51–53] Antibiotics are not easily degraded by wastewater treatment systems[54] and can persist post-treatment.[55] More than half of all antibiotic compounds produced are used in agricultural settings.[56] The combination of this anthropogenic input of resistant bacteria in proximity to the largest use of antibiotic compounds has the potential to be a major driver in the increased prevalence of clinically relevant ARG, that to date, remains largely uninvestigated.

This work supports the need to thoroughly examine human contributions to the antibiotic resistance crisis and demonstrates that multiple methods are required to comprehensively analyze antibiotic resistance profiles of bacterial strains. Cooccurrence networks of ARG allow for hypothesis generation for future studies of genetic exchange between biopesticides and clinically relevant pathogens. In conclusion, we anticipate the role of biopesticides acting as vectors and reservoirs of important antibiotic resistance genes to become part of the continued research regarding agricultural practices and the spread of antibiotic resistance in the environment.

Methods

We compiled a list of all bacterial biopesticide species approved for use in the US manually from databases published by the California Department of Pesticide Registration and United States Environmental Protection Agency databases (Table 1).[9,57] Publicly available whole genomes matching strain information (n=31) were queried against the Comprehensive Antibiotic Resistance Database[58] v3.0 (downloaded November 2019) using Blastn 2.10.0[59] using a 97% cut off for query coverage and 90% percent for percent identity match. Other settings were left as default. ARG data was collated from CARD, quantified and visualized using the R package ggplot2.[60] Genes listed in CARD entries with "no prevalence data" were not included. Resistome data from CARD was mapped in gene co-occurrence networks using the R package igraph.[61]

Four commercial *Bacillus*-based biopesticide products (cpb), Bt-kurstaki strain SA12*, B. subtilis* strain QST 713 and *B. amyloliquefaciens* strain D747, were purchased and coded by number and strain: cbp1-Bt -kurstaki, cbp2-Bt-kurstaki, cbp3-BsubQST, and cbp4-BamyD747. The two Bt products were the same strain, but from different companies and contained different suspension materials. Commercial biopesticide products were cultured in triplicate using standard culture methods as specified in the American Society of Microbiology Kirby-Bauer Disk Diffusion Susceptibility Test Protocol.[62] Minimum inhibitory concentration was determined using the standard Clinical and Laboratory Standards Institute guidelines[63] on replicates using Liofilchem (Liofilchem Inc. MA) antibiotic minimum inhibitory concentration test strips for cephazolin, clindamycin, ceftazidime, quinupristin/dalfpopristin, ertapenem, imipenem, erythromycin, and tetracycline antibiotics. DNA from biopesticide products was extracted directly from products and from LB cultures using Qiagen's DNeasy DNA Extraction kit (Qiagen NV, Germany) using a modified protocol. After performing the protocol's first step, samples were incubated at 90ºC for 10-15 minutes in order to account for Gram positive cell wall structure. DNA concentration was quantified using a Qubit fluorometer (Thermo Fisher Scientific, MA) and quality and purity quantified using an Eppendorf Biospec (Eppendorf, Germany). Libraries were generated using Illumina NextTera Flex kit with IDT set A Dual Indexes. DNA was sequenced on both an Illumina MiSeq and Illumina iSeq systems (Illumina Inc, CA).

CPB sequences were checked for quality using FastQC v0.11.8[64] and trimmed using Trimmomatic v0.36.[65] Genomes were assembled using SPAdes v3.11.1.2[66] and assessed for quality using Quast v5.0.0.[67] Genomes were annotated with RAST v4.0.2[68] and assemblies were queried against CARD and manually curated for antibiotic resistance annotations and verified against UniProt.[69] Annotated and CARD-identified ARG were processed in R and visualized in ggplot. A curated plasmid database[70] was used to identify plasmids hosted by biopesticide strains. The mined strain sequences were queried against this curated plasmid database and results scoring as 100% matches for query coverage with greater than 97% identity match and match length of greater than 2,500 base pairs were analyzed. NCBI Batch Entrez was used to apply taxonomy to the results. Identified plasmid sequences were combined and queried against CARD to determine their ARG content. Pathogens containing identical plasmid sequences to biopesticide plasmids were mapped in co-occurrence networks using R package igraph. Strains containing identical plasmids with identical ARG were manually curated.

Antibiotic resistance content of mined biopesticide genomes

A

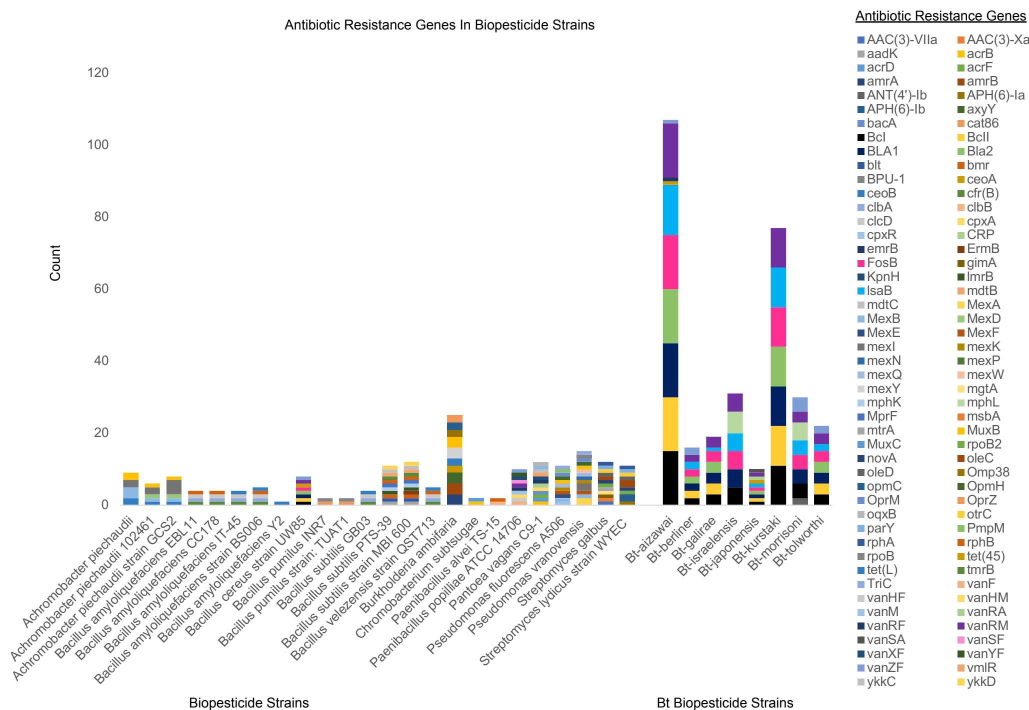


Figure 1 Mined genomes of biopesticide strains were analyzed for antibiotic resistance content. A. Unique ARG detected in biopesticide strains. Bt strains are grouped together.

Antibiotic resistance content of mined biopesticide genomes (cont.)

B

Frequency of Antibiotic Resistance Genes

Count

CARD-Identified Antibiotic Reisistance Gene

Figure 1 Mined genomes of biopesticide strains were analyzed for antibiotic resistance content. B. Gene frequency of individual antibiotic resistance genes across all mined genomes of available biopesticide strains.

Antibiotic resistance content of mined biopesticide genomes (cont.)

C

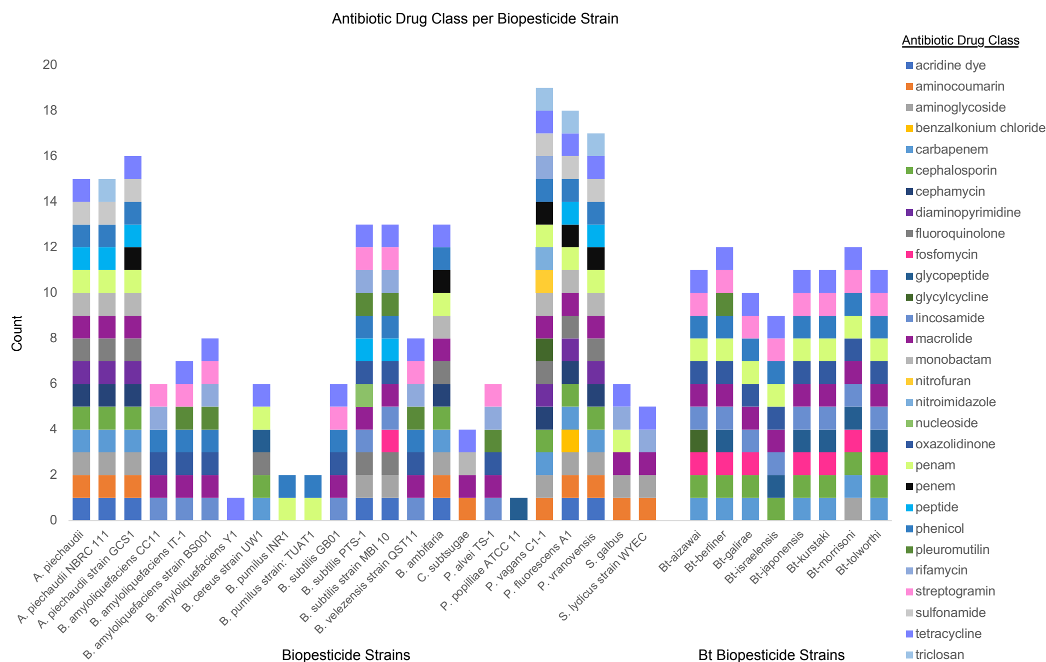


Figure 1 Mined genomes of biopesticide strains were analyzed for antibiotic resistance content. C. Antibiotic drug classes in each mined biopesticide strain. The two groups are split between bacterial strains and Bt-specific strains. Each strain contains genes conferring resistance to at least one antibiotic.

Antibiotic resistance content of mined biopesticide genomes (cont.)

D

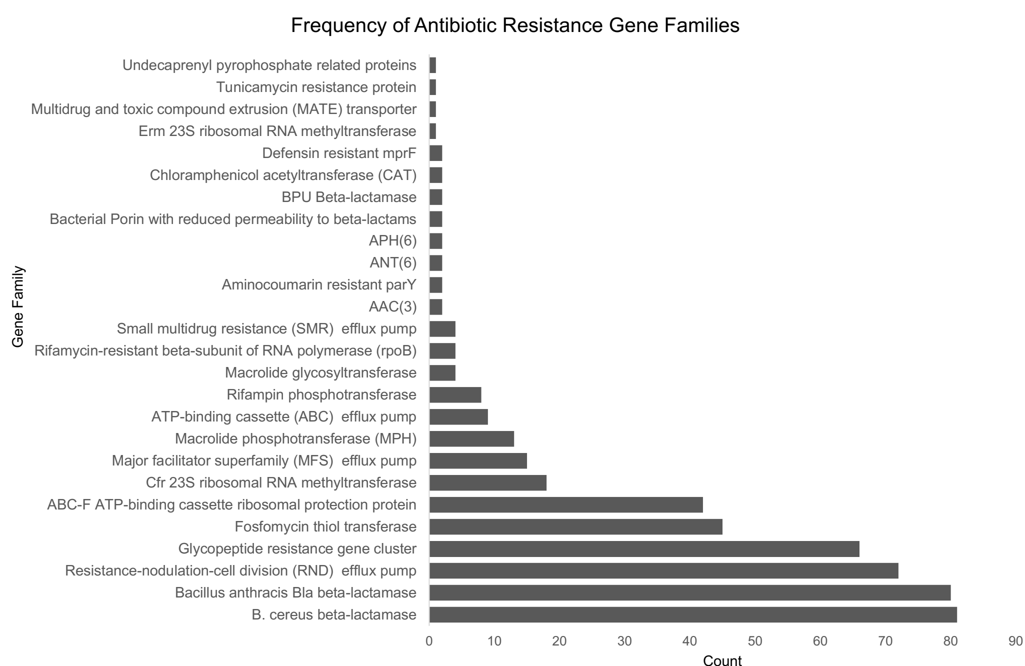


Figure 1 Mined genomes of biopesticide strains were analyzed for antibiotic resistance content. D. Frequency of ARG family across all mined genomes of available biopesticide strains. The majority of genes belonged to gene families encoding for enzymes.

Co-occurrence network of biopesticide resistomes of homologous ARG

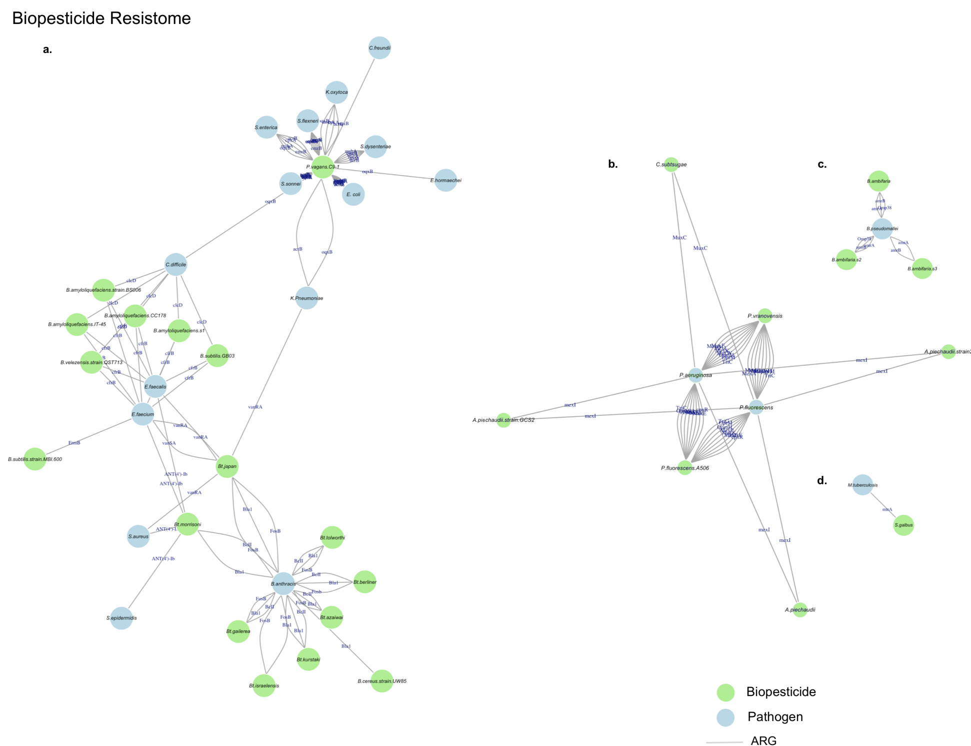


Figure 2. Co-occurrence network of biopesticide resistomes. Biopesticide nodes are in green and pathogen nodes are in blue. Edges are homologous antibiotic resistance genes. A. *Bacillus*-based strains cluster together in lower and middle of network and *Bacillus* ACT pathogens and biopesticides are grouped together in lower cluster. B. Both *Pseudomonas* strains and *A. piechaudii* strains connect to *Pseudomonas* pathogens, *P. aeruginosa* and *P. fluorescens*. C. *B. ambifaria* strains connect to pathogen *B. pseudomallei*.

Antibiotic resistance content of commercial biopesticide genomes

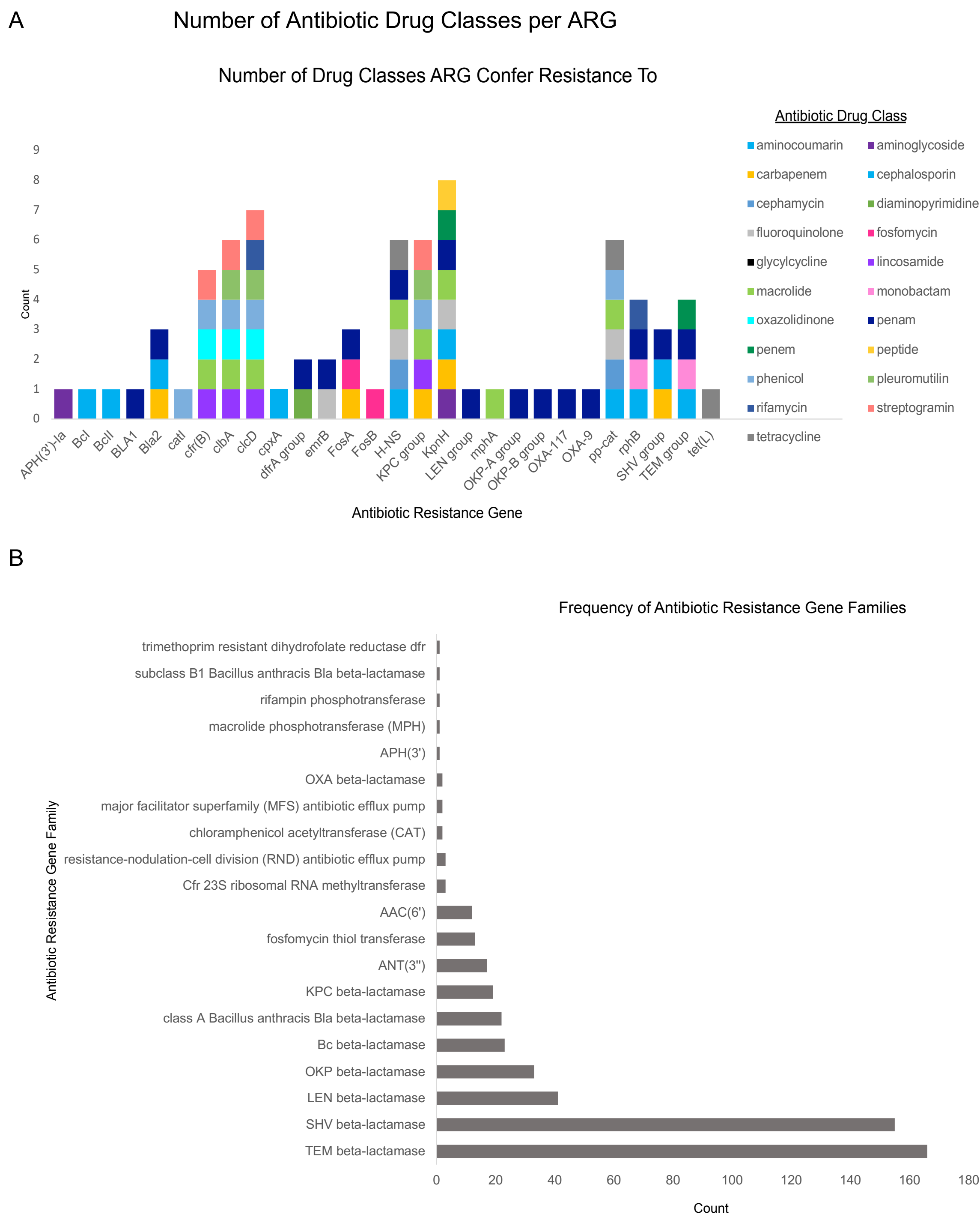


Figure 3. Commercially available *Bacillus*-based biopesticides were sequenced and analyzed. A. The number CARD-identified corresponding antibiotic resistance gene and its corresponding drug class across sequenced *Bacillus*-based biopesticide products. B. Frequency of antibiotic resistance gene family across all mined genomes of available biopesticide strains.

Antibiotic resistance content of commercial biopesticide genomes (cont.)

C

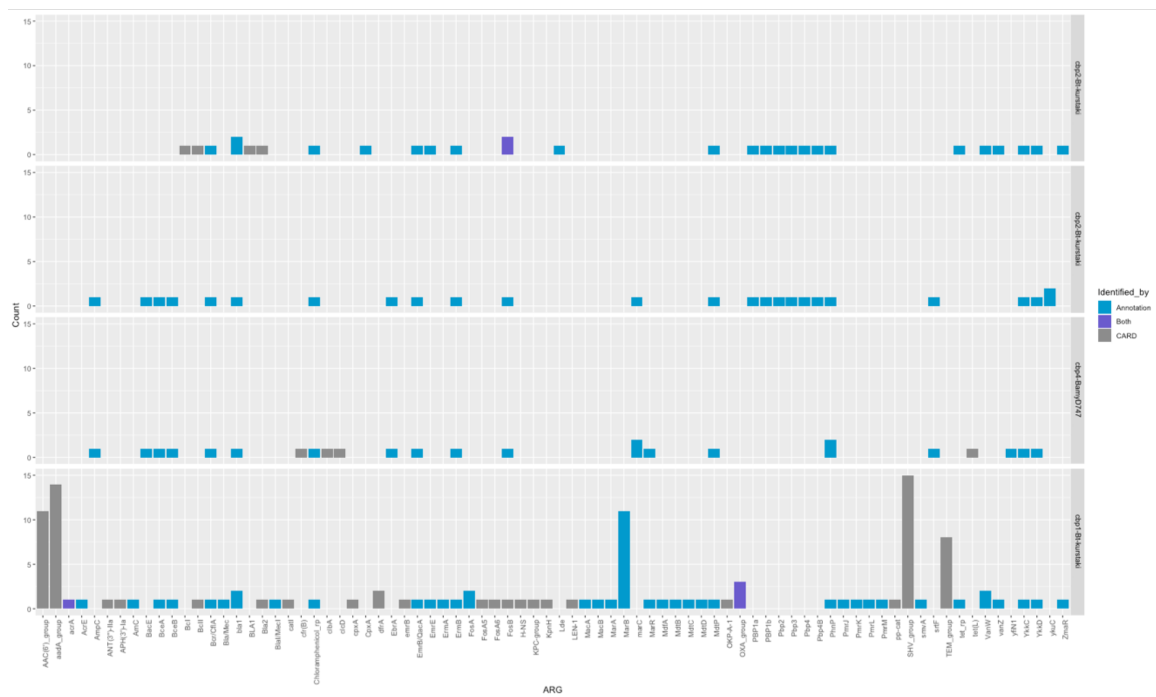


Figure 3. Commercially available *Bacillus*-based biopesticides were sequenced and analyzed. C. Comparison of antibiotic resistance genes identified by CARD and genes identified by annotation of sequenced genomes. Blue is annotation identified, gray is CARD-identified, and purple is identified by both.

Table 1 Minimum Inhibitory Concentrations of eight clinically relevant antibiotics tested on *Bacillus*-based biopesticide products

Antibiotic (µg/mL)	*B. amyloliquefaciens* (cbp4-BamyD747)		*B. thuringiensis* (cbp1-Bt-kurstaki)		*B. thuringiensis* (cbp2-Bt-kurstaki)		*B. subtilis QST* (cbp3-BsubQST)	
CAZ	nt	NA	256	**Resistant**	48	**Resistant**	nt	NA
CD	nt	NA	0.19	Susceptible	0.19	Susceptible	0.64	Susceptible
E	0.125	Susceptible	0.19	Susceptible	0.19	Susceptible	0.125	Susceptible
ETP	0.19	Susceptible	0.125	Susceptible	0.094	Susceptible	0.19	Susceptible
IMI	0.032	Susceptible	0.125	Susceptible	0.19	Susceptible	0.125	Susceptible
KZ	0.125	Susceptible	256	**Resistant**	256	**Resistant**	0.094	Susceptible
QDA	4	**Resistant**	nt	NA	nt	NA	3	Intermediate
TE	4	**Resistant**	nt	NA	nt	NA	3	Intermediate

Table 2 Disk diffusion assay of five clinically relevant antibiotics tested on *Bacillus* based biopesticide products

Biopesticide	DA2 (mm)		DO30		LZD30		STX25		VA30	
cbp4-BamyD747.r1	31 ± 1	Susceptible	19 ± 0.5	Susceptible	30 ± 1	Susceptible	31 ± 0.5	Susceptible	21 ± 0.5	Susceptible
cbp4-BamyD747.r2	30 ± 0.5	Susceptible	20 ± 0.5	Susceptible	29 ± 0.5	Susceptible	31 ± 0.5	Susceptible	21 ± 0.5	Susceptible
cbp4-BamyD747.r3	31 ± 0.5	Susceptible	21 ± 0.5	Susceptible	30 ± 0.5	Susceptible	31 ± 0.5	Susceptible	21 ± 0.5	Susceptible
cbp3-BsubQST.r1	30 ± 0.5	Susceptible	20 ± 0.5	Susceptible	31 ± 0.5	Susceptible	28 ± 0.5	Susceptible	21 ± 0.5	Susceptible
cbp3-BsubQST.r2	30 ± 0.5	Susceptible	19 ± 0.5	Susceptible	31 ± 0.5	Susceptible	27 ± 0.5	Susceptible	21 ± 0.5	Susceptible
cbp3-BsubQST.r3	30 ± 1	Susceptible	19 ± 0.5	Susceptible	28 ± 0.5	Susceptible	27 ± 0.5	Susceptible	21 ± 0.5	Susceptible
cbp2-Bt-kurstaki.r1	24 ± 0.5	Susceptible	18 ± 0.5	Susceptible	28 ± 0.5	Susceptible	11 ± 0.5	**Resistant**	19 ± 0.5	Susceptible
cbp2-Bt-kurstaki.r2	24 ± 0.5	Susceptible	19 ± 0.5	Susceptible	28 ± 0.5	Susceptible	10 ± 0.5	**Resistant**	20 ± 0.5	Susceptible
cbp2-Bt-kurstaki.r3	22 ± 0.5	Susceptible	19 ± 0.5	Susceptible	29 ± 0.5	Susceptible	11 ± 0.5	**Resistant**	19 ± 0.5	Susceptible
cbp1-Bt-kurstaki.r1	0	**Resistant**	20 ± 0.5	Susceptible	36 ± 0.5	Susceptible	20 ± 1	Susceptible	22 ± 0.5	Susceptible
cbp1-Bt-kurstaki.r2	0	**Resistant**	20 ± 0.5	Susceptible	35 ± 0.5	Susceptible	20 ± 0.5	Susceptible	22 ± 0.5	Susceptible
cbp1-Bt-kurstaki.r3	0	**Resistant**	20 ± 0.5	Susceptible	35 ± 0.5	Susceptible	20 ± 0.5	Susceptible	22 ± 0.5	Susceptible

Photographs of replicate plates of Minimum Inhibitory Concentrations assays

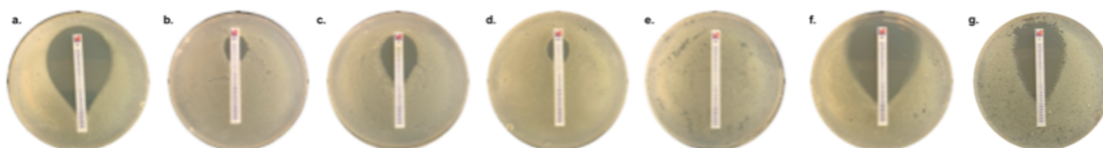


Figure 4 Photographs of various MIC results on Mueller Hinton agar for the *Bacillus*based biopesticide products. A. Cbp3-BsubQST and cephazolin B. Cbp3-BsubQST and quinupristin/dalfpopristin. C. Cbp3-BsubQST and tetracycline. D. Cbp4-BamyD747 and quinupristin/dalfpopristin. E. Cbp2-Bt-kurstaki and ceftazidime. F. Cbp2-Bt-kurstaki and cephazolin. G. Cbp1-Bt-kurstaki erythromycin.

Photographs of replicate plates of disk diffusion assays

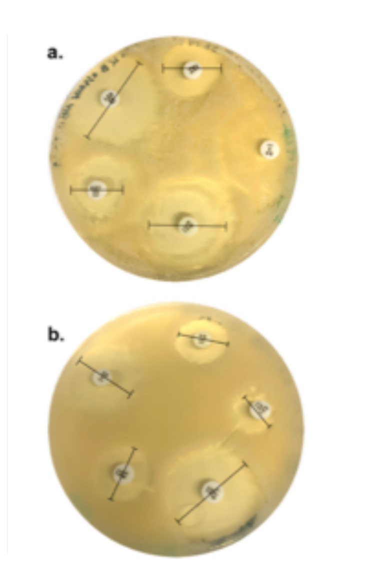


Figure 5. Disk diffusion assay. Black bars indicate where measurement was taken. A. Cbp1-Bt-kurstaki with clindamycin, doxycycline, linezolid, sulfonamide/trimoxazole, and vancomycin. B. Cbp1-Bt-kurstaki with clindamycin, doxycycline, linezolid, sulfonamide/trimoxazole, and vancomycin.

Co-occurrence network of commercial biopesticide resistomes

A

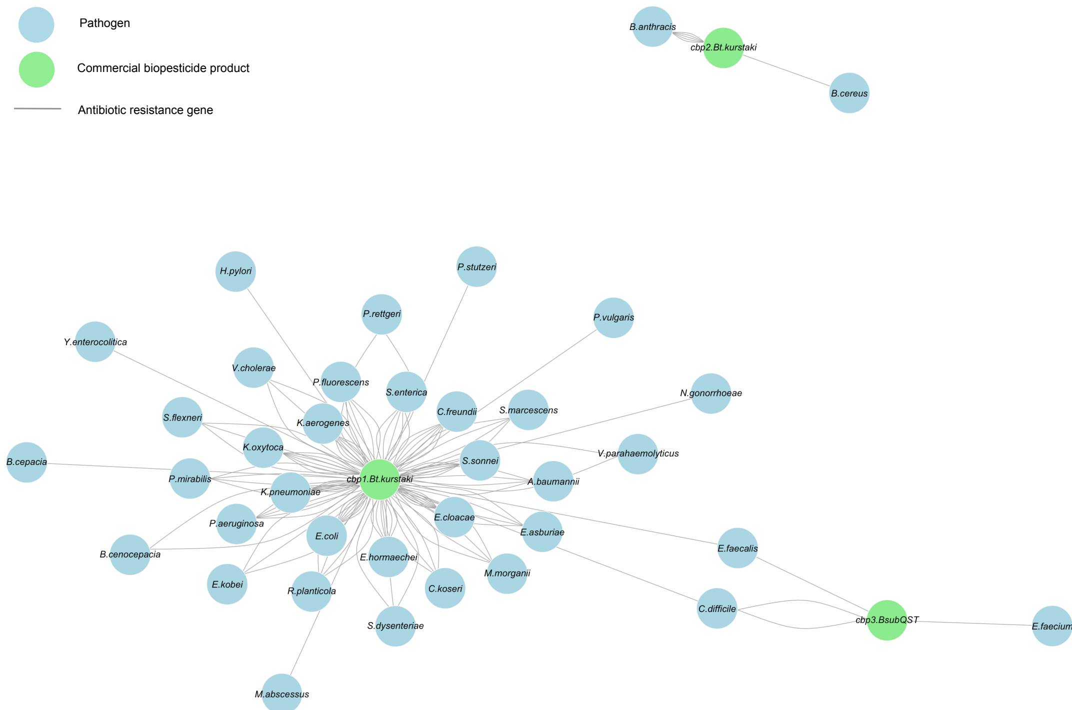


Figure 6. A. Co-occurrence network of CARD ARG of CBP sequences. Biopesticide nodes are in green and pathogen nodes are in blue. Edges are shared antibiotic resistance genes.

Co-occurrence network of commercial biopesticide resistomes (cont.)

B

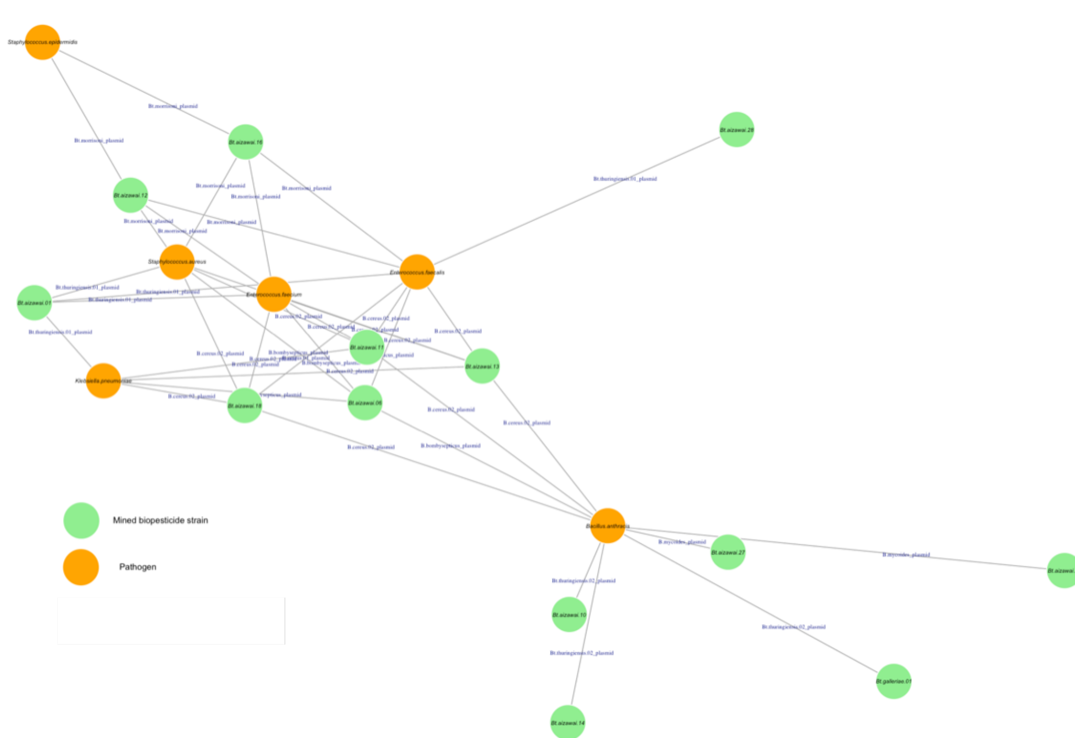


Figure 6. B. Co-occurrence network connecting biopesticide strains and pathogens via shared plasmid sequences. Orange circles represent newly identified pathogens. Green circles represent mined biopesticide strains. Connections are plasmids containing identical ARG.

Table 1 List of biopesticides compiled from EPA and CADPR records

Bacteria	Strains	Use sites	Aerial application	Types	Pest	EPA or CADPR ID	Product names
Achromobacter piechaudii	CBS, 133259		NA	Pesticide	Agrobacter infection		Galltrol - A
Agrobacterium radiobacter	K84	Almonds, Apples, Apricots, Berries, Cherries, Grapes, Walnuts, Kiwis, Nectarines, Peaches, Pears, Pecans, Plums	Yes	Pesticide	Crown gall	40230-1	Galltrol - A
Bacillus amyloliquefaciens	D747	Vegetables, lettuces, tubers, berries, melons, fruits, mushrooms,	Yes	Fungicide	Mildews, fungal disease	70051-108	Amylo-X, Bacstar
Bacillus cereus	UW85	Cotton, Soybeans, Water treatment	NA	Pesticide		66330-282, 7969-439	BPO1 1.7, BAFI SDN (MUP)
Bacillus firmus	I-1582	Legumes, tubers, vegetables, peppers, fruits, herbs, grains, grasses, tobacco, cotton	Yes	Insecticide	nematodes	7969-439, 264-1183	BAFI SDN (MUP), Votivo 240FS
Bacillus pumilus	INR7, TUAT1, I-I582	Nuts, fruits, grains, berries, vegetables, tubers, herbs, grassesm citrus, water treatment, melons, lettuces, legumes	Yes	Fungicide, Pesticide	Fungal lesions, Bacterial blight, Rot, Mold, Citrus scab, Crown gall, Fire blight, Gummy Stem, Powder mold, Smuts, Soilborne diseases, Xanthomonas infection	264-1153	Sonata ASO
Bacillus subtilis	MBI 600, QST 713	Crop seeds such as beans, corn, peas, soybeans, barley, wheat, peanuts and cotton. Grains, beans, tobacco, fruits, vegetables.	Yes	Insecticide,Fungicide, Pesticide	Fungal lesions, Bacterial Blight, Rots, Mold, Citrus scab, Crown gall, Fire blight, Gummy Stem, Powder mold, Smuts, Soilborne diseases, Xanthomonas infection, Mosquitos, Rusts, Mildew, Seedborne diseases	71840-1, 264-1151, 92564-69, 70127-6, 70051-47-707	Fourstar Mbg, Plant Guardian Biofungicide Wettable Powder, Armor Tech Sonnet, Serenade Max, Cease, Rhapsody Aso, Serenade Garden Disease Control, Taegro Technical, Ketch Df
Bacillus thuringiensis subsp. Israelensis	AM 65-52, EG2215	Water treatment	No	Insecticide, Pesticide	Mosquitos (larvae), " Black Flies, Flies, Fungus Gnat (Larvae), Fungus Gnats, Midges, Mosquitoes	4-455-40208, 4-455-84732, 4-455-84732, 4-455-85732	Moskete Defeat, Vectobac G Biological Larvicide Granules, , Microbe-Lift Biological Mosquito, Bmp 144, Dexol Mosquito Free Granules, Bayer Advanced Garden Mosquito Preventer Ready-To-Use Granules, Baritone Bio-Insecticide, Moskeeto(Tm) Biological Mosquito Control Briquets, Anti-Mos Briquets, Brazos Matalarva 10, Summit B.T.I. Briquets, Delta Foremost Fm 4808-Es Skeeter Defeater, No-Squito Granules, Summit Mosquito Bits
Bacillus thuringiensis subsp. kurstaki	SA-12, SA3A	Fruits, Vegetables, Nuts, Grains, Grasses, Lawns, Ornamental Plants, Herbs, Mushrooms, Oil Crops, Root Crops, Tobacco	Yes	Insect growth , Insecticide, Pesticide, Miticide	Armyworm, Bagworm, Budworm, Cabbageworm, Cankerworm, Caterpillar, Cloverworm, Cutworm, Earworm, Fruitworm, Hornworm, Leaffolder, Leafroller, Melonworm, Moths, Skeletonizer Worm, Worms	73049-406, 73049-405, 4-226, 4-252, 4-336, 4-405, 2724-688, 73327-9, 73049-403	Thuricide 76 Lv Bmp 123 Primary Powder, Bt 320 Dust, Bt 320 Sulfur Dust, Safer Brand Garden Dust, Safer R Brand Vegetable And Fruit Insect Dust Rtu, Ringer Vegetable Insect Attack, Safer B.T. Caterpillar Killer Concentrate, Bonide Bacillus Thuringiensis (Bt), Bonide Dipel 150 Dust For Vegetables, Dipel Wp Home & Garden Insecticide, Dipel 10G Sweet Corn Granules, Dipel Bio Garden Spray, Security Bt Dust Biological Insecticide, Green Light Bt Worm Killer, Teknar G
Bacillus thuringiensis subsp. Morrisoni, tolworthi	NA	Corn, grains, seeds.	NA	Insecticide	Rootworm, moths, caterpillars	73049-65	Agrisure
Bacillus thuringiensis subspecies galleriea	SDS-502	Corn, grains, seeds.	NA	Insecticide	Beetles, moths, rice leaf folder	NA	Ecogen. ,BTG, Valent Btg Slurry
Bacillus thuringiensis subspecies tenebrionis	NB-176	Grains, vegetables.	NA	Insecticide, Pesticide	Moths	73049-47	Novodor Btt Liquid Technical Concentrate
Bacillus thuringiensis, subsp. aizawai	ABTS 1857	vegetables, fruits, nuts, row crops, and turf, cotton, and corn	Yes	Insecticide, Pesticide	Moths, armyworm, caterpillars	73049-23	Xentari Biological Insecticide Water Dispersible Granule
Burkholderia ambifaria	AMMD		No	Insecticide, Pesticide, Growth promotion, Fungicide	Fungal disease, nematodes	No longer in use	Venerate
Chromobacterium subtsugae	PRAA41	Vegetables, fruit, flowers, bedding plants, root vegetables and tubers, ornamentals, lawn, grains, tobacco	No	Insecticide, Pesticide	corn borer, citrus rust mite, and stink bug, beetles armyworm, cabbage looper,	84059-10	Marrone MBI-203 EP Bioinsecticide
Lysinibacillus sphaericus	2362, C341	Water treatment	Yes	Insecticide	Mosquitos (larvae)	73049-20-52437	Dionne Larva Halt Ii Mosquito Control, Bacillus Sphaericus Slurry
Paenibacillus alvei	TS-15	Tomatoes	NA	Fungicide, Bacteriocide	*Salmonella*	NA	Patent pending
Paenibacillus lentimorbus	NA	Vegetables, lawn	No	Insecticide, Pesticide	Beetles	54501	Doom Milky Disease Spore Powder
Paenicillus popilliae	NA	Ornamental flowers, Lawn	No	Pesticide	Japanese beetle (Larvae)	63191-1-71803	Nature'S Kind Milky Spore
Pantoea agglomerans	C9-1, E325	Apples, pears,	Yes	Pesticide	Fire blight	71368-45, 73771-2	Blightban C9-1, Bloomtime Biological Fd Biopesticide
Pantoea vagans	C9-1	Fruit trees	Yes	Pesticide	Fire blight		Bloomtime
Pasteuria penetrans	Pr-3	Legumes	Yes	Pesticide	Nematodes	85004-U	Soyacyst Tech
Pseudomonas aureofaciens	Tx-1	Turf, ornamental plants and grasses	NA	Fungicide, Pesticide	Fungal disease, nematodes	75801-1	Spot-Less Biofungicide
Pseudomonas fluorescens	A506	Apples, pears, citrus, grapes, ornamental plants	Yes	Fungicide, Pesticide	Rots, Fire Blight, Rusts, Frost	228-710	Frostban B
Pseudomonas syringae	ESC-11	Apples, pears, citrus	Yes	Fungicide, Pesticide	Fungal disease, rot	81803-3, 81803-1	Esc-10 Biofungicide Technical, Bio-Save 11 Lp Biological Fungicide
Pseudomonas vranovensis	CBS 133252	Water treatment	No	Insecticide	Mosquitos (larvae)	006583-1	Mosquito Depth Charges Water Soluble Pouches, Gardenique Mosquito Treatment, Smartpond Mosquito Treatment, Bonide Mosquito Beater Ws
Streptomyces galbus strain	QST 6047	Apples	NA	Insecticide	Caterpillars, moths	69592-O	Agraquest QST 6047 Technical
Streptomyces griseoviridis	K6	Legumes, ornamentals	NA	Fungicide	Spots, Mold, Rots, Fungal Lesions, Wilts	64137-5-68539, 64137-9	Prefence Biofungicide, Mycostop G Biofungicide
Streptomyces lydicus	WYEC 108	Soil mixes, turf, ornamentals	NA	Fungicide	Root decay	73314-20	Actinovate Soluble

References

1. Schwarz, S. *et al.* Antimicrobial Resistance among Staphylococci of Animal Origin. *Microbiol. Spectr.* 6, (2018).
2. Jechalke, S., Heuer, H., Siemens, J., Amelung, W. & Smalla, K. Fate and effects of veterinary antibiotics in soil. *Trends Microbiol.* 22, 536–545 (2014).
3. Munk, P. *et al.* Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries. *Nat. Microbiol.* 3, 898–908 (2018).
4. Deng, S., Yan, X., Zhu, Q. & Liao, C. The utilization of reclaimed water: Possible risks arising from waterborne contaminants. *Environ. Pollut.* 254, 113020 (2019).
5. FDA. *Antimicrobials Sold or Distributed for Use in Food-Producing Animals*. *Center for Veterinary Medicine* vol. 2 (2018).
6. EPA. Pseudomonas syringae strain ESC-10 (006441) Pseudomonas syringae strain ESC-11 (006451) Fact Sheet. 10, 10–11 (2008).
7. Parnell, J. J. *et al.* From the lab to the farm: An industrial perspective of plant beneficial microorganisms. *Front. Plant Sci.* 7, 1–12 (2016).
8. Akinrinlola, R. J., Yuen, G. Y., Drijber, R. A. & Adesemoye, A. O. Evaluation of Bacillus Strains for Plant Growth Promotion and Predictability of Efficacy by In Vitro Physiological Traits. *Int. J. Microbiol.* 2018, (2018).
9. CADPR. Summary of pesticide use report data - 2017. (2017).
10. EFSA. Risks for public health related to the presence of Bacillus cereus and other Bacillus spp. including Bacillus thuringiensis in foodstuffs. *EFSA J.* 14, (2016).
11. Yul, J., Choi, J.A.E.Y., Li, M. S., Jin, B.R.E. & Je, Y.H.O. BT as a specific, safe, and effective tool for insect pest control. *J Microbiol. Biotechnol.* 17, 547– 559 (2007).
12. Federici, B. A. & Siegel, J. P. Safety of Bacillus thuringiensis and Bt Crops Used in Insect Control. (2008).
13. Siegel, J. P. The Mammalian Safety of Bacillus thuringiensis- Based Insecticides. *J. Invertebr. Pathol.* 77, 13–21 (2001).
14. Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P. & Hens, L. Chemical Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture. *Front. Public Heal.* 4, 1–8 (2016).
15. Hernández, A. F. *et al.* Toxic effects of pesticide mixtures at a molecular level: Their relevance to human health. *Toxicology* 307, 136–145 (2013).
16. FDA. *Substances Generally Recognized as Safe*. vol. 81 (2016).
17. Thakore, Y. The biopesticide market for global agricultural use. *Ind. Biotechnol.* (2006).
18. Aujoulat, F. *et al.* Multilocus sequence-based analysis delineates a clonal population of Agrobacterium (Rhizobium) radiobacter (Agrobacterium tumefaciens) of human origin. *J. Bacteriol.* 193, 2608–2618 (2011).

19. From, C., Hormazabal, V. & Granum, P. E. Food poisoning associated with pumilacidin-producing Bacillus pumilus in rice. *Int. J. Food Microbiol.* (2007) doi:10.1016/j.ijfoodmicro.2006.11.005.
20. Tena, D. *et al.* Cutaneous Infection Due to Bacillus pumilus: Report of 3 Cases. *Clin. Infect. Dis.* 44, e40–e42 (2007).
21. Alejandra Bravo, et al. Bacillus thuringensis: A story of a sucessful bioinsecticide. *Insect Biochem. Mol. Biol.* 41, 423–431 (2013).
22. Reyes-Ramírez, A. *et al.* Plasmid Patterns of Bacillus thuringiensis Type Strains. *Appl. Environ. Microbiol.* 74, 125–129 (2008).
23. Frost, L. S., Leplae, R., Summers, A. O. & Toussaint, A. Mobile genetic elements: The agents of open source evolution. *Nat. Rev. Microbiol.* 3, 722–732 (2005).
24. Grohmann, E., Muth, G. & Espinosa, M. Conjugative plasmid transfer in grampositive bacteria. *Microbiol. Mol. Biol. Rev.* 67, 277–301 (2003).
25. Courvalin, P. Transfer of Antibiotic Resistance Genes between Gram-Positive and Gram-Negative Bacteria. *Antimicrob. Agents Chemother.* 38, 1447–1451 (1994).
26. Brooks, L. E., Kaze, M. & Sistrom, M. Where the plasmids roam: large-scale sequence analysis reveals plasmids with large host ranges. 1–10 (2019) doi:10.1099/mgen.0.000244.
27. McSpadden Gardener, B. B. & Driks, A. Overview of the nature and application of biocontrol microbes: Bacillus spp. *Phytopathology* 94, 1244 (2004).
28. Wright, G. D. The antibiotic resistome: The nexus of chemical and genetic diversity. *Nature Reviews Microbiology* (2007) doi:10.1038/nrmicro1614.
29. Belbahri, L. *et al.* Comparative genomics of Bacillus amyloliquefaciens strains reveals a core genome with traits for habitat adaptation and a secondary metabolites rich accessory genome. *Front. Microbiol.* 8, 1–15 (2017).
30. Patel, R., Piper, K., Cockerill, F. R., Steckelberg, J. M. & Yousten, A. A. The biopesticide Paenibacillus popilliae has a vancomycin resistance gene cluster homologous to the enterococcal VanA vancomycin resistance gene cluster. *Antimicrob. Agents Chemother.* 44, 705–709 (2000).
31. Sanchis, V., Agaisse, H., Chaufaux, J. & Lereclus, D. A recombinase-mediated system for elimination of antibiotic resistance gene markers from genetically engineered Bacillus thuringiensis strains. *Appl. Environ. Microbiol.* 63, 779–784 (1997).
32. Coenye, T. *et al.* Burkholderia ambifaria sp. nov., a novel member of the Burkholderia cepacia complex including biocontrol and cystic fibrosis-related isolates. *Int. J. Syst. Evol. Microbiol.* (2001) doi:10.1099/00207713-51-4-1481.
33. Guglierame, P. *et al.* Efflux pump genes of the resistance-nodulation-division family in Burkholderia cenocepacia genome. *BMC Microbiol.* (2006) doi:10.1186/1471-2180-6-66.

34. Partridge, S. R., Kwong, S. M., Firth, N. & Jensen, S. O. Mobile genetic elements associated with antimicrobial resistance. *Clin. Microbiol. Rev.* 31, 1–61 (2018).
35. Shintani, M. The behavior of mobile genetic elements (MGEs) in different environments. *Biosci. Biotechnol. Biochem.* 81, 854–862 (2017).
36. Bender, J. K. *et al.* Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: Towards a common nomenclature. *Drug Resist. Updat.* (2018) doi:10.1016/j.drup.2018.10.002.
37. Mühlberg, E. *et al.* Renaissance of vancomycin: Approaches for breaking antibiotic resistance in multidrug-resistant bacteria. *Canadian Journal of Microbiology* (2020) doi:10.1139/cjm-2019-0309.
38. Adeolu, M., Alnajar, S., Naushad, S. & Gupta, R. S. Genome-based phylogeny and taxonomy of the 'Enterobacteriales': Proposal for enterobacterales ord. nov. divided into the families Enterobacteriaceae, Erwiniaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morganellaceae fam. nov., and Budviciaceae fam. nov. *Int. J. Syst. Evol. Microbiol.* (2016) doi:10.1099/ijsem.0.001485.
39. Kintses, B. *et al.* Phylogenetic barriers to horizontal transfer of antimicrobial peptide resistance genes in the human gut microbiota. *bioRxiv* (2018) doi:10.1101/385831.
40. Caro-Quintero, A. & Konstantinidis, K. T. Inter-phylum HGT has shaped the metabolism of many mesophilic and anaerobic bacteria. *ISME J.* (2015) doi:10.1038/ismej.2014.193.
41. Fondi, M. & Fani, R. The horizontal flow of the plasmid resistome: Clues from inter-generic similarity networks. *Environ. Microbiol.* (2010) doi:10.1111/j.14622920.2010.02295.
42. Von Wintersdorff, C. J. H. *et al.* Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. *Frontiers in Microbiology* (2016) doi:10.3389/fmicb.2016.00173.
43. Vilas-Boas, G., Sanchis, V., Lereclus, D., Lemos, M. V & Bourguet, D. Genetic differentiation between sympatric populations of Bacillus cereus and Bacillus thuringiensis. *Appl. Environ. Microbiol.* 68, 1414–1424 (2002).
44. CADPR. *A Guide to Pesticide Regulation in California*. (2018).
45. Bernstein, I. L. *et al.* Immune responses in farm workers after exposure to Bacillus thuringiensis pesticides. *Environ. Health Perspect.* 107, 575–582 (1999).
46. Hu, R., Shi, L., Lee, D. C. & Haile, G. P. Access to and disparities in care among migrant and seasonal farm workers (MSFWs) at U.S. health centers. *J. Health Care Poor Underserved* (2016) doi:10.1353/HPU.2016.0107.
47. Flocks, J. *et al.* Lessons learned from data collection as health screening in underserved farmworker communities. *Prog. Community Heal. Partnerships Res. Educ. Action* (2018) doi:10.1353/cpr.2018.0024.

48. Raymond, B., Federici, B. A. & Raymond, B. In defence of Bacillus thuringiensis, the safest and most successful microbial insecticide available to humanity — a response to EFSA. 1–8 (2017) doi:10.1093/femsec/fix084.
49. Naik, G. A., Bhat, L. N., Chopade, B. A. & Lynch, J. M. Transfer of broad-hostrange antibiotic resistance plasmids in soil microcosms. *Curr. Microbiol.* 28, 209– 215 (1994).
50. D'Costa, V. M., McGrann, K. M., Hughes, D. W. & Wright, G. D. Sampling the antibiotic resistome. *Science (80-.).* (2006) doi:10.1126/science.1120800.
51. Intriago, J. C. *et al.* Agricultural reuse of municipal wastewater through an integral water reclamation management. *J. Environ. Manage.* 213, 135–141 (2018).
52. Han, X. M. *et al.* Antibiotic resistance genes and associated bacterial communities in agricultural soils amended with different sources of animal manures. *Soil Biol. Biochem.* 126, 91–102 (2018).
53. Metcalf, W. W. *et al.* Synthesis of methylphosphonic acid by marine microbes: A source for methane in the aerobic ocean. *Science (80-.).* 337, 1104–1107 (2012).
54. Maillard, J.-Y. Resistance of Bacteria to Biocides. *Microbiol. Spectr.* 6, (2018).
55. Pazda, M., Kumirska, J., Stepnowski, P. & Mulkiewicz, E. Antibiotic resistance genes identified in wastewater treatment plant systems – A review. *Sci. Total Environ.* 697, 134023 (2019).
56. Teuber, M. Veterinary use and antibiotic resistance. *Curr. Opin. Microbiol.* 4, 493– 499 (2001).
57. EPA Office of Pesticide Programs, U. Biopesticides Fact Sheet for Chromobacterium subtsugae strain PRAA4-1T. 1–3.
58. Jia, B. *et al.* CARD 2017: Expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 45, D566– D573 (2017).
59. NCBI. National Center for Biotechnology Information (NCBI)[Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [2019] – [cited 2019 Jan 20]. Available from: https://www.ncbi.nlm.nih.gov/. (2019).
60. Ginestet, C. ggplot2: Elegant Graphics for Data Analysis. *J. R. Stat. Soc. Ser. A (Statistics Soc.* (2011) doi:10.1111/j.1467-985x.2010.00676_9.x.
61. Csardi, G. & Nepusz, T. The igraph software package for complex network research. *InterJournal Complex Syst.* (2006).
62. Hudzicki, J. Kirby-Bauer disk diffusion susceptibility test protocol. *Kirby-BauerDisk-Diffusion-Susceptibility-Test-Protocol.*(2009).
63. CLSI. Minimum inhibitory concentration guidelines. (2015).
64. Andrews, S. FastQC. *Babraham Bioinforma.* (2010) doi:citeulike-articleid:11583827.

65. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* (2014) doi:10.1093/bioinformatics/btu170.
66. Bankevich, A. *et al.* SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* (2012) doi:10.1089/cmb.2012.0021.
67. Gurevich, A., Saveliev, V., Vyahhi, N. & Tesler, G. QUAST: Quality assessment tool for genome assemblies. *Bioinformatics* 29, 1072–1075 (2013).
68. Aziz, R. K. *et al.* The RAST Server: Rapid annotations using subsystems technology. *BMC Genomics* (2008) doi:10.1186/1471-2164-9-75.
69. Bateman, A. *et al.* UniProt: The universal protein knowledgebase. *Nucleic Acids Res.* (2017) doi:10.1093/nar/gkw1099.
70. Brooks, L., Kaze, M. & Sistrom, M. A Curated, Comprehensive Database of Plasmid Sequences

Mixing with a Bad Crowd: ARG exchange between *Klebsiella pneumoniae* and

Introduction

The causes of the global antibiotic resistance crisis are complex and the development of effective responses depends on a comprehensive understanding of these factors. Many agricultural practices have been implicated in the evolution and dissemination of antibiotic resistance genes.[1–3] Up to 80% of antibiotics produced In the US are for agricultural use[4] and exposure of bacteria to antibiotics has selected resistant strains.[5] Biopesticides - microorganism-based insecticides, are applied globally in large quantities and have recently been connected to the presence of ARG in the environment. Commercial *Bacillus*-based biopesticides are safe and effective, and increasing in popularity[6-8] however, previous work indicates that widely used biopesticides are reservoirs of ARG.[9]

An important, multi-drug resistant pathogen that has arisen globally in the last 30 years is *Klebsiella pneumoniae*. A Gram negative, soil-dwelling bacterium that causes pneumonia, septicemia, and meningitis, and can be both community and hospital acquired. *K. pneumoniae*-based pneumonia is a common nosocomial infection often associated with ventilator use.[10] Carbapenem resistant *K. pneumoniae* appeared in the late 1990's and is currently a serious issue in antibiotic resistant nosocomial infections.[11] *K. pneumoniae* is an important reservoir and vector of ARG in the environment and there are explicit environment to clinic connections.[12] One such known environmental-clinical association is between *K. pneumoniae* contaminated meat and urinary tract infections.[13] Multiple agricultural practices, such as prophylactic and overuse of antibiotics as growth promoters rather than therapeutics, have been well-characterized as origins antibiotic resistant genes (ARG). Antibiotic resistant *K. pneumoniae* has been shown to enter food production streams via multiple processes such as the application of fertilizer from manure[14] or untreated wastewater to crops.[15] *K. pneumoniae* has been found in the feces of dairy cows[16,17] and occurs in multiple areas of feedlots and dairies[18]

Large quantities of biopesticides, such as globally popular *Bacillus thuringiensis*, applied to agricultural settings increases the likelihood of interaction between biopesticides and antibiotic resistant *K. pneumoniae* strains. *K. pneumoniae* and the widely used biopesticide *B. thuringiensis* both have unusual plasmid permissivity and genomic plasticity[19], making their potential for exchange an intriguing and important interaction to investigate. This potential genetic exchange between reservoirs for ARG, *Bacillus*-based biopesticides, and pathogenic *K. pneumoniae*, raises the concern of generation of novel pathogenicity and ARG spread. Confirmation of the location of ARG, on plasmids or chromosome, can provide important information about which genes are most likely to be exchanged.

This study tests the hypothesis that horizontal gene exchange via plasmid conjugation can occur between commercial, *Bacillus*-based biopesticide products and a carbapenemase-positive (bla_{KPC}) strain of *K. pneumoniae,* and that this

exchange results in the acquisition of resistant phenotypes by commercial biopesticide strains. We used Hi-C proximity ligation to identify the location - chromosome or plasmid, of specific ARG in biopesticides isolated from commercial products, and MIC tests to determine their resistance phenotypes. We then conducted conjugation pairwise between biopesticide strains and carbapenemase-positive (*bla*$_{KPC}$) *K. pneumoniae.* We again used Hi-C genome sequencing and MIC techniques to asses horizontal gene transfer between strains, demonstrating that *K. pneumoniae* and *Bacillus*-based biopesticides can exchange carbapenem antibiotic resistance genes after contact resulting the acquisition of carbapenem resistant phenotypes in biopesticides. This critically evidences the role that biopesticides can play as vectors and reservoirs of clinically relevant antibiotic resistance and highlights the need for further investigation of the role that these commonly used agricultural processes play in the evolution and spread of antibiotic resistance.

Results

Characterization of ARG In commercial biopesticides

Hi-C sequencing is a chromosome conformation capture method developed originally to analyze the spatial organization of chromatin in eukaryotic cells.[20] When used in prokaryotic organisms, the proximity ligation step produces chimeric sequencing reads that combine chromosomal and extrachromosomal gene content present In the same cell - thus allowing the determination of plasmid and host from a mixed bacterial sample.[21] In this study, we used Hi-C sequencing to map antibiotic resistance genes to the chromosome or plasmid contained within each of four *Bacillus* strains isolated from commercial biopesticide products: (cbp1-Bt -kurstaki, cbp2-Bt-kurstaki, cbp3-BsubQST, and cbp-BamyD747). Cbp1-Bt-kurstaki contained two plasmids, both containing vancomycin genes. (Fig 1C) One plasmid, identified as *Bacillus thuringiensis* strain Bt185 (GenBank: CP014283.1) contained three vancomycin ARGs; vanRA, vanSA, and vanYF and two tetracycline ARGs: tetL and tet45. The second plasmid identified as *Bacillus cereus* strain AR156 (GenBank: CP015592.1) contained two vancomycin ARGs; vanRA and vanSA. The cbp1-Bt-kurstaki chromosome contained seven ARG, three of which are class A Bacillus beta-lactamases (BcI, BcII, Bla1) along with IsaB, InuC, FosB, and mel. Cbp2-Bt-kurstaki contained plasmid *Bacillus cereus* C1L plasmid pC1L1 (RefSeq: NZ_CP022446.1) with vanRA, vanSA and tet45 ARG. (Fig 1D) Its chromosome contained the beta-lactamases BcI, Bla2, and FosB. Cbp3-BsubQST did not contain any plasmids, however four ARGs were identified in its chromosome; three encoding tetracycline resistance (tet40, tetW, tetWNW), and an efflux regulator (ykkC). (Fig 1A) Cpb4-BamyD747 contained four chromosomally encoded ARG. (Fig 1B) A vancomycin resistance variant was identified along with three other genes belonging to the Cfr 23S Ribsomal RNA methyltransferase antimicrobial resistance gene family: cfr(B), clbA, and clcD. No carbapenem ARGs were Identified in the biopesticide strains prior to co-culturing.

Phenotypic resistance before and after co-culturing

Biopesticides were isolated before and after co-culturing with *K. pneumoniae* and tested against two carbapenem antibiotics (i.e., ertapenem and imipenem) to determine changes to their carbapenem resistance phenotypes. (Fig 2) All biopesticide products demonstrated susceptibility to ertapenem before co-culture. Two (i.e., cbp2-Bt-kurstaki and cpb3-BsubQST) changed to complete resistance carbapenem phenotypes after coculture (Fig 2A). Cbp2-Bt-kurstaki changed from an ertapenem MIC of 0.094 – 0.125 µg/mL to complete resistance with an ertapenem MIC of 32 µg/mL. (Table 1). Cpb3BsubQST changed from an ertapenem MIC of 0.094 – 0.125 µg/mL to complete resistance with an MIC of 32 µg/mL. The other two biopesticide products demonstrated modest changes to resistance; cbp1-Bt-kurstaki changed from an ertapenem MIC of 0.064 – 0.125 µg/mL to 2.0 µg/mL and cbp4-BamyD747 changed from an ertapenem MIC of 0.019 – 0.125 µg/mL to 1.5-3.0 µg/mL. There were also changes to imipenem resistance after exposure to *K. pneumoniae*. (Fig 2B) Cbp2-Bt-kurstaki became completely resistant imipenem after co-culture, 0.19 – 4.0 µg/mL to 32 µg/mL. Cbp4BamyD747 changed phenotype from susceptible to imipenem, MIC of 0.19 – 0.125 µg/mL, to completely resistant with an MIC of 32 µg/mL. Cpb3-BsubQST did not change phenotype and its imipenem MIC before was 0.19–0.38 µg/mL and after had an MIC of 0.19 µg/mL for all replicates. Representative plates show *K. pneumoniae* tested against ertapenem (Fig 2C) and imipenem (Fig 2D). A representative plate for cbp4-BamyD747 demonstrating susceptibility to imipenem before co-culture (MIC 0.032 µg/mL) (Fig 2E) and resistance to imipenem after co-culture (MIC 32 µg/mL) (Fig 2F).

Genetic exchange between K. pneumoniae and biopesticides

Carbapenem resistance associated genes transferred from *K. pneumoniae* to the *Bacillus*-based biopesticide products were confirmed using the sequenced genomes of the “after” co-cultured strains using gene alignments, annotations, and functional orthologs. (Fig 3A) The carbapenem resistance gene KPC-2[19] was transferred from *K. pneumoniae* to cbp1-Bt-kurstaki and cbp4-BamyD747. Another carbapenem resistance gene - SHV-18,[22] was transferred to *K. pneumoniae* cbp4-BamyD747. Carbapenem resistance gene ompC was transferred from *K. pneumoniae* to all four biopesticides.

Genes AcrA and AcrB were present in cbp1-Bt-kurstaki “before” sequence and only appeared in the reference genomes for “before” cpb3-BsubQST and cbp4-BamyD747 (IMG). In the “after” biopesticide sequences this pair was present. None of the biopesticide genomes contained soxS in the “before” but all contained this gene “after.” MarA was also transferred from *K. pneumoniae* to all four biopesticides. A TEM-2 beta lactamase was identified in cbp4-BamyD747 “after.” TolC was transferred to cpb3BsubQST and cbp4-BamyD747.

Additional genes encoding antibiotic resistance associated proteins also transferred from *K. pneumoniae* to biopesticide strains and were identified by functional orthology. Beta-lactamase induction transducer ampG and antimicrobial peptide arnC, were taken up by cbp1-Bt-kurstaki, cbp2-Bt-kurstaki and cpb3-BsubQST. Only cbp4BamyD747 took up genes for multidrug resistance

proteins mdtG, mtdH, mdtL, mdtO, and antimicrobial peptide associated signal transduction protein pmrD. Multidrug transporter mtdK/norM and small multidrug resistance pump QacE were transferred to cbp2-Btkurstaki andcbp4-BamyD747. Multidrug resistance efflux pump smvA was transferred to all four biopesticide products.

Biopesticides also exchanged antibiotic resistance genes with each other. Cbp1Bt-kurstaki contributed multiple ARG to the other biopesticides. (Fig 3B) Vancomycin resistant variants, vanR, vanS, and vanW, two of which were identified on plasmids, were taken up by cpb3-BsubQST and cbp4-BamyD747. Three components of a multidrug efflux system, MtdABC and gene sapA were transferred from cbp1-Bt-kurstaki to the other biopesticides. Multidrug transporter protein mdlb was transferred cbp1-Bt-kurstaki to cpb3-BsubQST and cbp4-BamyD747. Class C beta-lactamase component ampH was transferred cbp1-Bt-kurstaki to cbp2-Bt-kurstaki and cpb3-BsubQST. Not all gene exchanges were clear; beta-lactam resistance associated transport system substrate binding protein mppA was present in the "before" cbp1-Bt-kurstaki sequence as well as the "before" *K. pneumoniae* indicating either may have supplied this gene to cbp2-Btkurstaki and cpb3-BsubQST. Multidrug resistance transporter protein mdtD was present in the "before" sequences for cbp1-Bt-kurstaki, cbp4-BamyD747, and *K. pneumoniae* and in "after" sequences for cbp2-Bt-kurstaki and cpb3-BsubQST.

Discussion

This study demonstrates the ability of widely used *Bacillus*-based biopesticide products to act as vectors for clinically important ARG. Acceptance of carbapenem resistance genes from a carbapenemase positive strain of pathogenic *K. pneumoniae* lead to alterations in carbapenem susceptibility phenotypes in the biopesticide products. Using multiple bioinformatic methods for analysis, specific carbapenem related genes were identified as having been transferred. Genetic exchange occurred bidirectionally between *K. pneumoniae* and the biopesticide products, and between the biopesticides themselves. Contact and exchange between these species in the environment is especially concerning when considering the large amounts of biopesticides that are added to the environment and the potential for biopesticides to transfer clinically important antibiotic resistance to other environmental microbes.

Chromosome conformation capture provided important information regarding the location of ARG on the biopesticide strains. This is the first study that we know that uses Hi-C for known individual strains. However, Hi-C has been previously used to survey environmental soil samples for ARG. Stadler et al 2019 used Hi-C to identify ARG locations on mobile genetic elements in wastewater samples.[23] This allowed for a culture independent analysis and identification of ARG in integrons and plasmids and connected the "resistome and plasmidome" of a complex community sample. While there are known issues with complex community sampling [24] which likely impact Hi-C output as well,

working with low-complexity, known strains allows for a greater depth of sequencing and higher confidence in deconvolution and alignment to reference databases. Previous work has demonstrated that ARGs are commonly present in bacteria used as biopesticides.[9] While it is unsurprising that the *Bacillus*-based biopesticides exchanged ARG with each other, it is concerning that components for vancomycin resistance, as identified by Hi-C analysis, are located on plasmids indicating an increased likelihood for transfer. The presence of a plasmids identified as belonging to *Bacillus cereus* is also unsurprising given that *B. thuringiensis* is a member of the *Bacillus*-ACT group which includes pathogens *Bacillus anthracis* and *B. cereus.* These three siblings share a common genome and are known to bear each other's plasmids.[25,26] The product that transferred the most genes to the other pesticides, cbp1-Bt-kurstaki, contained more plasmids than the other strains. As biopesticide products are often mixed in multi-strain consortia this increases the opportunity for exchange and uptake of ARG before application.

Straightforward exposure to carbapenemase positive *K. pneumoniae* resulted in changes in phenotype from complete susceptibility to carbapenem antibiotics to complete resistance in more than one of the tested biopesticide products. *K. pneumoniae* has unique plasmid permissivity which offers the opportunity for uptake and exchange of various ARG and some clones have been found to host as many as ten plasmids.[12,27] Despite both *B. thuringiensis* kurstaki products ostensibly containing the same strain, they did not demonstrate indistinguishable genetic exchange behavior or genetic makeup. Each was isolated from different suspension media, and sequencing showed different plasmid content between isolates - potentially demonstrating variation in quality control and handling between biopesticide products that could influence their capacity to harbor and disseminate ARGs. *K. pneumoniae* triggers horizontal gene transfer when exposed to low levels of antibiotics.[28] It is possible that the biopesticides had prior exposure to antibiotics or there were trace amounts of antibiotics in the suspension materials causing this evolutionary strategy to impact exchange.

Genes encoding carbapenem resistance KPC-2 and SHV-18 were transferred to the biopesticides along with other genes that participate in resistance to imipenem and ertapenem. Some genes transferred completed multicomponent antibiotic efflux pumps that conferred resistance to carbapenems that were not associated with the KPC or SHV beta-lactamase gene families. *K. pneumoniae*'s loss of its characteristic membrane penetrability leads to an upregulation of AcrAB efflux pumps that ejects imipenem.[29] This genomic flexibility also allows for the insertion of and maintenance of ARG in its chromosome.[30] Both genes for AcrA and AcrB were present in cbp1-Bt-kurstaki prior to co-culturing, and also only appeared in the reference genomes for cpb3-BsubQST and cbp4-BamyD747 prior to co-culturing (IMG). After co-culturing, this gene pair was present in biopesticide sequences. AcrAB is part of a trio that includes tolC, a gene that was transferred to cpb3-BsubQST and cbp4-BamyD747 from *K. pneumoniae*. Global regulator soxS was transferred from *K.*

pneumoniae to all four products and functions to upregulate AcrAB efflux pumps.[31] Interestingly, *K. pneumoniae* also picked up genes from the biopesticide strains cbp1-Bt-kurstaki. While these genes were not related to antibiotic resistance, they demonstrate horizontal transfer between biopesticides and *K. pneumoniae* Is bidirectional, and thus once biopesticides acquire ARG, they are presumably able to disseminate them to clinically relevant pathogens.

Exchange of clinically important ARG between pathogens and microbial biopesticides presents a serious issue and requires inclusion of biopesticide use in the list of agricultural practices that generate and maintain ARG in the environment. This study demonstrates that biopesticides can act as vectors for carbapenem resistance genes and acquire resistance via simple contact with a carbapenemase positive strain of *K. pneumoniae*. Strains of *K. pneumoniae* have been tested as potential fertilizers and agricultural soil enhancers[32,33] indicating an interest in using this species as a soil additive. Without a strategy or solution to prevent ARG uptake, this presents a potentially misguided agricultural practice. *K. pneumoniae*-based pneumonia is a common nosocomial infection, often associated with ventilator use.[10] As the Covid-19 pandemic continues, ventilator use has increased[34] as has prophylactic application of multiple broad-spectrum antibiotics, indicating the need for oversight in order to prevent ARG generation and spread.[35] Multi-drug resistant *K. pneumoniae* has been found in vegetables, cow, pig, seafood, and chicken meats products [36–38] *K. pneumoniae* has been isolated from dust and air samples from dairy farms[39] suggesting that inhalation of community-acquired *K. pneumoniae* may be an issue. Our results indicate the clear need for scaling up in order to investigate additional enteric and Gram positive pathogens and a larger suite of bacterial biopesticide products. Having demonstrated that biopesticides can act as vectors of ARG *in vitro*, it is important to consider the use of biopesticides among the agricultural practices that contribute to the spread of antibiotic resistance and follow up with field-based investigations of Its role.

Methods

Four commercial Bacillus-based biopesticide products (cbp), *B. thuringiensis* kurstaki strain SA12*, B. subtilis* strain QST 713 and *B. amyloliquefaciens* strain D747, were purchased and coded by number and strain: cbp1-Bt -kurstaki, cbp2-Bt-kurstaki, cbp3-BsubQST, and cbp-BamyD747. The two *B. thuringiensis* products were the same strain but from separate companies and contained different suspension materials. Cultures were all performed in triplicate with controls of un-inoculated media incubated along with each experimental co-culture. Carbapenemase-positive *K. pneumoniae* ATCC® BAA-1705™ was obtained from ATCC. *Bacillus* cultures were passaged and incubated at increasing temperatures and moved from LB medium to Mueller Hinton medium. Freeze-dried *K. pneumoniae* was revived by adding cells to 10 mL of LB broth, *K. pneumoniae* cells were passaged and incubated at decreasing temperatures from 37C to 30C in 2-degree steps and moved from LB to MH media. Incubations were performed at 30C for 18 hours unless otherwise

specified. Liquid cultures of the four *Bacillus*-based biopesticides and *K. pneumoniae* were separately plated by spreading 200uL of liquid culture evenly onto MH agar plates with either an imipenem or ertapenem Lilofilchem Minimum Inhibitory Concentration test strip. 200 uL of Liquid culture of each Bacillus-based biopesticide was added to 5 mL of MH broth along with 100 uL of *K. pneumoniae* liquid culture and incubated. An additional culture was prepared with 100 uL of all Bacillus-based biopesticides along with 100 ul of *K. pneumoniae*. Each mixed culture was plated on MH agar and streaked for isolation. Colonies were selected by morphology and re-plated. Individual colonies were selected again via colony morphology, Gram stained, and visually verified as Gram positive bacilli. Verified colonies were double checked by re-plating, incubating and Gram staining to ensure only Gram positive cells were present. Verified Gram positive cells were incubated in 5 mL of MH broth. Liquid cultures were Gram stained to check for Gram positive cells and to rule out contamination in controls. 200 uL of liquid cultures were spread on MH agar plates and with MIC test strips of imipenem and ertapenem antibiotics. Minimum inhibitory concentration was determined using the standard Clinical and Laboratory Standards Institute guidelines on replicates and recorded post-incubation. In this work we do not use the term "co-culturing" as no additional material was added to the nutrient broth and no alterations to other conditions were made.

Sequencing

A 10 µL culture loop was used to remove growth from the MIC lawns and DNA was extracted using Qiagen's DNeasy DNA Extraction kit with a modified protocol. (Qiagen NV, Germany) After protocol Step 1, samples were incubated at 90°C for 10 minutes to account for *Bacillus* cell wall structure. Extracted DNA concentration was quantified using a Qubit fluorometer (Thermo Fisher Scientific, MA). Libraries were generated using Illumina's NexTerra Flex kit with IDT set A Dual Indexes. DNA was sequenced on an Illumina iSeq system (Illumina Inc., CA). Library prep for extracted DNA was also performed with the Proximeta Hi-C kit and sent to Phase Genomics for sequencing and quality assurance (Phase Genomics, WA).

Bioinformatics

Sequences were checked for quality using FastQC[43] and trimmed using Trimmomatic.[44] Sequences were assembled using SPAdes[45] and assessed for quality using Quast[46] and annotated with RAST.[47] Final sequence assemblies were queried against CARD. Sequencing of the four biopesticide products were completed in a previous study,[9] where they were cultured and acclimated under the same conditions and sequenced on the same platform. These prior sequences are the "before" and were used to compare to the *K. pneumoniae-Bacillus*-based products post co-culture sequences, referred to as the "after" sequences. Both before and after sequences of the biopesticide products were queried against CARD for ARG and ARG-associated functional annotations were manually verified. The ATCC *K. pneumoniae* genome was obtained from NCBI

and also queried against CARD. Hi-C sequence data was demultiplexed using a custom reference sequence and curated plasmids database[48] and aligned with BWA.[49] Output was manually verified. The “after” sequences were queried against the curated plasmid database and results scoring as 100% matches for query coverage with greater than 97% identity match were analyzed. NCBI Batch Entrez was used to apply taxonomy to the results. Identified plasmid sequences were combined and queried against CARD to determine their ARG content. Reference sequences for “before” biopesticides were obtained from NCBI and IMG[50] and UniProt[51] KEGG[52] orthologies used to verify presence of carbapenem resistance genes and proteins. Additional genes not specific to antibiotic resistance that were transferred were not included in mapping.

Hi-C results of the biopesticides indicate location of antibiotic resistance genes

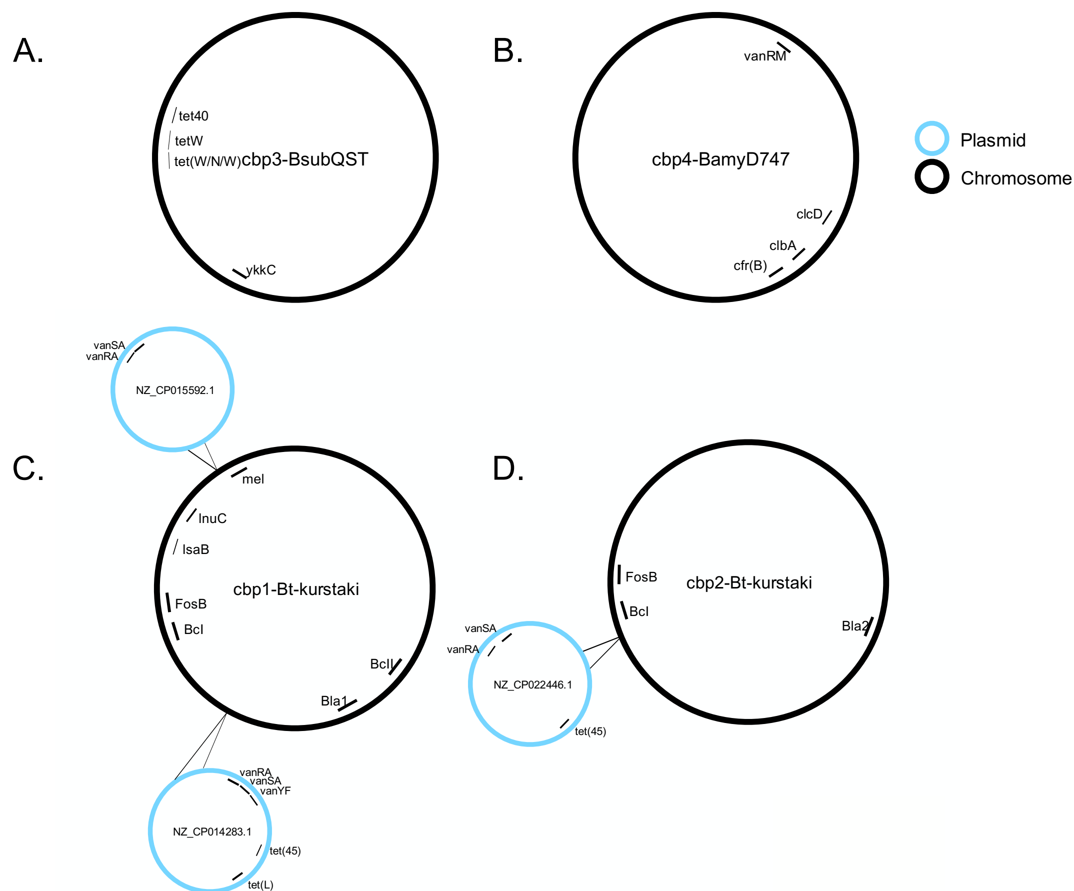


Figure 1. Hi-C results of the biopesticides indicating location of antibiotic resistance genes. Blue indicates a plasmid and black indicates the chromosome. A. Cpb3-BsubQST contained four genes on its chromosome. B. Cpb4-BamyD747 contained four genes. C. Cpb1-Bt-kurstaki contained genes for vancomycin and tetracycline resistance genes on plasmids and additional genes on its chromosome. D. Cpb2-Bt-kurstaki had similar genes for vancomycin and tetracycline resistance genes on a plasmid with additional genes on its chromosome.

Minimum Inhibitory Concentration Results

	Minimum Inhibitory Concentration			
	Before		**After**	
	ETP	IMI	ETP	IMI
B. thuringiensis (cbp1-Bt-kurstaki)	0.064	0.125	2.0	3.0
	0.125	0.125	2.0	3.0
	0.125	0.125	2.0	3.0
B. thuringiensis (cbp2-Bt-kurstaki)	0.094	0.19	32	32
	0.094	0.19	32	32
	0.125	4.00	32	32
B. subtilis QST (cbp3-BsubQST)	0.19	0.064	32	0.19
	0.19	0.064	32	0.19
	0.38	0.064	32	0.19
B. amyliloqufaciens (cbp4-BamyD747)	0.19	0.032	1.5	32
	0.125	0.032	3.0	32
	0.125	0.047	3.0	32

* 0.016 - 32 µg/mL

Table 1. Results of testing two carbapenem antibiotics on biopesticides co-cultured with a carbapenem resistance strain of *K. pneumoniae*.

Representative plates showing resistant phenotypes

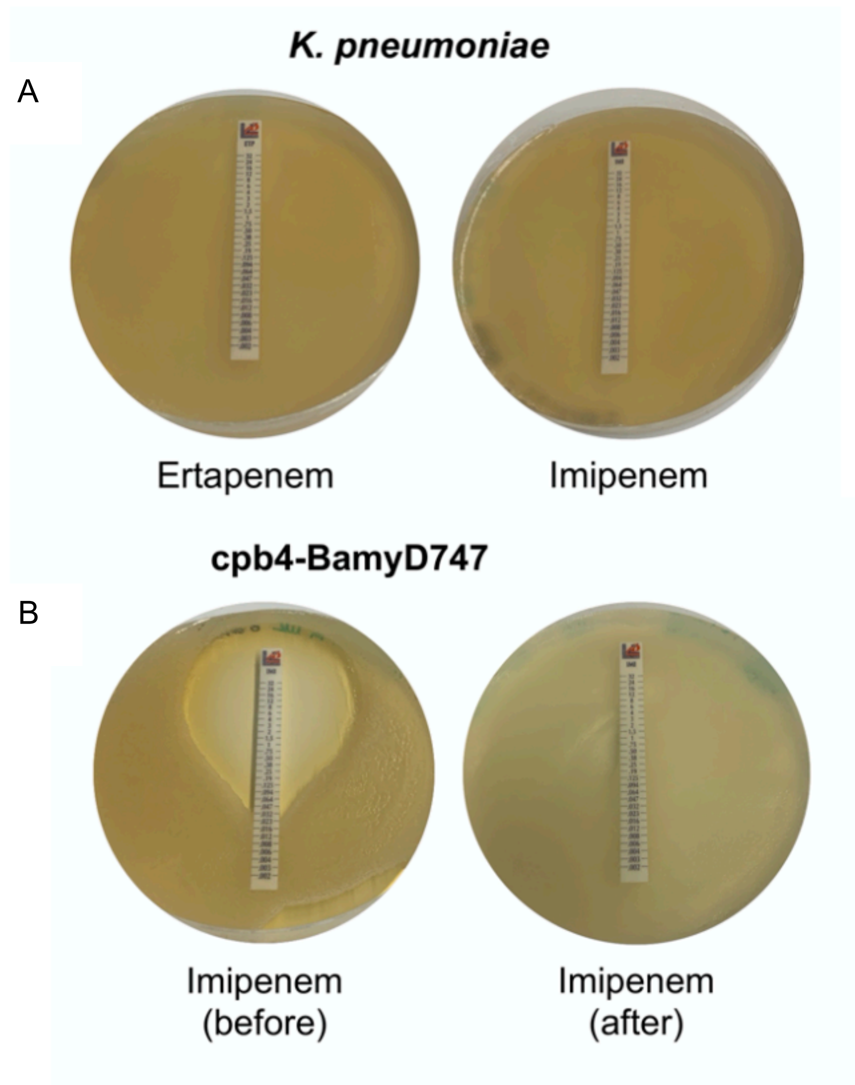
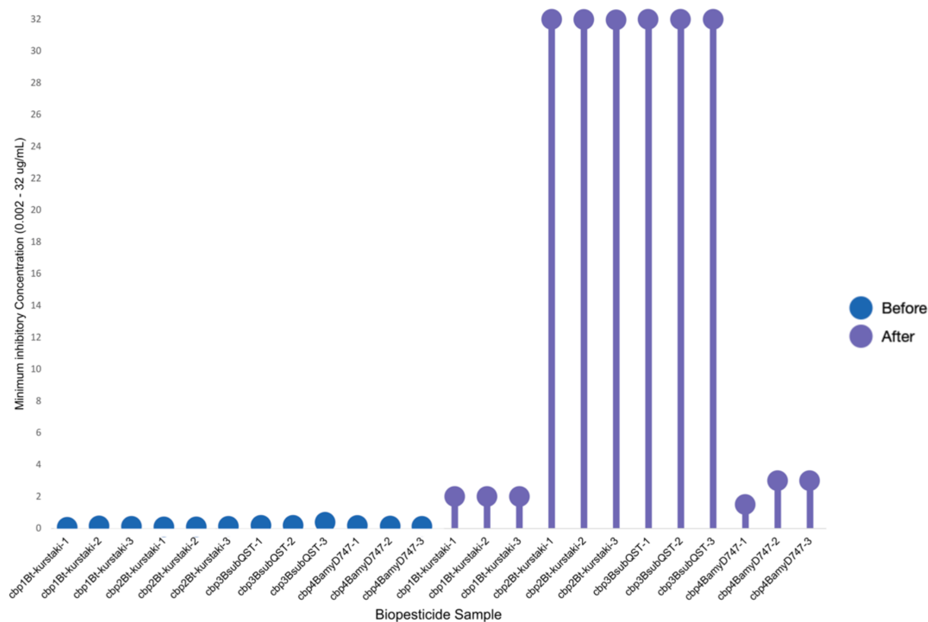


Figure 2 Example replicate plates showing carbapenem resistance. A. Carbapenem resistant *K. pneumoniae* demonstrating complete resistance to ertapenem and imipenem. B. Representative result of one replicate demonstrating susceptibility to imipenem before co- culturing with carbapenem resistant *K. pneumoniae* and demonstrating resistance to imipenem after co-culturing.

Alteration to resistance phenotypes before and after co-culturing

A. Ertapenem MIC of Biopesticides (Before & After Co-culture)



B. Imipenem MIC of Biopesticides (Before & After Co-culture)

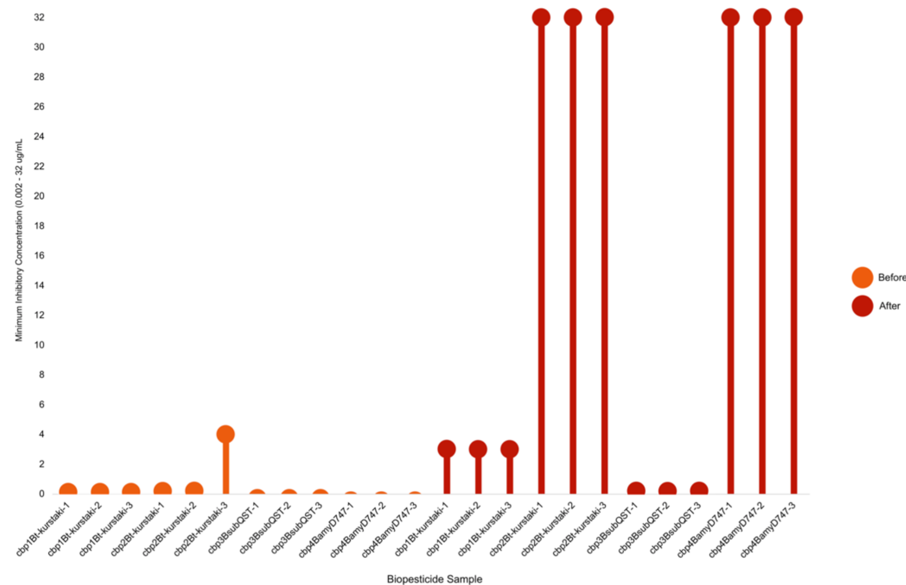


Figure 3. Change in resistance phenotypes before and after co-culturing *Bacillus*-based biopesticide products. A. Two products', cbp1-Bt-Kurstaki and cbp3-BsubQST, replicates show a phenotype change from susceptible to resistant to antibiotic ertapenem when co-cultured with *K. pneumoniae*. B. Two products', cbp2-Bt-Kurstaki and cbp4-BamyD747, replicates show a change from susceptible to resistant to antibiotic imipenem when co-cultured with *K. pneumoniae*.

Antibiotic resistance genes exchanged during co-culture of *K. pneumoniae* and *Bacillus*-based biopesticides

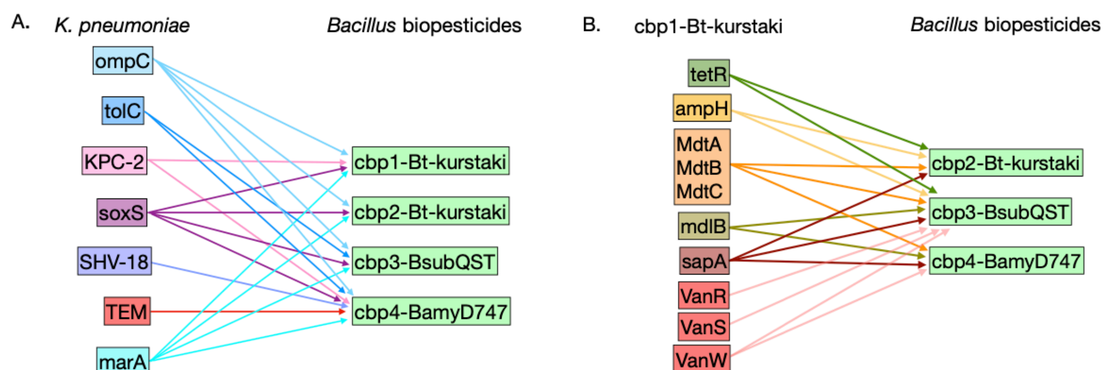


Figure 4. Antibiotic resistance genes exchanged during co-culture of carbapenem resistant *K. pneumoniae* and *Bacillus*-based biopesticide products. A. Carbapenem resistance associated genes transferred from *K. pneumoniae* to the *Bacillus*-based biopesticide products. B. Antibiotic resistance genes transferred from cbp1-Bt-kurstaki to the other *Bacillus*-based biopesticide products.

REFERENCES

1. Stefan Schwarz, 1, Andrea T. Feßler, 1 Igor Loncaric, 2 Congming Wu, 3, Kristina Kadlec, 4 & Yang Wang, 3 and Jianzhong Shen3. Antimicrobial Resistance among Staphylococci of Animal Origin. *Antimicrob. Resist. Bact. from Livest. Companion Anim.* (2018) doi:doi:10.1128/microbiolspec.ARBA-0010-2017.
2. Jechalke, S., Heuer, H., Siemens, J., Amelung, W. & Smalla, K. Fate and effects of veterinary antibiotics in soil. *Trends Microbiol.* 22, 536–545 (2014).
3. Munk, P. *et al.* Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries. *Nat. Microbiol.* 3, 898–908 (2018).
4. Cully, M. Public health: The politics of antibiotics. *Nature* (2014) doi:10.1038/509S16a.
5. FDA. *Antimicrobials Sold or Distributed for Use in Food-Producing Animals.Center for Veterinary Medicine* vol. 2 (2018).
6. Federici, B. A. & Siegel, J. P. Safety of Bacillus thuringiensis and Bt Crops Used in Insect Control. (2008).
7. Raymond, B., Federici, B. A. & Raymond, B. In defence of Bacillus thuringiensis, the safest and most successful microbial insecticide available to humanity — a response to EFSA. 1–8 (2017) doi:10.1093/femsec/fix084.
8. Yul, J., Choi, J. A. E. Y., Li, M. S., Jin, B. R. a E. & Je, Y. H. O. BT as a specific, safe, and effective tool for insect pest control. *J Microbiol. Biotechnol.* 17, 547– 559 (2007).
9. Kaze, M., Brooks, L. E. & Sistrom, M. This GRAS Isn't Greener: Biopesticides as Unintentional Reservoirs of Antibiotic Resistance. 5 (2020).
10. Zander, D. S. & Farver, C. F. *Pulmonary Pathology: A Volume in the Series: Foundations in Diagnostic Pathology*. *Pulmonary Pathology: A Volume in the Series: Foundations in Diagnostic Pathology* (2017).
11. Navon-Venezia, S., Kondratyeva, K. & Carattoli, A. Klebsiella pneumoniae: A major worldwide source and shuttle for antibiotic resistance. *FEMS Microbiol. Rev.* 41, 252–275 (2017).
12. Wyres, K. L. & Holt, K. E. Klebsiella pneumoniae as a key trafficker of drug resistance genes from environmental to clinically important bacteria. *Curr. Opin.Microbiol.* 45, 131–139 (2018).
13. Davis, G. S. *et al.* Intermingled Klebsiella pneumoniae Populations between Retail Meats and Human Urinary Tract Infections. *Clin. Infect. Dis.* 61, 892–899 (2015).
14. Wei, R. *et al.* Occurrence of seventeen veterinary antibiotics and resistant bacterias in manure-fertilized vegetable farm soil in four provinces of China. *Chemosphere* 215, 234–240 (2019).
15. Ibenyassine, K. *et al.* Bacterial pathogens recovered from vegetables irrigated by wastewater in Morocco. in *Journal of Environmental Health* (2007).

16. Munoz, M. A., Ahlström, C., Rauch, B. J. & Zadoks, R. N. Fecal shedding of Klebsiella pneumoniae by dairy cows. *J. Dairy Sci.* 89, 3425–3430 (2006).
17. Munoz, M. A., Welcome, F. L., Schukken, Y. H. & Zadoks, R. N. Molecular epidemiology of two Klebsiella pneumoniae mastitis outbreaks on a dairy farm in New York State. *J. Clin. Microbiol.* 45, 3964–3971 (2007).
18. Zadoks, R. N. *et al.* Sources of Klebsiella and Raoultella species on dairy farms: Be careful where you walk. *J. Dairy Sci.* 94, 1045–1051 (2011).
19. Gomez-Simmonds, Angela, and Anne-Catrin Uhlemann. "Clinical implications of genomic adaptation and evolution of carbapenem-resistant Klebsiella pneumoniae." *The Journal of infectious diseases* 215.suppl_1 (2017): S18-S27.
20. Ay, F. & Noble, W. S. Analysis methods for studying the 3D architecture of the genome. *Genome Biology* (2015). doi:10.1186/s13059-015-0745-7
21. Woo, Sheridan L., and Olimpia Pepe. "Microbial consortia: promising probiotics as plant biostimulants for sustainable agriculture." *Frontiers in plant science* 9 (2018): 1801.
22. Nordmann, P. & Poirel, L. Emerging carbapenemases in Gram-negative aerobes. *Clinical Microbiology and Infection* (2002) doi:10.1046/j.1469-0691.2002.00401.x.
23. Rasheed, J. K. *et al.* Characterization of the extended-spectrum β-lactamase reference strain, Klebsiella pneumoniae K6 (ATCC 700603), which produces the novel enzyme SHV-18. *Antimicrob. Agents Chemother.* (2000) doi:10.1128/AAC.44.9.2382-2388.2000.
24. Stalder, T., Press, M. O., Sullivan, S., Liachko, I. & Top, E. M. Linking the resistome and plasmidome to the microbiome. *ISME J.* 13, 2437–2446 (2019).
25. Bharti, R. & Grimm, D. G. Current challenges and best-practice protocols for microbiome analysis. *Brief. Bioinform.* (2019) doi:10.1093/bib/bbz155.
26. Rasko, D. A. *et al.* The genome sequence of Bacillus cereus ATCC 10987 reveals metabolic adaptations and a large plasmid related to Bacillus anthracis pXO1. *Nucleic Acids Res.* (2004) doi:10.1093/nar/gkh258.
27. Amadio, A. F., Benintende, G. B. & Zandomeni, R. O. Complete sequence of three plasmids from Bacillus thuringiensis INTA-FR7-4 environmental isolate and comparison with related plasmids from the Bacillus cereus group. *Plasmid* (2009) doi:10.1016/j.plasmid.2009.07.005.
28. Rozwandowicz, M. *et al.* Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *J. Antimicrob. Chemother.* 73, 1121–1137 (2018).
29. Kim, S. *et al.* Transfer of antibiotic resistance plasmids in pure and activated sludge cultures in the presence of environmentally representative microcontaminant concentrations. *Sci. Total Environ.* (2014) doi:10.1016/j.scitotenv.2013.08.100.
30. Nicolas-Chanoine, M. H., Mayer, N., Guyot, K., Dumont, E. & Pagès, J. M. Interplay between membrane permeability and enzymatic barrier leads to

antibiotic-dependent resistance in Klebsiella pneumoniae. *Front. Microbiol.* (2018) doi:10.3389/fmicb.2018.01422.

31. Huang, W. *et al.* Emergence and evolution of multidrug-resistant Klebsiella pneumoniae with both blaKPC and blaCTX-M integrated in the chromosome. *Antimicrob. Agents Chemother.* (2017) doi:10.1128/AAC.00076-17.
32. Ma, D., Alberti, M., Lynch, C., Nikaido, H. & Hearst, J. E. The local repressor AcrR plays a modulating role in the regulation of acrAB genes of Escherichia coli by global stress signals. *Mol. Microbiol.* (1996) doi:10.1046/j.13652958.1996.357881.x.
33. Riggs, P. J., Chelius, M. K., Iniguez, A. L., Kaeppler, S. M. & Triplett, E. W. Enhanced maize productivity by inoculation with diazotrophic bacteria. *Aust. J. Plant Physiol.* 28, 829–836 (2001).
34. Motamedi, H., Azizi, A. & Ahmadi, M. Nutritive value of treated Quercus infectoria and Quercus libani leaves with the tannin-degrading bacterium Klebsiella pneumoniae for ruminant feeding in vitro. *J. Appl. Microbiol.* 127, 1339–1348 (2019).
35. Póvoa, H. C. C., Chianca, G. C. & Iorio, N. L. P. P. COVID-19: An Alert to Ventilator-Associated Bacterial Pneumonia. *Infectious Diseases and Therapy* (2020) doi:10.1007/s40121-020-00306-5.
36. Clancy, C. J. & Nguyen, M. H. Coronavirus Disease 2019, Superinfections, and Antimicrobial Development: What Can We Expect? *Clin. Infect. Dis.* (2020) doi:10.1093/cid/ciaa524.
37. Kim, S. H., Wei, C. I., Tzou, Y. M. & Haejung, A. N. Multidrug-resistant Klebsiella pneumoniae isolated from farm environments and retail products in Oklahoma. *J. Food Prot.* 68, 2022–2029 (2005).
38. Garza-Ramos, U. *et al.* Phenotypic and molecular characterization of Klebsiella spp. isolates causing community-acquired infections. *New Microbes New Infect.* 23, 17–27 (2018).
39. Bowring, B. G., Fahy, V. A., Morris, A. & Collins, A. M. An unusual culprit: Klebsiella pneumoniae causing septicaemia outbreaks in neonatal pigs? *Vet. Microbiol.* 203, 267–270 (2017).
40. Lopes, R. *et al.* Draft genome sequence of a CTX-M-15-producing endophytic Klebsiella pneumoniae ST198 isolate from commercial lettuce. *Journal of Global Antimicrobial Resistance* (2017) doi:10.1016/j.jgar.2017.03.005.
41. Boehme, S., Werner, G., Klare, I., Reissbrodt, R. & Witte, W. Occurrence of antibiotic-resistant enterobacteria in agricultural foodstuffs. *Mol. Nutr. Food Res.* 48, 522–531 (2004).
42. Abd-Elall, A. M. M., Mohamed, M. E. M. & Awadallah, M. A. I. Potential airborne microbial hazards for workers on dairy and beef cattle farms in Egypt. *Vet. Ital.* 45, 275–27585 (2009).
43. Andrews, S. FastQC. *Babraham Bioinforma.* (2010) doi:citeulike-articleid:11583827.

44. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* (2014) doi:10.1093/bioinformatics/btu170.
45. Bankevich, A. *et al.* SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* (2012) doi:10.1089/cmb.2012.0021.
46. Gurevich, A., Saveliev, V., Vyahhi, N. & Tesler, G. QUAST: Quality assessment tool for genome assemblies. *Bioinformatics* 29, 1072–1075 (2013).
47. Aziz, R. K. *et al.* The RAST Server: Rapid annotations using subsystems technology. *BMC Genomics* (2008) doi:10.1186/1471-2164-9-75.
48. Brooks, L., Kaze, M. & Sistrom, M. A Curated, Comprehensive Database of Plasmid Sequences. *Microbiol. Resour. Announc.* 85, 1–11 (2018).
49. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* (2009) doi:10.1093/bioinformatics/btp324.
50. Huntemann, M. *et al.* The Standard Operating Procedure of the DOE-JGI Microbial Genome Annotation Pipeline (MGAP v . 4). *Stand. Genomic Sci.* 10, 1–6 (2015).
51. Bateman, A. *et al.* UniProt: The universal protein knowledgebase. *Nucleic Acids Res.* (2017) doi:10.1093/nar/gkw1099.
52. Kanehisa, M. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* (2000) doi:10.1093/nar/28.1.27.

The Phage, Prophage, AMG and IME Content of Biopesticides

Introduction

Biopesticides are any bacterial, fungal, or viral product used in agricultural practices such as pest and pathogen control, and soil enhancements. Use of these microorganismal products has been steadily increasing in the last 70 years[1]. Biopesticides have a global market value of close to $3 billion and make up 5% of the crop enhancement market.[2] Over 200 commercial biopesticide products are on the market in the United States alone.[3] Bacterial products used to control insects can be applied aerially and are applied to a large variety of crops.[4] One of the most successful bacterial biopesticide is *Bacillus thuringiensis*, an aerobic, soil-dwelling bacteria, identified by the presence of its characteristic plasmids containing *cry* and *cyt* genes.[5,6] *B. thuringiensis'* unique insecticidal properties are due to these important toxin encoding genes.[7] Biopesticides are easy, inexpensive, and efficient to produce and are used globally. These commercial products are not considered pathogens and are safe for consumption.[8,9] Biopesticide products are an excellent substitute for artificially generated chemical compounds.[10] Synthetic pesticides have been well characterized as carcinogenic and tumorigenic. Traditional chemical pesticides are harmful to wildlife and can persist in the environment.[11] Bacterial are classified as "Generally recognized as safe" (GRAS) by multiple organizations in the United States such as the FDA, EPA, and USDA.[12,13] These bacterial products are considered safe for many reasons.

Chromobacterium subtsugae strain PRAA4,[14] an insect, fly, and mite repellant and *Bacillus subtilis* strain GB03,[15] an antifungal. do not produce harmful toxins. *Pseudomonas syringae* strain ESC-10 cannot grow at human body temperature and poses no risk to human health.[16] Use of *Pantoea agglomerans*, which can outcompete many plant pathogens, leads to decreased needs for bacteriocidic and fungicidal applications.[17,18] In California alone, nearly 9 million pounds live bacteria were distributed as biopesticide in 2018.[4] Despite the success of these bacterial products, recent research has demonstrated that multiple biopesticides are currently reservoirs and vectors of antibiotic resistance genes.[19,20]

Genetic exchange between bacteria in the environment is been a critical evolutionary process, however, there remain systems that have not been investigated comprehensively.[21] Bacteria can transfer genes horizontally and this transmission is the main method of movement and dispersal of antibiotic resistance, pathogenicity, and virulence factors.[22] Plasmids and bacteriophages, extrachromosomal mobile genetic elements, contribute to the movement of genetic material between individuals and communities.[23] Genome plasticity confers essential and rapid adaptation to changing conditions.[24] While plasmid exchange and their broad host ranges has been characterized,[25] less is known about ancillary mechanisms of genetic movement such integrative and mobilizable elements (IME). These elements contain genes that encode self-excision and integration systems and enable transfer of IME and conjugative

mechanisms.[26] We are also now beginning to understand the way viruses influence and control bacterial communities and their functions.[27–29] One method of control is through bacteriophage auxiliary metabolic genes (AMG). Auxiliary metabolic genes are bacterial in origin and have been identified in many phages.[30] AMG contribute to the pirating of host metabolism in order to ensure functions related to infection and genetic replication are controlled by the phage. It is important to look at these additional processes in order to have a comprehensive view of genetic movement in the environment.

The addition of biopesticides to the environment creates increased opportunities for horizontal genetic transfer and raises the possibility of movement and generation of novel pathogenicity in agricultural environments. Extensive research into bacterial products as pest control and soil enhancements has provided insight into their functional genetic composition, however, the viral content of these biopesticides has yet to be investigated. There is an urgent need to understand the role biopesticides play in the transmission of antibiotic resistance genes and generation of novel pathogenicity as biopesticides come in contact with pathogens in the environment. This study uses multiple bioinformatics approaches and whole genome sequencing to investigate the phage and prophage content of widely used biopesticides. Using a protein homology and machine learning combinatory tool we quantify the number of phage genomes present in mined biopesticide genomes, identify phage and prophage proteins, IMEs, and AMGs in commercial biopesticide products. Understanding genetic movement of widely dispersed, live bacterial product

Results

Phage and Prophage Content of Biopesticides

Multiple phage genomes were identified within mined biopesticide strains that belonged to different phyla. (Fig 1). A *Pseudomonas* phage, originally isolated from a Gram negative *P. syringae* strain from phyla Proteobacteria, was identified in three biopesticide genomes. It was identified with strict cutoff values when queried against the NCBI viral genomes database and was located within *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Paenibacillus popillae* genomes. A *Moraxella* phage genome, isolated from host a Gram negative *M. catarrhails* from phyla Proteobacteria, was identified in a *C. subtsugae* (phyla Proteobacteria) and *B. subtilis* (phyla Firmicutes) genome. Mined strains *of B. thuringiensis* serovar aizawai contained *Bacillus* phage genomes that were originally isolated from four different hosts: *Bacillus anthracis, Bacillus cereus, B. thuringiensis*, and *Staphylococcus pasteuri*. The majority of the phages identified in the Bt-aizawai strains were from pathogens *B. anthracis* and *B. cereus*. Bt-aizawai strains were also hosting an *S. pasteuri* phage SpaA1.

Plasmid sequences from mined biopesticide strains contained viral genomes and genes encoding phage associated proteins. (Fig 2) Queried against the ViruSite gene database, mined *B. thuringiensis* plasmids contained phage associated functional genes from five host genera and two phyla: genera *Bacillus, Enterobacteria, Klebsiella, Lactococcus, Staphylococcus* and Firmicutes

and Proteobacteria phyla. The majority of phage proteins were tape measure related proteins with 76% from *Bacillus* and 24% from *Staphylococcus.* Eight tail assembly proteins identified, half from *Enterobacteria* and half from *Klebsiella* phages. In *P. agglomerans* strains multiple *Escherichia* prophage associated functional proteins were identified by PHASTER. They were evenly divided with 12-13% of identified proteins associated with membrane penetrance, metabolic control, protein enzymes, recombination and regulation. The largest group when combined together (26%) are the protease and peptidase protein degrading enzymes. (Fig 2B) Sixty-four viral genomes were found in seven *B. thuringiensis* plasmids, with some plasmid sequences containing phage genomes from multiple viral genera identified by the ViruSite curated genome database. *B. thuringiensis* serovar israelensis HD789 plasmids contained 7 phage genomes: 5 from *B. thuringiensis* hosts, one *Enterobacteria* phage isolated from *Escherichia coli*, and one filamentous Ralstonia phage isolated from *Ralstonia solanacearum*. Escherichia hosted phages were 58% (37) of the phages found in the mined *B. thuringiensis* plasmids and *Salmonella* phages were 28% (18). Two *B. thuringiensis* plasmids contained genomes from two *Spiroplasma* phages. *B. thuringiensis* strain CTC plasmid contained five different phage genomes from *Enterobacteria, Escherichia, Salmonella* and *Spiroplasma*.

Functional annotation using VIBRANT

Commercial biopesticide products, purchased, cultured, and sequenced, along with publicly available biopesticide sequences were analyzed for phage presence and viral functional annotation using VIBRANT. (Fig 4) Commercially biopesticide products' (cbp) whole genomes were analyzed for phage content. Cbp1-Bt-kurstaki contained sequences for 52 total phages while it is an identical strain but different product cpb2-Btkurstaki contained 24 total phages. VIBRANT indicated that cbp-Bt-kurstaki had 15,452 total sequences with 752 "correct size" sequences and cp2-Bt-kurstaki had nearly 10% of cbp-Bt-kurstaki's total sequences with 382 "correct size" sequences. Commercial *B. subtilis* product cbp3-BsubQST contained 14 phages from 358 total sequences. Commercial *B. amyloliquefaciens* product contained 7 total phages from a total of 128 sequences. Two hundred twenty publicly available mined *B. thuringiensis* genomes contained 609 phages. Additional biopesticide strains (see supplemental Table 1) were combined together and contained 1,471 total sequences with 705 determined as correct and contained 81 phages. The sequences of widely used biopesticides were annotated individually by VIBRANT and annotated using phage and prophage protein orthology databases: Viral Orthologous Groups (VOG), Protein Families (Pfam), and the Kyoto Encyclopedia of Genes and Genome (KO). (Fig 4) Proteins were quantified into twenty-three phage and prophage associated categories: Antirepressor, Baseplate, Capsid, Endopeptidase, Expression factor, Head, Holin, Hypothetical protein, Integrase, Mu protein, PBSX, Recombinase, Regulatory protein, Ribonucleoside-diphosphate, Serine protease, SPBc2, Spike, Tail, Tape measure protein, Terminase, and Uncharacterized. Biopesticide strains from *B.*

amyloliquefaciens, Lysinbacillus sphaericus, A. radiobacter, P. fluorescens, C. subtsugae, and *B. subtilis* were run through VIBRANT. Functional protein content was quantified and separated by orthology database (Supplemental Table 2). *B. amyloliquefaciens* contained 22 viral proteins. Fifty percent of the proteins identified by Pfam were phage tail associated such as XkdN-like tail assembly chaperone proteins (PF08890.11), tail tube proteins (PF09393.10), and tail sheath protein subtilisin-like domains (PF04984.14). The main proteins identified by VOG were probable portal (VOG00236) and hypothetical proteins. KO identified two terminase large subunit proteins (K06909). *L. sphaericus* had 19 total proteins identified with 67% integrase (PF00589.22) and 24% tail associated proteins (PF09684.10) making up the main results from Pfam. VOG protein results comprised mainly, 31%, of very late expression factor (VOG00041).

Nine KO results contained orthologous proteins for terminases (K21512), integrases (K14059), and recombinases (K04763). *A. radiobacter* had 8 Pfam results: capsid, headtail joining, integrase, portal, tail tube and terminase protein families. VOG results were capsid, expression factor, head, portal and terminase proteins. KO results comprised only integrases (K14059), and recombinases (K04763). *P. fluorescens* strains contained the largest amount of phage and prophage associated proteins with 41 Pfam entries, with 20 tail associated proteins. VOG results were also mainly tail associated with 31% of the total results. KO results mainly uncharacterized proteins. C. substugae had tail and baseplate proteins identified by both Pfam and VOG as the most abundant in this strain. The *B. subtilis* strain had 22 Pfam results that were also mainly tail associated protein families. The VOG results were similar but had seven (31%) uncharacterized orthologous groups. There was only one KO group identified, terminases.

<u>Auxiliary metabolic genes and integrative and mobilizable elements</u>

Auxiliary metabolic gene categories were identified by VIBRANT in sequenced commercial biopesticide. (Fig 5) Cbp1-Bt-kurstaki contained one AMG category amino acids. Cbp2-Bt-kurstaki contained two AMGs from metabolic categories: aromatic compounds and nucleotides. Cbp3-BsubQST contained two AMGS from the cofactors and vitamins metabolic group. Cbp4 had one AMG from five different AMG metabolic categories: lipids, nucleotides, cofactors and vitamins, secondary metabolites, and aromatic compounds. AMG categories were identified by VIBRANT in mined *B. thuringiensis* and additional biopesticide genomes. (Fig 6) *B. thuringiensis* strains had AMGs from all of the metabolic categories except terpenoids ad polyketides. Lipids and cofactors and vitamins were the most abundant AMGs and together comprise 47% (36 AMGs each) of the metabolic categories. The largest group with 14 (30%) AMGs is the amino acids metabolic category followed by 11 (23%) cofactors and vitamins.

Integrative and mobilizable elements were identified in the commercial biopesticides by the ICEberg database, which includes predicted and experimental IMEs. (Fig 7) One hundred and fifty IMEs total originating from four species were found. The four species *Morganella morganii, Proteus mirabilis,*

Salmonella enterica, and Vibrio cholerae are all Gram negative pathogens. The *M. morganii* IME was a genomic island. *P. mirabilis* made up 51% of the total IMEs and of these 77, 90% were GTPase associated genes tdhF and tdhE. S. enterica genomic islands were 46% of the total IMEs (69). This *B. thuringiensis* genome contained a single *V. cholerae* genomic island and 8 *P. mirabilis* multidrug-resistant genomic island sequence IMEs. Cbp2-Bt-kurstaki contained 74 total IMEs, 40 *P. mirabilis* associated genomic islands, 25 GTPase genes and 5 multi-drug resistant genomic islands and 5 *S. enterica* genomic islands. Cbp3-BsubQST had 48 total IMEs: 18 *P. mirabilis* associated genomic islands, 21 GTPase IMEs, 5 multidrug-resistant genomic island sequences, and 5 *S. enterica* genomic islands. Cbp4-BamyD747 had 35 total IMEs: 12 *P. mirabilis* associated genomic islands, 17 GTPase IMEs, 5 multi-drug resistant genomic island sequences, and one *S. enterica* genomic island. Alignment of the multidrug antibiotic resistant genomic island sequences identified an Lnu gene cassette cbp1-Bt-kurstaki. Manual analysis of the sequences of the multidrug-resistant genomic island IMEs of the two *B. thuringiensis* products found a three gene group, FosBBla1-Bla2, was found in identical locations. (Fig 8) Forty-four complete *B. thuringiensis* genomes were aligned and these three antibiotic resistance genes occurred together in the same order in all aligned genomes indicating the presence of a gene cassette.

Discussion

In this study we determine the presence and identity of phages, prophages, AMGs and IMEs in widely distributed bacteria based biopesticides and measure functional gene content capable of transfer to and integration from other species. The application of large amounts of live bacterial species containing mobile genetic elements and previously unknown gene cassettes raises the possibility of introduction of novel pathogenicity, virulence or antibiotic resistance to environmental species or soil-dwelling pathogens. Studying the mobile genetic elements present in biopesticide strains may provide insights into clinically important antibiotic resistance gene movement and viral movement in the environment. This work demonstrates that biopesticides may be providing transportation for bacteriophages and begins the foundational work of determining the role of AMGs, IMEs and gene cassettes in genetic movement in biopesticides. The role of viral control of bacterial activities and community structure is evident in these species which account the millions of pounds of live bacteria added to the environment.

Biopesticides as antibiotic resistance gene reservoirs has been previously demonstrated[19] and this work shows viral genes and genomes belonging to different phyla are also present in biopesticide genomes. Biopesticide products have been demonstrated to behave as vectors of ARG and complete genetic exchange with pathogens across phyla.[20] Our results show the presence of Gram negative phages present in Gram positive bacterial genomes and species that are not closely related confirming finding that that many bacteriophages have broad host ranges are not limited to specific genera and genetic exchange can

occur between Gram negative and Gram positive species.[25,31] *P. syringae* is used as frost prevention and its bacteriophage's infection of other biopesticide strains is counter to the narrow host range of *P. syringae* phages defined by Frampton et. al.[32] *Moraxella* is not characterized as a human pathogen and viral exchange may have occurred during contact in the environment. Two of the phage hosts of *B. thuringiensis* aizawai strains are members of the Bacillus-ACT group. *B. thuringiensis, B. anthracis,* and *B. cereus* share a common genome and only differ from each other based on their extrachromosomal toxin genes.[33] The other phage genome is from *Staphylococcus* phage Spa1. This phage genome has been found in *B. thuringiensis* phages and *B. cereus* phages and can infect both species.[34]

Viral genes and genomes identified on plasmids point to opportunity for expanding host range. Bacteriophage genes encoding functional proteins from other phyla is more evidence of broader host ranges and is curious as CRISPR/Cas systems also target and degrade plasmids bearing recognized viral sequences.[35] The presence of cross-phyla phage genes appearing in *B. thuringiensis* plasmids, such as the Enterobacteria tape measure chaperone and tail assembly genes from Enterobacteria and Klebsiella makes sense as the tail tape measure proteins control attachment and insertion during infection. *P. agglomerans* prophage content are associated with attachment, cell entry and host metabolic control. A *Serratia* phage was previously found to have infected a strain of *P. agglomerans* [36] and the presence of an *Escherichia* prophage in a *Pantoea* strain may be due to their phylogenetic relatedness. A potential explanation for the presence of viral genomes from different species of phage is infectivity occurring after phage-phage interactions which can trigger genetic movement and exchange of genes encoding attachment to various membrane receptors.[37] The presence of *Salmonella* and *Escherichia* associated phage genomes in in *B. thuringiensis* plasmids is concerning. However, the presence of two *Spiroplasma* phages could be a helpful inclusion. *Spiroplasma* phages infect *Spiroplasma citri*, a bacteria that causes Citrus Stubborn Disease.[38] The presence of this phage in a biopesticide could aid in preventing citrus disease as it causes the death of pests that consume *B. thuringiensis* and are the normal hosts of *Spiroplasma citri*.

Output of relative abundances for phages counts by VIBRANT appeared inconsistent between the commercial biopesticide products. The relative abundance is based on the identification of total sequences identified in the analyzed genome, however, the designation of "correct size" is not necessarily an accurate assessment. The two commercial *B. thuringiensis* kurstaki products were nearly identical when annotated with PROKKA and RAST, however, VIBRANT indicated cp2-Bt-kurstaki had 10% of cbp1-Bt-kurstaki's total "correct size" sequences. Despite similar sequence content VIBRANT indicated that 28% of the sequences for cp2-Bt-kurstaki were "correct" and cbp1-Bt-kurstaki, with 10 times fewer, sequences only had 5% "correct." There were also some discrepancies between the non-Bt mined biopesticide strains that had only half of its NCBI verified sequences determined as "correct". The 31 mined strains

contained 81 phages which seems low when compared to the 52 phages identified in the single genome of cbp1-Bt-kurstaki. There are limitations to what VIBRANT can return in terms of HMM profiles as the three databases were reduced in size to increase processing efficiency, which likely explains the differences between the results provided by Pfam and KO. The phage results also differed from the results from the NCBI virus database.

This work is the first look at AMGs and IMEs in biopesticides and shows a diverse group of metabolic categories and mobilizable elements. Integrating host metabolic genes is an important evolutionary mechanism for phages. AMGs are expressed in order to redirect host metabolism and resources to the virus in order to support viral reproduction and infection.[39] Cyanophages bearing host derived AMGs for photosynthesis have been characterized in marine environments.[30] Two products cbp3 and cb4 contained AMGs related to cofactor and vitamin metabolism. Cofactors and vitamins play an important role in stimulation of viral replication by its host. They are often produced by the bacteria themselves and then must be redirected for virion construction.[40] The mined biopesticide strains offer a more comprehensive view of the metabolic requirements of infecting phage. The main AMGs in mined B. thuringiensis genomes are lipids and cofactors and vitamins. Carbohydrates, aromatic compounds and lipids are membrane components and many viruses use these host cell materials for their own construction.[41] *B. subtilis* phage has been recently been suggested as rerouting host lipids in order to enable entry through membrane of nearby cells.[42] Glycans are an essential mechanism for interactions between viruses and their hosts and are the main component of the outer surface of viruses.[43] Sulfur relay AMGs are required by viruses hijacking protein synthesis, tRNA molecules are made of nucleosides with sulfur compounds and are a necessity in viral reproduction.[44] Iron-sulfur clusters are required for many metabolism processes such as respiration.[45] Cofactors, like iron, are highly regulated in bacterial cells as sulfide and iron compounds loose in a cell can lead to cell death.[46] Viruses have to pirate these systems immediately in order to proceed with host control. Maintaining viral sulfur relay systems is an excellent evolutionary strategy when compared to the role that sulfur application plays in agriculture. Application of sulfur dioxide directly to crops can act as a soil additive, fungicide, and pesticide.[47,48] This use could be assisting phages, such as the *Enterobacteria*, *Salmonella* and *Escherichia* phages found in the biopesticides, which require sulfur for protein synthesis. It is not clear why only single categories of AMGs were results for three of the biopesticides and multiple categories for the *B. amyloliquefaciens* product's genome. It is possible this product had a higher concentration of phage; however, VIBRANT results indicated fewer phages were present. Yet, a likely explanation is related to the phage and prophage proteins. VIBRANT reported a larger number and more diverse set of viral proteins and possibly accounts for this disparity between commercial biopesticide products. There was a larger set of AMG found in the mined strains as there were 3-10 times as many genomes analyzed. As AMG research expands beyond studies of

marine bacteria,[30,49,50,51,52] there are many exciting discoveries to made about the presence and role AMGs play in environmental and industrial bacteria.

Integrative and mobilizable elements are an important mechanism of evolution and genetic movement and commercial biopesticide genomes bear a large number. For the four products GTPase associated genes, thdF, thdE and tmrE, were 42% of identified IMEs. GTPases function as molecular switches and tmrE is associated with tRNA modifications of GTPase,[21] however the function of these genes is not entirely understood. It's possible these switches are involved in metabolic activities or trigger excision from or integration into chromosomes. Genomic islands made up 51% across all the commercial biopesticide genomes. Genomic islands, like pathogenicity islands, can carry antibiotic resistance genes, virulence factors, metabolic capabilities and move around via HGT. These islands enhance genome plasticity and advance adaptive evolution. The genomic islands of the commercial strains, despite being identified as originating in *M. morganii*, *V. cholerae*, *P. mirabilis*, appear to be pathogenicity islands originating from *Salmonella*.[53] Investigation into the sequences show all the genomic islands are SGI1 a multidrug resistance IME that harbors a wide range of antimicrobial resistance gene clusters.[53] A *Proteus* genomic island was identified in 2018 by Bie et. all. And confirmed as a variant of the SGI1.[54] IMEs contribution to genome plasticity is seen by the way SGI1 can integrate multiple copies of itself into a chromosome.[55] *B. thuringiensis'* unique ability to bear several plasmids without a fitness cost makes it an ideal target to host numerous IMEs. A previous study[20] showed the presence of LnuC genes on the chromosome of cbp1-Bt-kurstaki. LnuC is a member of the lincosamide resistance group Lnu.[56] An Lnu gene cassette appears towards the end of the multidrug resistant islands found in cbp1-Bt-kurstaki. The identification of this gene cassette lead to manual examination for additional gene cassette patterns. The antibiotic resistance trio FosB-Bla1-Bla2 is a potential gene cassette and genes inserted within this cassette could be transferred to other species. Experimental analysis of this process would confirm this hypothesis and potentially explain the presence of this trio in *B. thuringiensis* genomes and its role as a reservoir of antibiotic resistance genes. While conjugation is still considered the main mechanism of HGT and the source of bacterial genomic plasticity, plasmid contributions to genetic movement is well characterized, as more IMEs are documented the part they play as conduits for genetic movement and bacterial adaptation will be better understood.

As we continue to understand viral control over bacterial activities and community structure we can connect contributions biopesticide viral content make to bacterial processes in the environments they are added to. This study characterizes the presence of mechanisms of genetic movement contained within biopesticides. Transfer of pathogen associated bacteriophages and IMEs likely play a larger role than previously known. Large quantities of live bacteria to the environment allows for opportunities for genetic movement via multiple mechanisms. Bacteria used for insect, fungal, and frost damage control confers many benefits and these "organic" products are considered much healthier and safer for humans and the environment. However, these products also bring

antibiotic resistance genes, bacteriophages with large host ranges, prophages, AMGs and IMEs that are primed to participate in genetic exchange with bacteria physically interact with. As the global health crisis of antibiotic resistant bacteria continues contamination of food production streams steadily increases, it is essential to identify solutions to prevent the transfer genetic material that can lead to the generation of novel pathogens. Biopesticides are not considered as a facet of agricultural practices contributing to this generation, however, it is becoming increasingly clear that they require further assessment as safe for use.

Methods

Four commercial *Bacillus*-based biopesticide products (cpb), Bt-kurstaki strain *B. subtilis* strain QST 713 and *B. amyloliquefaciens* strain D747, were purchased and coded by number and strain: cbp1-Bt -kurstaki, cbp2-Bt-kurstaki, cbp3-BsubQST, and cbp4BamyD747. The two Bt products were the same strain, but from different companies and contained different suspension materials. DNA from biopesticide products was extracted directly from products using Qiagen's DNeasy DNA Extraction kit (Qiagen NV, Germany) using a modified protocol. After performing the protocol's first step, samples were incubated at 90oC for 10-15 minutes in order to account for Gram positive cell wall structure. DNA concentration was quantified with a Qubit fluorometer (Thermo Fisher Scientific, MA). Libraries were generated using Illumina NextTera Flex with IDT set A Dual Indexes. DNA was sequenced on an Illumina MiSeq (Illumina Inc, CA). Sequences were checked for quality using FastQC v0.11.8and trimmed using Trimmomatic v0.36. Genomes were assembled using SPAdes v3.11.1.2 and assessed for quality using Quast v5.0.0. I compiled a list of all bacterial biopesticide species approved for use in the United States manually from databases published by the California Department of Pesticide Registration[57] and United States Environmental Protection Agency databases. [58] The mined publicly available whole genomes matching strain information (Bt = 220, non-Bt strains n=31) and the four purchased biopesticide product sequences were queried against multiple databases using Blast tools 2.10.0[59] using a 97% cut off for query coverage and 90% percent for percent identity match. Other settings were left as default. A databased of all viral genomes was generated from NCBI in November 2019. PHASTER[60] database downloaded November 2019 (release Aug 14, 2019) was used to identify phages. ViruSite[61] gene and genome databases (Release 2019.1) were downloaded February 1, 2020. ICEBerg[62] v 2.0 IME database was downloaded January 2020. A curated plasmids database[63] was used to identify plasmids from biopesticide strains and also queried against these databases. All sequences were analyzed by VIBRANT[64] in the discovery environment of CyVerse.[65] Data visualizations were performed in BioRender[66], and alignments of sequences were done with BWA[67] and clustalW[68] and visualized in R studio[69] version 3.6.1 with packages ggmsa and ggplot.[70]

Phage genomic content identified in biopesticide strains

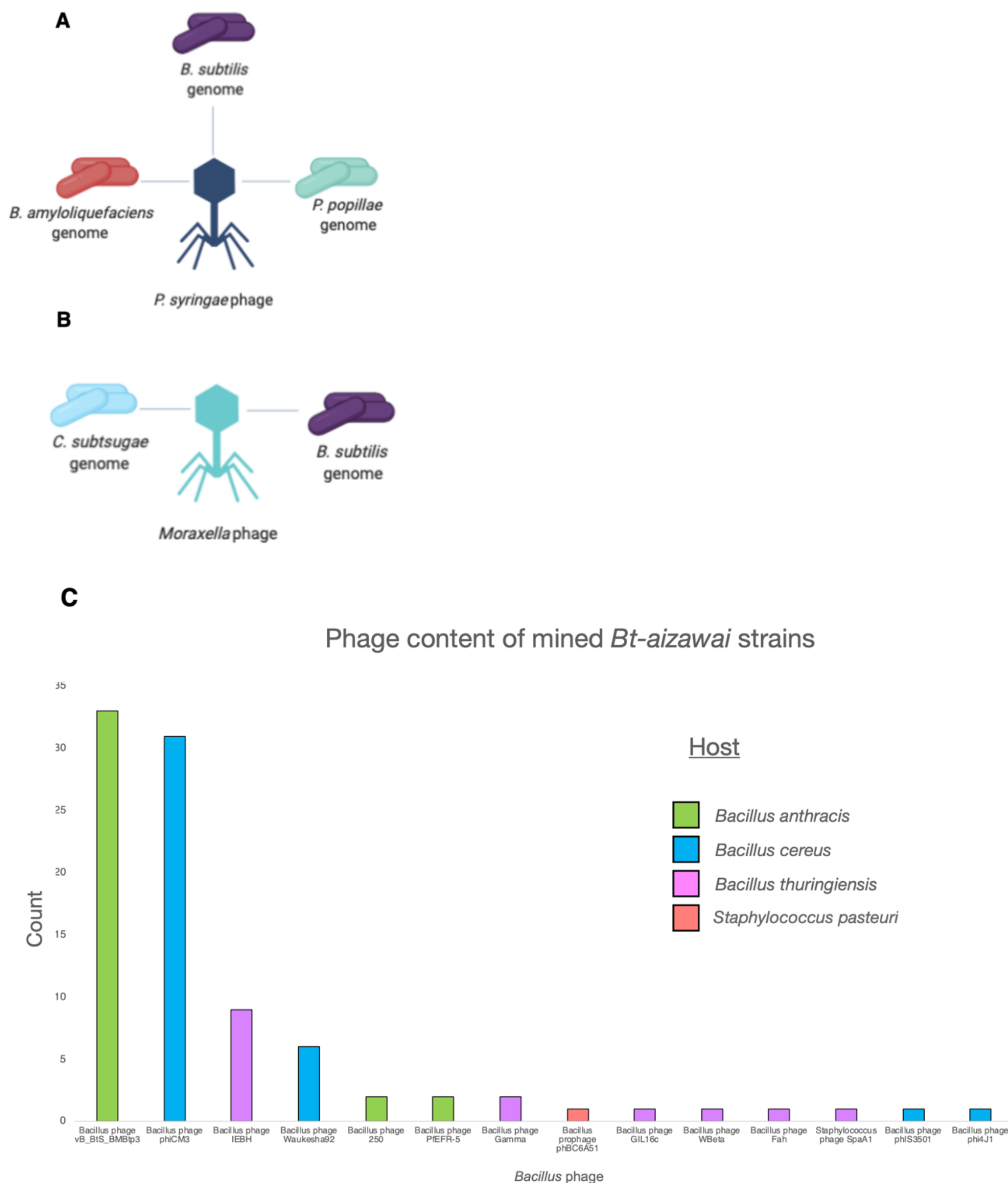


Figure 1. Phage genomes identified in biopesticides. A. *Pseudomonas* phage PN05, isolated from host *Pseudomonas syringae*, was isolated in *B. amyloliquefaciens, B. subtilis,* and *P. popillae* genomes. B. A *Moraxella* phage Mcat16, isolated from host *Moraxella catarrhalis* was found in *C. subtsugae* and *B. subtilis* genomes. C. Phage content of mined *B. thuringiensis* strain aizawai by host species.

Bacteriophage genomes and genes identified in plasmids

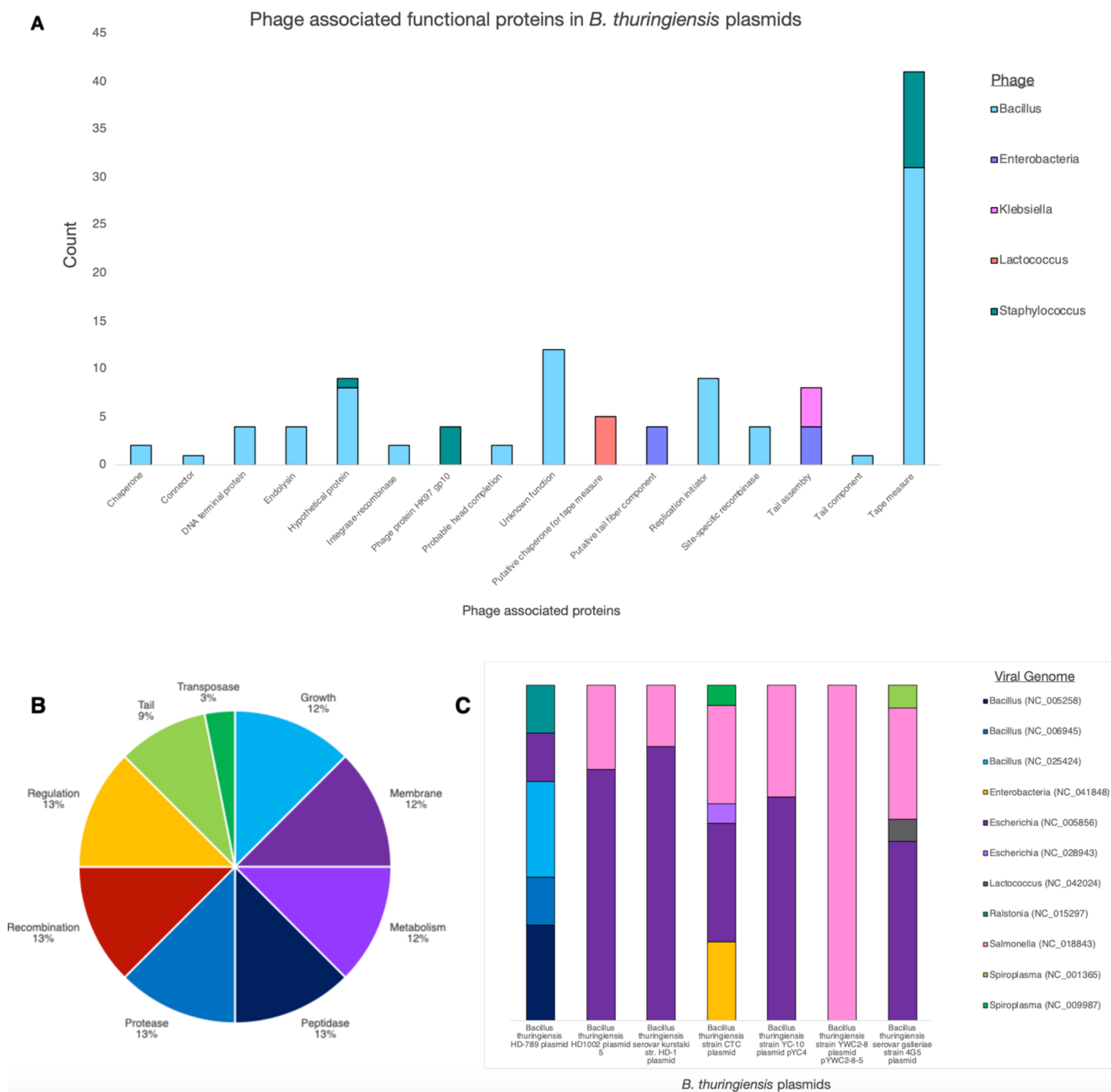


Figure 2. Plasmid sequences from mined biopesticide strains contained viral genomes and genes encoding phage associated proteins. A. Multiple phage associated proteins were found in *B. thuringiensis* plasmids with five different phage genera. B. Breakdown of *Escherichia* prophage associated functional proteins found in *P. agglomerans* strains. C. Relative abundances of viral genomes identified in *B. thuringiensis* plasmids.

VIBRANT phage analysis of mined and commercial biopesticide genomes

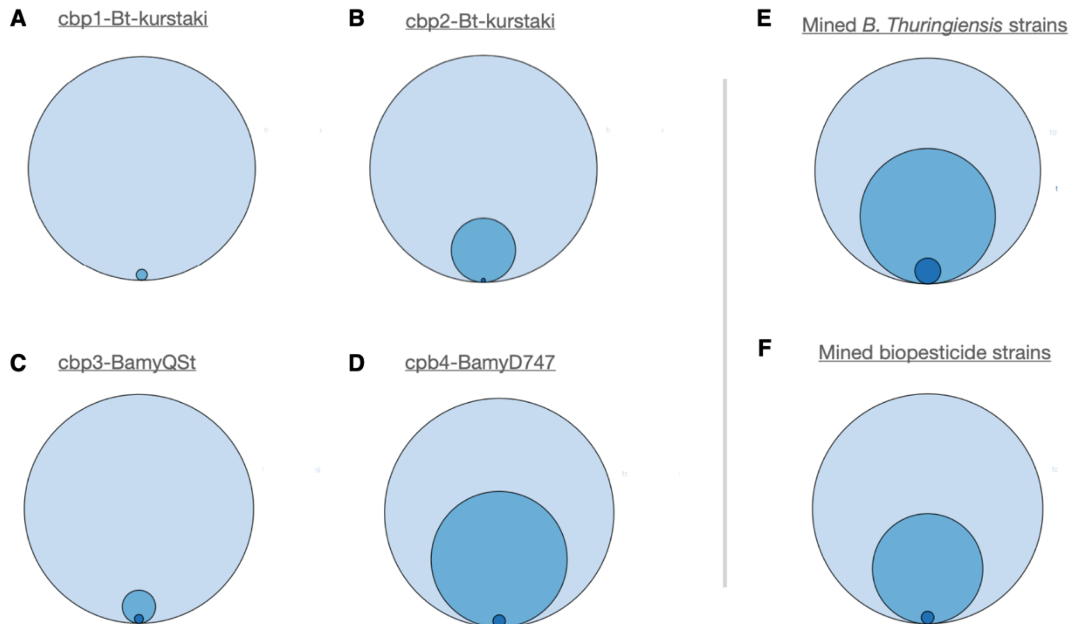


Figure 3. Commercial biopesticide products and mined biopesticide analyzed by VIBRANT for sequence count, quality, and phage genome as relative abundances. A. Commercial Bt-kurstaki 1 product contained 52 total phages from 15,452 sequences. B. Commercial Bt-kurstaki 2 product contained 24 total phages from 1,350 sequences. C. Commercial *B. subtilis* product contained 14 phages form 358 sequences. D. Commercial *B. amyloliquefaciens* product contained 7 total phages from 128 sequences. E. Two hundred twenty publicly available *B. thuringiensis* genomes contained 609 phages. F. Additional biopesticide strains contained 81 phages total from 1,471 sequences. Light blue indicates the total number of sequences, medium blue indicated the number of sequences determined as correct, dark blue indicates the relative abundance of phage genomes.

Comparison of orthology databases for phage and prophage associated genes

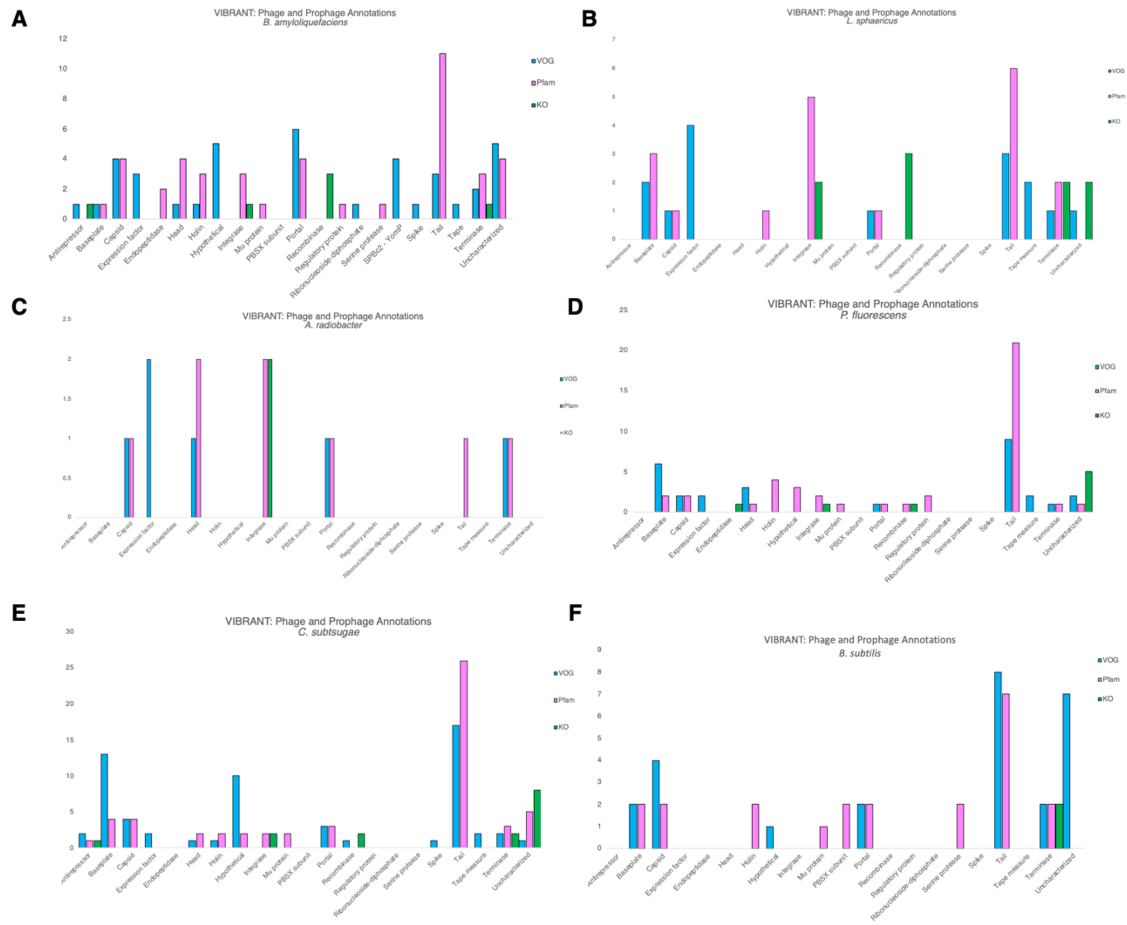


Figure 4. VIBRANT generated phage and prophage associated functional annotations by orthology database for six mined biopesticide strains. A. Green represents Virus Orthologous Groups database (VOG), pink represents the protein family database (Pfam), and blue shows results from the Kyoto Encyclopedia of Genes and Genomes (KEGG). A) Twenty-three functional annotation groups quantified in *B. amyloliquefaciens*. B) *L. sphaericus*. C) *A. radiobacter.* D) *P. fluorescens.* E) *C. subtsugae.* F) *B. subtilis.*

VIBRANT identified auxiliary metabolic gene categories for commercial biopesticides

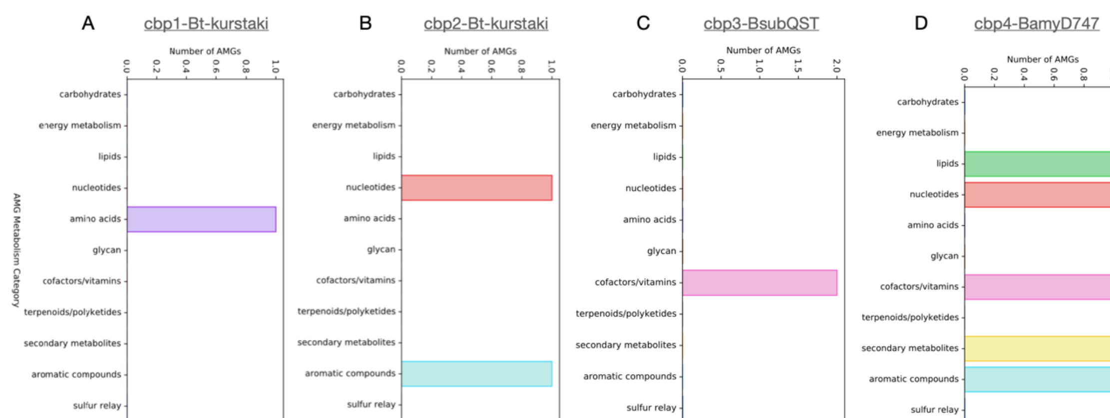


Figure 5. Auxiliary metabolic gene categories identified by VIBRANT in four commercial biopesticide products, *B. thuringiensis* kurstaki #1, *B. thuringiensis* kurstaki #2. *B. subtilis*, and *B. amyloliquefaciens*.

VIBRANT identified auxiliary metabolic gene categories for mined biopesticides

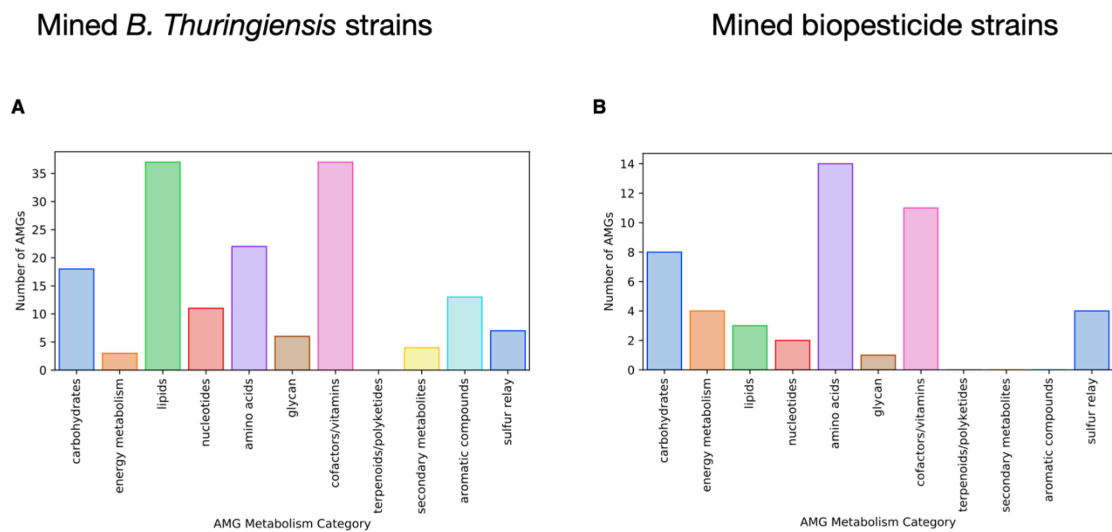


Figure 6. Comparison of auxiliary metabolic gene categories identified by VIBRANT. A. Results for combined *mined B. thuringiensis* genomes. B. Results for combined mined biopesticide genomes.

Integrative and mobilizable elements identified in commercial biopesticides

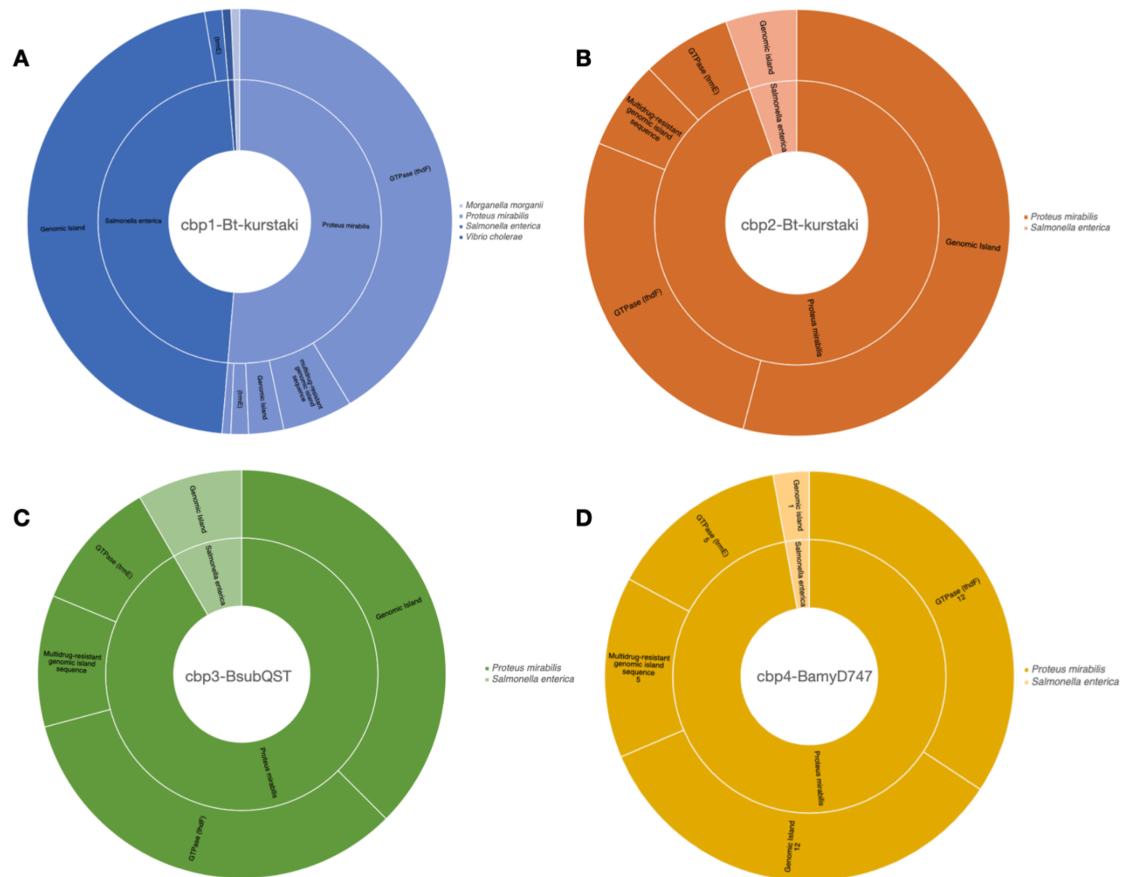


Figure 7. Integrative and mobilizable elements (IMEs) identified in commercial biopesticides. A. Cpb1-Bt-kurstaki contained 150 IMEs originating from four species. B. Cbp2-Bt-kurstaki contained 74 IMEs from two species. C. Cbp3-BsubQST contained 48 IMEs from two species. D. Cbp4-BamyD747 contained 35 IMEs from two species of origin. Inner ring is species of origin and outer ring are IME categories.

Antibiotic resistance gene pattern found in forty-four *B. thuringiensis* genomes

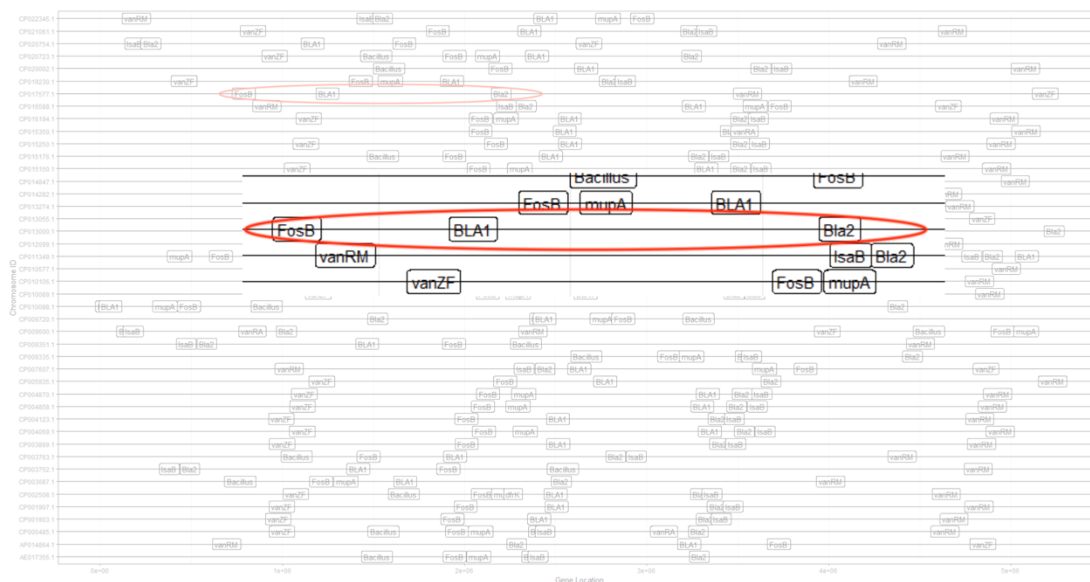


Figure 8. A potential gene cassette containing the same three genes, FosB, Bla1, and Bla2 was identified in 44 mined *B. thuringiensis* strains. The red circle indicates the trio of antibiotic resistance genes and represents the order in which they occur in mined *B. thuringiensis* genomes.

References

1. Parnell, J. J. *et al.* From the lab to the farm: An industrial perspective of plant beneficial microorganisms. *Front. Plant Sci.* 7, 1–12 (2016).
2. Damalas, C. A. & Koutroubas, S. D. Current status and recent developments in biopesticide use. *Agriculture (Switzerland)* (2018) doi:10.3390/agriculture8010013.
3. Kumar, S. & Singh, A. Biopesticides: Present Status and the Future Prospects. *J. Biofertilizers Biopestic.* 06, (2015).
4. CADPR. Summary of pesticide use report data - 2017. (2017).
5. Federici, B. A. & Siegel, J. P. Safety of Bacillus thuringiensis and Bt Crops Used in Insect Control. (2008).
6. Alejandra Bravo, et al. Bacillus thuringensis: A story of a sucessful bioinsecticide. *Insect Biochem. Mol. Biol.* 41, 423–431 (2013).
7. Lereclus, D., Agaisse, H., Grandvalet, C., Salamitou, S. & Gominet, M. Regulation of toxin and virulence gene transcription in Bacillus thuringiensis. *Int. J. Med. Microbiol.* 290, 295–299 (2000).
8. Yul, J., Choi, J. A. E. Y., Li, M. S., Jin, B. R. a E. & Je, Y. H. O. BT as a specific, safe, and effective tool for insect pest control. *J Microbiol. Biotechnol.* 17, 547– 559 (2007).
9. Siegel, J. P. The Mammalian Safety of Bacillus thuringiensis- Based Insecticides. *J. Invertebr. Pathol.* 77, 13–21 (2001).
10. Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P. & Hens, L. Chemical Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture. *Front. Public Heal.* 4, 1–8 (2016).
11. Hernández, A. F. *et al.* Toxic effects of pesticide mixtures at a molecular level: Their relevance to human health. *Toxicology* 307, 136–145 (2013).
12. FDA. *Substances Generally Recognized as Safe*. vol. 81 (2016).
13. EFSA. Risks for public health related to the presence of Bacillus cereus and other Bacillus spp. including Bacillus thuringiensis in foodstuffs. *EFSA J.* 14, (2016).
14. EPA Office of Pesticide Programs, U. Biopesticides Fact Sheet for Chromobacterium subtsugae strain PRAA4-1T. 1–3.
15. Thakore, Y. The biopesticide market for global agricultural use. *Ind. Biotechnol.* (2006).
16. EPA. Pseudomonas syringae strain ESC-10 (006441) Pseudomonas syringae strain ESC-11 (006451) Fact Sheet. 10, 10–11 (2008).
17. EPA. Pantoea agglomerans strain C9-1 (006470) Fact Sheet. 1, 20460.
18. Dutkiewicz, J., Mackiewicz, B., Lemieszek, M. K., Golec, M. & Milanowski, J. Pantoea agglomerans: A mysterious bacterium of evil and good. Part IV. Beneficial effects. *Annals of Agricultural and Environmental Medicine* (2016) doi:10.5604/12321966.1203879.
19. Kaze, M., Brooks, L. E. & Sistrom, M. This GRAS Isn't Greener: Biopesticides as Unintentional Reservoirs of Antibiotic Resistance. (2020).

20. Kaze, M., Brooks, L. E. & Sistrom, M. Mixing with a bad crowd: ARG exchange between Klebsiella pneumoniae and Bacillus based-Biopesticides Leads to Carbapenem Resistant Phenotype. (2020).
21. Guédon, G., Libante, V., Coluzzi, C., Payot, S. & Leblond-Bourget, N. The obscure world of integrative and mobilizable elements, highly widespread elements that pirate bacterial conjugative systems. *Genes* (2017) doi:10.3390/genes8110337.
22. Partridge, S. R., Kwong, S. M., Firth, N. & Jensen, S. O. Mobile genetic elements associated with antimicrobial resistance. *Clin. Microbiol. Rev.* 31, 1–61 (2018).
23. Shintani, M. The behavior of mobile genetic elements (MGEs) in different environments. *Biosci. Biotechnol. Biochem.* 81, 854–862 (2017).
24. Cury, J., Oliveira, P. H., De La Cruz, F. & Rocha, E. P. C. Host range and genetic plasticity explain the coexistence of integrative and extrachromosomal mobile genetic elements. *Mol. Biol. Evol.* (2018) doi:10.1093/molbev/msy123.
25. Brooks, L. E., Kaze, M. & Sistrom, M. Where the plasmids roam: large-scale sequence analysis reveals plasmids with large host ranges. 1–10 (2019) doi:10.1099/mgen.0.000244.
26. Coluzzi, C. *et al.* A glimpse into the world of integrative and mobilizable elements in Streptococci reveals an unexpected diversity and novel families of mobilization proteins. *Front. Microbiol.* (2017) doi:10.3389/fmicb.2017.00443.
27. Paez-Espino, D. *et al.* Uncovering Earth's virome. *Nature* 536, 425–430 (2016).
28. Roux, S. *et al.* Ecogenomics of virophages and their giant virus hosts assessed through time series metagenomics. *Nat. Commun.* 8, (2017).
29. Roux, S. & Brum, J. R. A viral reckoning: Viruses emerge as essential manipulators of global ecosystems. *Environ. Microbiol. Rep.* 00, 1–4 (2018).
30. Thompson, L. R. *et al.* Phage auxiliary metabolic genes and the redirection of cyanobacterial host carbon metabolism. *Proc. Natl. Acad. Sci. U. S. A.* (2011) doi:10.1073/pnas.1102164108.
31. Courvalin, P. Transfer of Antibiotic Resistance Genes between Gram-Positive and Gram-Negative Bacteria. *Antimicrob. Agents Chemother.* 38, 1447–1451 (1994).
32. Frampton, R. A. *et al.* Identification of bacteriophages for biocontrol of the kiwifruit canker phytopathogen Pseudomonas syringae pv. actinidiae. *Appl. Environ. Microbiol.* (2014) doi:10.1128/AEM.00062-14.
33. Bruce, D. *et al.* Pathogenomic Sequence Analysis of Bacillus cereus and Bacillus thuringiensis Isolates Closely Related to Bacillus anthracis. *J. Bacteriol.* 188, 3382–3390 (2006).
34. Swanson, M. M. *et al.* Novel bacteriophages containing a genome of another bacteriophage within their genomes. *PLoS One* 7, (2012).

35. Garneau, J. E. *et al.* The CRISPR/cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* 468, 67–71 (2010).
36. Evans, T. J. *et al.* Characterization of a broad-host-range flagellum-dependent phage that mediates high-efficiency generalized transduction in, and between, Serratia and Pantoea. *Microbiology* (2010) doi:10.1099/mic.0.032797-0.
37. Boyd, E. F., Davis, B. M. & Hochhut, B. Bacteriophage-bacteriophage interactions in the evolution of pathogenic bacteria. in *Trends in Microbiology* (2001). doi:10.1016/S0966-842X(01)01960-6.
38. Shi, J. *et al.* Novel diagnosis for citrus stubborn disease by detection of a spiroplasma citri-secreted protein. *Phytopathology* (2014) doi:10.1094/PHYTO06-13-0176-R.
39. Warwick-Dugdale, J., Buchholz, H. H., Allen, M. J. & Temperton, B. Host-hijacking and planktonic piracy: How phages command the microbial high seas. *Virology Journal* (2019) doi:10.1186/s12985-019-1120-1.
40. Ankrah, N. Y. D. *et al.* Phage infection of an environmentally relevant marine bacterium alters host metabolism and lysate composition. *ISME J.* (2014) doi:10.1038/ismej.2013.216.
41. Banerjee, N. & Mukhopadhyay, S. Viral glycoproteins: biological role and application in diagnosis. *VirusDisease* (2016) doi:10.1007/s13337-015-0293-5.
42. Godinho, L. M. *et al.* The revisited genome of bacillus subtilis bacteriophage SPP1. *Viruses* (2018) doi:10.3390/v10120705.
43. Amon, R., Reuven, E. M., Leviatan Ben-Arye, S. & Padler-Karavani, V. Glycans in immune recognition and response. *Carbohydr. Res.* (2014) doi:10.1016/j.carres.2014.02.004.
44. Čavužić, M. & Liu, Y. Biosynthesis of sulfur-containing tRNA modifications: A comparison of bacterial, archaeal, and eukaryotic pathways. *Biomolecules* (2017) doi:10.3390/biom7010027.
45. Ayala-Castro, C., Saini, A. & Outten, F. W. Fe-S Cluster Assembly Pathways in Bacteria. *Microbiol. Mol. Biol. Rev.* (2008) doi:10.1128/mmbr.00034-07.
46. Selbach, B. P., Pradhan, P. K. & Dos Santos, P. C. Protected sulfur transfer reactions by the Escherichia coli Suf system. *Biochemistry* (2013) doi:10.1021/bi4001479.
47. Craig, K. A review of the chemistry, pesticide use, and environmental fate of sulfur dioxide, as used in California. in *Reviews of Environmental Contamination and Toxicology* (2019). doi:10.1007/398_2018_11.
48. Gammon, D. W., Moore, T. B. & O'Malley, M. A. A Toxicological Assessment of Sulfur as a Pesticide. in *Hayes' Handbook of Pesticide Toxicology* (2010). doi:10.1016/B978-0-12-374367-1.00088-4.
49. Crummett, L. T., Puxty, R. J., Weihe, C., Marston, M. F. & Martiny, J. B. H. The genomic content and context of auxiliary metabolic genes in marine cyanomyoviruses. *Virology* (2016) doi:10.1016/j.virol.2016.09.016.

50. Hurwitz, B. L. & U'Ren, J. M. Viral metabolic reprogramming in marine ecosystems. *Current Opinion in Microbiology* (2016) doi:10.1016/j.mib.2016.04.002.
51. Rosenwasser, S., Ziv, C., Creveld, S. G. van & Vardi, A. Virocell Metabolism: Metabolic Innovations During Host–Virus Interactions in the Ocean. *Trends in Microbiology* (2016) doi:10.1016/j.tim.2016.06.006.
52. Hurwitz, B. L., Hallam, S. J. & Sullivan, M. B. Metabolic reprogramming by viruses in the sunlit and dark ocean. *Genome Biol.* (2013) doi:10.1186/gb-2013-14-11r123.
53. Doublet, B., Boyd, D., Mulvey, M. R. & Cloeckaert, A. The Salmonella genomic island 1 is an integrative mobilizable element. *Mol. Microbiol.* (2005) doi:10.1111/j.1365-2958.2005.04520.x.
54. Bie, L., Fang, M., Li, Z., Wang, M. & Xu, H. Identification and Characterization of New Resistance-Conferring SGI1s (Salmonella Genomic Island 1) in Proteus mirabilis. *Front. Microbiol.* 9, 1–9 (2018).
55. Grohmann, E., Muth, G. & Espinosa, M. Conjugative plasmid transfer in grampositive bacteria. *Microbiol. Mol. Biol. Rev.* 67, 277–301 (2003).
56. Noli, C. & Boothe, D. Macrolides and lincosamides. *Vet. Dermatol.* (1999) doi:10.1046/j.1365-3164.1999.00176.x.
57. CADPR. *A Guide to Pesticide Regulation in California*. (2018).
58. U.S. Environmental Protection Agency Office of Pesticide Programs & Division, B. and P. P. Biopesticides Registration Action Document. *Epa* 1–30 (1984). 59.
59. Rédei, G. P. Blast (basic local alignment search tool). in *Encyclopedia of Genetics, Genomics, Proteomics and Informatics* (2008). doi:10.1007/978-14020-6754-9_1879.
60. Arndt, D. *et al.* PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* (2016) doi:10.1093/nar/gkw387.
61. Stano, M., Beke, G. & Klucar, L. ViruSITE - Integrated database for viral genomics. *Database* (2016) doi:10.1093/database/baw162.
62. Liu, M. *et al.* ICEberg 2.0: An updated database of bacterial integrative and conjugative elements. *Nucleic Acids Res.* (2019) doi:10.1093/nar/gky1123.
63. Brooks, L., Kaze, M. & Sistrom, M. A Curated, Comprehensive Database of Plasmid Sequences. *Microbiol. Resour. Announc.* 85, 1–11 (2018).
64. Kristopher Kieft, Zhou1, Z. & Anantharaman, K. VIBRANT: Automated recovery, annotation and curation of microbial viruses, and evaluation of virome function from genomic sequences. *bioRxiv* (2019).
65. Merchant, N. *et al.* The iPlant Collaborative: Cyberinfrastructure for Enabling Data to Discovery for the Life Sciences. *PLoS Biol.* (2016) doi:10.1371/journal.pbio.1002342.
66. Aoki, S. BIORENDER. *Biorender* (2017).
67. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* (2009) doi:10.1093/bioinformatics/btp324.

68. Clustalw, U., To, C. & Multiple, D. O. ClustalW and ClustalX. *Options* (2003) doi:10.1002/0471250953.bi0203s00.
69. R Studio Team. R Studio. *R.S. ed. http://www.rstudio.com/.* (2015) doi:http://www.rstudio.com/.
70. RStudio. Data Visualization with ggplot2: Cheat Sheet. 2 (2015) doi:10.1080/15228959.2015.1060147.

<u>Conclusion</u>

This work represents the first investigation into bacteria biopesticides as reservoirs and vectors of antibiotics. It characterizes the viral, metabolic auxiliary gene, and integrative and mobilizable elements content of biopesticide genomes. Genomes were generated from cultured purchased biopesticide products and mined from public databases. Incorporating both methods provides a comprehensive view of face of antibiotic resistance gene movement and persistence in the environment.

Chapter 1 addresses the question of whether or not biopesticides contain antibiotic resistance genes and characterizes the resistomes of mined biopesticide genomes and commercial products. The presence of clinically relevant antibiotic resistance genes raises the possibility that contact with other biopesticides or bacteria in the environment they are added to will result in genetic exchange. This exchange could lead to the generation of novel pathogenicity and further the spread of antibiotic resistance genes. Chapter 2 builds upon the results from Chapter 1 and answers the question of whether or not *Bacillus*-based biopesticide products can act as vectors of antibiotic resistance genes. Genes location was verified by use of high throughput chromosome conformation capture and use of our curated plasmid database. A straightforward co-culture experiment that mixed the biopesticides with a carbapenemase positive *Klebsiella pneumoniae* strain resulted in a changed resistance phenotype. Biopesticides that were completely susceptible to two carbapenem antibiotics before mixing became completely resistance after contact with *K. pneumoniae* after 18 hours. Not only did the biopesticide products take up antibiotic resistance genes from *K. pneumoniae* they exchanged genes between each other. *K. pneumoniae* also took up genes from the biopesticides. This demonstrates the role that Bacillus-based biopesticides can play as vectors of antibiotic resistance genes Chapter 3 investigates additional mechanisms for genetic exchange that may be in use by biopesticides. This work characterizes their phage, prophage, and metabolic auxiliary gene, and integrative and mobilizable elements content. Bioinformatic approaches, including a protein homology and machine learning combinatory tool, identified mobile genetic elements and a new antibiotic resistance gene cassette.

This project contributes to the understanding of genetic movement in the environment by investigating important commercial bacterial products that are added in large quantities to highly processed agricultural environments. It includes support for consideration as biopesticides as participants in the generation, spread, and maintenance of antibiotic resistance genes in the environment. This work raises the possibility that these widely used products are not nearly as safe or as healthy as they are currently designated.