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FINE FOCUS

AN INTERNATIONAL MICROBIOLOGY JOURNAL FOR UNDERGRADUATE RESEARCH

MISSION

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

SCOPE

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

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OBJECTIVE LENS

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

UG: MILESTONES & OUR PEER REVIEW SYSTEM

This issue begins our fourth year publishing original undergraduate research from around the globe. As a product-based "immersive learning" course, the *Fine Focus* student editors have achieved several important milestones over the last several months, including:

- Developed and launched a crowd source funding page
- Print and digital issues now in the collections of university libraries in the Czech Republic, Finland, Iceland, Germany and the U.S.
- Created consumer & user surveys, now available on our website
- Established an Executive Committee
- Created an interactive and user friendly map of Editorial Board, manuscript submissions, and library locations

Our consumer and user surveys will be administered to past and potential *Fine Focus* authors, Editorial Board members, and the general readership to improve the content and presentation of our website and print journal. The survey links are found at finefocus.org and will also be promoted and administered during our conference presentations, and will also effectively quantify the impact *Fine Focus* has on undergraduate research in microbiology internationally.

The formation of our Executive Committee is especially important for the current group of *Fine Focus* student editors. This Committee, consisting (for now) of three carefully selected individuals, will help us develop a vision for success and planning our long-term goals in the dynamic and competitive arena of academic journal publishing. Our niche of undergraduate microbiology research, and managing the journal by an ever-changing group of student editors in a product-based course, makes *Fine Focus* unique and rapidly able to adapt to a changing marketplace, but many new challenges are created using this model. I welcome the new inaugural Executive Committee members who will address these opportunities and challenges:

- Kyla Adamson – former *Fine Focus* alum and current M.S. student in biotechnology/microbiology at Ball State University – will serve as the Managing Consultant
- Saara-Maria Kallio - former *Fine Focus* alum and international marketing student at the University of Tampere in Finland will serve as the Marketing Consultant
- Emma Kate Fittes – Education Reporter for the Muncie (IN) *Star Press* – who will be serving in the role as the Design Consultant
- I (JLM) will be serving as the overall Executive Director

Over the last several conferences, many of you have inquired about our rigor in peer-review of undergraduate manuscripts, and how we manage submissions. I am glad to be able to share with our readers the system we use for our double-blind peer-review of manuscripts, as this is one of the unique aspects of *Fine Focus*.

Completed and correctly formatted manuscripts are submitted to us through our Open Journal System (OJS) site: finefocussubmissions.org and are received by one of our student Lead Editors. This editor will screen the manuscript for appropriate formatting, complete figures, tables, legends, and references. This 1-2 day vetting also ensures that the work is original hypothesis-driven research by one or more undergraduates that fits within the scope of *Fine Focus*.

Notification/acknowledgement of this process is then communicated to the corresponding author by the Lead Editor via OJS. In order to ensure a double-blind peer review, the Lead Editor will remove all identifiers from the manuscript, and secure at least two expert external reviewers (ideally from our Editorial Board) who will be invited to complete their reviews within approximately 21 days. This communication also takes place through the OJS platform, ensuring professionalism, and confidentiality. To assist in standardizing high-quality constructive critiques from our reviewers, we offer the following list of queries, that I share with you here, to perhaps facilitate journal club article discussions or classroom article critiques you may teach in your courses or research:

Suggested/optional queries to address when reviewing:

- Is the topic of the paper within the general scope of *Fine Focus*?
- Does this research represent an original contribution to the greater body of knowledge within this sub discipline of microbiology?
- Does the title accurately reflect the contents?
- Does the abstract contain sufficient information?
- Does the title page contain appropriate keywords?
- Does the Introduction section contain sufficient information without being verbose?
- Are the objectives and hypothesis/ hypotheses clearly stated and justified?
- Are the Materials & Methods detailed enough to allow for replication?
- Are the statistical methods correct and adequate?
- Do authors clearly present and interpret their results?
- Is the overall organization of the paper satisfactory?
- Are all figures & tables necessary, and properly shown?
- Are the references appropriately cited (no unnecessary citations)?
- Does the manuscript read well in terms of English/grammar/syntax?
- Are conclusions properly supported with appropriate results?
- Overall recommendation: Accept, Accept with revisions, Revise & Resubmit, or Reject?

The completed external reviews are assessed in relationship to our own class discussion of the manuscript by our *Fine Focus* review team, where we arrive at a conclusion and render a final decision on the acceptability of the submission.

We hope you find this issue interesting and informative. If you are nearing completion of an UG research project, or have a manuscript underway, we hope you will consider submitting to *Fine Focus* when ready. As always, we welcome your input in ways we can improve, or how *Fine Focus* has helped you or your students achieve success.

–JLM

CUTTING WEDGE: BACTERIAL COMMUNITY DIVERSITY AND STRUCTURE ASSOCIATED WITH THE CHEESE RIND AND CURD OF SEVEN NATURAL RIND CHEESES

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- cheese microbiome
- rind
- curd
- diversity
- antibiotic resistance
- 16S rRNA gene

ABSTRACT

The microorganisms that inhabit cheese contribute greatly to the flavor and development of the final product. While the rind and curd microbiota have been characterized separately, there is limited information on how the structure and function of microbial communities in rinds and curds vary within and amongst cheeses. To better understand the differences in community structure and function between communities of cheese rinds and curds, we combined culture-based methods with culture-independent community profiling of curds and rinds. Rinds contained greater taxonomic diversity than curds. *Lactobacillales* dominated curd communities while members from the order *Actinomycetales* were found in high abundance in rind communities. Communities varied more between rinds and curds than among cheeses produced from different milk types. To better understand microbial community functions, we cultured and assayed isolates for antibiotic susceptibility and carbon source utilization. Among European and U.S. cheeses, 70% of all susceptible isolates were cultured from U.S. cheeses. Overall, our study explored the differences within and between rind and curd microbial communities of natural rind cheeses, provided insights into the environmental factors that shape microbial communities, and demonstrated that at the community and isolate level the cheese microbiome was diverse and metabolically complex.

INTRODUCTION

Cheese production exemplifies a reproducible succession of microbial communities (10, 48, 49). Microbes execute the biochemical transformation of milk from a liquid suspension of lactose, casein, whey, and fat into cheese which is a solid aggregate of amino acids, lactate and volatile flavor compounds and pigments (6). Over centuries, humans optimized the cheese-making process to select for distinct and reproducible microbial communities that give cheeses their individual tastes, consistencies, colors and other desirable properties (6, 14, 43, 49). In natural-rind cheeses, endogenous microorganisms or the addition of bacterial starter culture to milk are needed to acidify and coagulate the milk into

curds and enable future colonization by fungi and bacteria on the cheese surface (26, 49).

The early process of curd formation is well characterized (11, 16) . Ripening begins in milk, where lactic acid bacteria (LAB) such as *Streptococcus thermophilus*, *Lactobacillus helveticus*, and *Lactobacillus casei* ferment lactose into lactate, acidify the medium and digest proteins and milk (11, 25, 35, 36). LAB can be introduced by a starter culture, or the milk can be permitted to acidify naturally from the microbial community in it (6). Regardless of the addition of starter culture, most curds become inhabited by a simple community, dominated by lactose fermenters that may include organisms belonging to genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Staphylococcus*, *Enterobacter*, and *Streptococcus* (1, 6, 16).

After the initial curd formation, the rind begins to form. Throughout the ripening, excess lactate accumulates and dissolves into the medium, eventually migrating upward to the curd surface (22). Once lactate becomes accessible at the outer surface of the cheese, aerobic fungal taxa including *Candida*, *Penicillium*, and *Scopulariopsis* colonize the surface and metabolize lactate, leading to an increase in pH at the surface environment (22, 49). De-acidification facilitates the colonization and succession of microbes that prefer more alkaline and salty conditions including coryneforms such as *Corynebacterium*, *Brevibacterium* and *Brachybacterium* (27, 37, 41).

The composition of the external rind communities is governed by factors beyond pH, including microbe-microbe interactions. Fungal species can contribute to cheeses' unique characteristics, such as the blue-vein appearance of Roquefort by the fungus *Penicillium roqueforti*, and can be crucial to the survival of rind bacteria like *Corynebacterium*, *Halomonas*, *Pseudomonas*, *Pseudoalteromonas*, and *Vibrio* spp. (4, 29, 49). Fungi such as those belonging to the spore-forming *Penicillium* species can also produce antibacterial compounds that can lead to selection for more antibiotic-resistant bacterial strains (28). Likewise, bacteria on the rind such as those belonging to the antibiotic-producing *Actinomycetes* group can have a similar effect (32). The possibility of cheese bacteria developing resistance mechanisms has warranted the characterization of antibiotic-susceptibility of bacteria on various types of cheeses (4, 29).

While many studies have characterized the succession of early cheese community development from curd to rind in individual natural rind cheeses (1, 16, 20, 21), less is known about the comparison of microbial composition of a mature rind to a mature curd within and across natural rind cheese varieties. Here we compare microbial community structure between the rinds and curds of seven natural-rind cheeses that differ by pH, moisture content, and milk source. To obtain a detailed understanding of the rind and curd microbial communities, we used culture-independent, high-throughput Illumina sequencing of 16S rRNA genes. We also sought to characterize specific bacteria cultured from the cheeses. These isolates were identified using Sanger sequencing of the 16S rRNA gene (40) and were assayed for antibiotic susceptibility and carbon utilization profiles using BIOLOG's Ecoplates. Through these analyses, we aimed to evaluate the following hypotheses: 1) cheese rind communities would exhibit higher taxonomical diversity than curd communities; 2) antibiotic susceptibility of cheese isolates would differ between cheeses and between the two regions where these cheeses originated, Europe and the U.S.; and 3) cheeses with higher taxonomical diversity would be more metabolically active and can utilize more carbon sources. This study contributes to the characterization of the curd- and rind-associated communities of natural rind cheeses and reveals patterns of microbial diversity according to cheese type as well as the overall metabolic and antibiotic resistance profile of isolates from the different cheeses.

METHODS

SAMPLE COLLECTION AND ENVIRONMENTAL PARAMETERS

Seven natural rind cheeses—Vermont Shepherd, Stichelton, Sonnet, Missouri Truckle, Maggie's Round, Comte, and Alpage Gruyere—were obtained from Wasik's Cheese Shop in Wellesley, Massachusetts. A 100 +/– 10 mg sample from each of the rinds and curds of the examined cheeses was collected aseptically and processed immediately. Rind samples were scraped using a sterile razor blade and curd samples were taken from the cheese center after scraping off the exposed curd layer. A solid-state pH meter (S175CD/BNC; Sensorex, Garden Grove, CA) was used to determine the pH of each cheese rind and curd. Samples were dried for 7 days and moisture was determined by subtracting the dry weight of the sample from the original wet weight of a 1 g sample.

BACTERIAL ISOLATION AND CHARACTERIZATION

The 100 +/– 10 mg of fresh rind and curd samples were crushed using a pellet pestle and diluted in sterile water to 10^{-3}, 10^{-4}, and 10^{-5} of their original concentrations. All diluted samples were grown aerobically at room temperature on three different media: Plate Count Agar (PCA) with Milk (PCAM: 5g Peptone, 2.5 g yeast extract, 1 g dextrose, 1 g of whole milk powder, 15 g agar), Nutrient Agar (NA: 0.5% Peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl) and PCA with fermentation indicators for lactose (5 g Peptone, 2.5 g yeast extract, 1 g dextrose, 10 g lactose, 0.03 g neutral red, 15 g agar). Isolates were re-streaked from single colonies three times in order to obtain pure cultures.

DNA ANALYSIS OF ISOLATES

The genomic DNA of selected bacterial isolates were extracted by suspending a colony in polymerase chain reaction (PCR)–grade water and freezing for 20 min at –80°C and then thawing. A 1465 base pair (bp) sequence of the 16S rRNA gene was PCR-amplified from genomic DNA (10–30 ng). Mastermix reagents consisted of 1 µg/µL bovine serum albumin, 200 µM dNTP mix, 1x buffer w/ $MgCl_2$, 300 pM 27F primer (AGA GTT TGA TCC TGG CTC AG), 300 pM 1492R primer (ACG GCT ACC TTG TTA CGA CTT) (IDT Integrated DNA Technologies, Inc., Coralville, IA) (30), and 0.2 U Takara Ex. *Taq* polymerase (Takara Clontech, Mountain View, CA). DNA extracts from *Escherichia coli* and sterile PCR-grade water were used as positive and negative controls, respectively. The thermal cycler program ran for 34 cycles in the following order: 1 cycle of initial denaturation (3 min at 95°C); 32 cycles of denaturation (30 sec at 95°C), annealing (25–30 sec at 50°C), and extension (1.5 min at 72°C); 1 cycle of final extension (10 min at 72°C); and hold (4°C). Amplicons were detected from banding patterns on 1.5% agarose gel electrophoresis and quantified on the NanoDrop 2000 UV-Vis spectrophotometer (NanoDrop Products, Thermo Fisher Scientific, Inc., Wilmington, DE). Excess *TAQ* polymerase, primer and nucleotides were precipitated from the amplicon solution by adding 1µL USB ExoSap-iT reagent (Affymetrix, Inc., Santa Clara, CA) to 9µL of amplicon. Purified products were identified via Sanger sequencing of the 16S rRNA gene using 27F primer (5'-AGAGTTTGATCCTGGCTCAG-3') (GeneWiz, Madison, WI, USA). From each sequence we extracted >750 consecutive nucleotides with quality score Q>20 and each chromatogram was visually inspected to insure there were no base caller errors (Supplementary Table 1).

Supplementary Table 1

ISOLATE NAME	SEQUENCE IDENTIFICATION	MILK TYPE	CHEESE NAME	COUNTRY	GENUS
LWHW2-27F	Uncultured *Brevibacterium* sp. Clone PtS-1-cl11	cow	Alpage Gruyere	Switzerland	*Brevibacterium*
LWHW3-27F	Uncultured *Brevibacterium* sp. Clone PtS-1-cl11	cow	Alpage Gruyere	Switzerland	*Brevibacterium*
LWHW4-27F	*Brevibacterium aurantiacum*, isolate 0911TES25Y3	cow	Alpage Gruyere	Switzerland	*Brevibacterium*
LWHW6-27F	*Brevibacterium* sp. EP11 Strain EP11	cow	Alpage Gruyere	Switzerland	*Brevibacterium*
LWHW7-27F	*Corynebacterium glycinophilum*	cow	Alpage Gruyere	Switzerland	*Corynebacterium*
ZMBB1-27F	*Staphylococcus equorum*	cow	Stichelton	England	*Staphylococcus*
ZMBB2-27F	*Brachybacterium alimentarium*	cow	Stichelton	England	*Brachybacterium*
ZMBB3-27F	*Brachybacterium alimentarium*	cow	Stichelton	England	*Brachybacterium*
ZMBB4-27F	*Brachybacterium alimentarium*	cow	Stichelton	England	*Brachybacterium*
ZMBB5-27F	*Lactobacillus plantarum* or *casei*	cow	Stichelton	England	*Lactobacillus*
ZMBB6-27F	*Brevibacterium linens* or *epidermidis*	cow	Stichelton	England	*Brevibacterium*
ZMBB7-27F	*Staphylococcus simulans*	cow	Stichelton	England	*Staphylococcus*
ZMBB8-27F	*Staphylococcus pasteuri*	cow	Stichelton	England	*Staphylococcus*
ZMBB9-27F	*Staphylococcus equorum*	cow	Stichelton	England	*Staphylococcus*
ZMBB10-27F	*Staphylococcus equorum*	cow	Stichelton	England	*Staphylococcus*
AX1-27F	*Staphylococcus equorum*	cow	Missouri Truckle	USA	*Staphylococcus*
AX2-27F	*Brachybacterium aimentarium*	cow	Missouri Truckle	USA	*Brachybacterium*
AX5-27F	*Brachybacterium alimentarium*	cow	Missouri Truckle	USA	*Brachybacterium*
AX7-27F	*Staphylococcus warneri*	cow	Missouri Truckle	USA	*Staphylococcus*
RRSH1-27F	*Staphylococcus xylosus*	cow	Maggies Round	Massachusetts	*Staphylococcus*
RRSH3-27F	*Brachybacterium* sp.	cow	Maggies Round	Massachusetts	*Brachybacterium*
RRSH4-27F	*Bacillus* sp.	cow	Maggies Round	Massachusetts	*Bacillus*
RRSH6-27F	*Staphylococcus xylosus*	cow	Maggies Round	Massachusetts	*Staphylococcus*
RRSH7-27F	*Staphylococcus xylosus*	cow	Maggies Round	Massachusetts	*Staphylococcus*
RRSH8-27F	*Bacillus mojavensis*	cow	Maggies Round	Massachusetts	*Bacillus*
CH1-27F	*Staphylococcus* sp. U1371-101227-XH136	sheep	Vt. Shepherd	USA	*Staphylococcus*
CH3-27F	*Brevibacterium* sp. EP11	sheep	Vt. Shepherd	USA	*Brevibacterium*
CH7-27F	*Staphylococcus equorum*	sheep	Vt. Shepherd	USA	*Staphylococcus*

DIRECT DNA ANALYSIS OF CHEESE MICROBIAL COMMUNITIES

Total DNA from the cheese rind and curd samples was extracted using the MO BIO Power® Soil DNA Isolation Kit (MoBio, Carlsbad, CA). The 16S rRNA gene from community genomic DNA was amplified, detected, quantified, and purified as described for the isolates. Samples were sequenced on a MiSeq instrument at the Forsyth Institute, Cambridge, MA. The V3/V4 region of the 16S rRNA gene was amplified from each sample using the forward primer 341F 5'-*AATGATACGGCGACCACCGAGATCTACAC* **TATGGTAATT *GT*** CCTACGGGAGGCAGCAG-3'; where italicized text indicates Illumina adaptor, bold text indicates primer pad, italicized bold indicates primer linker and an underlined text a conserved bacterial primer 314F. The reverse primer was 806R 5'- *CAAGCAGAAGACGGCATACGAGAT* **XXXXXXXXXXXX AGTCAGTCAG *CC*** GGACTACHVGGGTWTCTAAT-3'; where italicized text indicates reverse complement of the Illumina adaptor, 12 X-letters in bold are the Golay barcode primer followed by barcode primer and primer linker (italicized bold letters). The conserved bacterial primer 806R is indicated by underlined letters. PCR products from respective samples were each tagged by a sample-specific 12-base barcode (9). All samples were amplified in triplicates with 5 Prime Hot Master PCR Mix (Five Prime) on an Eppendorf Master Cycler Pro PCR Thermocycler using 0.2 µM of each primer and 10 ng template. Reaction conditions were: 94°C for 3 min, followed by 35 cycles at 94°C for 45 secs, 50°C for 1 min and 72°C for 1.5 min. Following the 35th cycle, samples were incubated at 72°C for 10 min. Amplicons were purified using Ampure magnetic beads according to the manufacturer's instructions (Agencourt from Beckman Coulter, Danvers, MA), quantified by Nanodrop, and further purified using the Qiagen MiniElute Gel Extraction Kit (Qiagen, Valencia, CA). Libraries were quantified on a Bioanalyzer instrument according to the Bioanalyzer manual using a DNA High Sensitivity chip, pooled, and sequenced on a MiSeq Illumina sequencer (Illumina, San Diego, CA). Forward and reverse reads were joined using Flash software (31). Libraries were demultiplexed and filtered using a q-score cutoff of 20 using split_libraries_fasq.py in Quantitative Insights into Microbial Ecology (QIIME) v1.8.0 (9). Any reads that did not assemble or meet the q-score threshold were removed and were not used in subsequent analyses. Sequences were clustered into operational taxonomic units (OTUs) using the UCLUST algorithm (15) at 97% sequence identity level with the generation of new clusters with sequences that match the reference, and classified using the Greengenes 97% reference dataset released on May 2013 (12, 33). Raw sequence data were submitted to Sequence Read Archive in Genbank under accession number PRJNA354727.

ANTIBIOTIC DISC SUSCEPTIBILITY ASSAY

We used a modified version of antibiotic disc diffusion susceptibility test to compare fungal and bacterial-derived antibiotic susceptibility of cheese bacteria isolates, focusing on antibiotic susceptibility trends among isolates cultured from cheeses of different regions (5, 7). Ten morphologically diverse isolates were cultured in nutrient broth (NB; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 40 – 48 hours. The liquid culture of bacteria was diluted using NB to match the turbidity of a 0.5 McFarland standard to ensure roughly equivalent densities of each inoculum. Bacterial culture was evenly streaked onto the dried surface of a nutrient agar plate using a sterile swab and allowed to be absorbed into the agar for at least 3 – 5 minutes. Six antimicrobial-

Table 1. Characteristics of the seven investigated natural rind cheeses. ND = Not Determined.

Cheese	*Origin*	*Country*	*Milk*	*Curd pH*	*Rind pH*	*Curd % Moisture*	*Rind % Moisture*
Alpage Gruyere	Gruyere	Switzerland	Cow	7.45	6.42	9.71	28.43
Comte	French Alps	France	Cow	7.14	6.5	21	26
Maggie's Round	MA	USA	Cow	5.34	5.04	4	26
Missouri Truckle	MO	USA	Cow	6.01	5.73	27	24
Vermont Shepherd	VT	USA	Sheep	ND	ND	ND	ND
Sonnet	VT	USA	Goat	6.44	5.55	ND	ND
Stichelton	Nottingham	England	Cow	8.17	Blue – 8.24 White – 6.96	ND	ND

impregnated discs (AM10: Ampicillin 10 μg; P10: Penicillin 10 IU/IE/UI; E15: Erythromycin 15 μg; RA5: Rifampin 5 μg; N30: Neomycin 30 μg; NB30: Novobiocin 30 μg) were evenly pressed onto the bacterial-containing agar surface using a disc dispenser. Plates were incubated for one week at room temperature prior to examination for antibiotic susceptibility. Diameter measurements in millimeters of the zone of clearance around the individual antibiotic discs for cheese isolates were used to categorize each cheese isolate into one of the three susceptibility levels based on the following zone clearance interpretation: Resistant (13 mm or less); Intermediate Susceptible (14 – 16 mm); and Susceptible (17 mm or more) (45).

CARBON SOURCE UTILIZATION PROFILING

Community-level physiological profiling (CLPP), a metabolic profile, of both the cheese rind and selected curd communities and individual bacterial isolates were analyzed using BIOLOG EcoPlate™ assay (Biolog, Hayward, CA). The capacity of either a bacterial community or a single bacterial isolate to utilize 31 distinct carbon sources over a 7-day period was examined and compared between the community sample and isolates of the same cheese and among different cheese types. The 100 +/– 10 mg of fresh rind and curd samples were crushed with a pestle and diluted in sterile water to 10^{-3} in 10 mM phosphate buffer. Subsequently, 100 μL of the solution was inoculated into separate BIOLOG Ecoplate™ wells. Individual isolates were incubated in NB overnight at room temperature and diluted to 10^{4} cells/ml and 100 μl was inoculated into each well of the new BIOLOG EcoPlate™. Growth was measured using the Molecular Devices SpectraMax 190 and the SOFTmaxPRO6.3™ program at A_{590} absorbance, for six consecutive days where day 1 was the day of inoculation. Metabolic diversity (CMD) was defined as the number of carbon sources utilized by the sample. Top carbon sources were defined as carbon sources that exhibited the maximum absorbance value for from fluorescence of tetrazolium salt reduction in the BIOLOG ECOPLATE™ assay.

RESULTS

ENVIRONMENTAL PARAMETERS OF NATURAL RIND CHEESES

To explore the relationship between the rind and curd within and between natural rind cheeses, and to determine how the physical factors of cheese environments (pH and moisture) correlate with microbial community diversity, we analyzed the microbial communities from seven different cheeses. The seven natural rind cheeses varied in appearance, place of origin, and type of milk used in the cheese-making process. Four cheeses came from the United States and three came from Switzerland, France, and England. All of the European cheeses and two of the U.S. cheeses were made from cow milk. The remaining two U.S. cheeses were made from sheep (Vermont Shepherd) and goat (Sonnet) milk. In each cheese the curd was slightly more acidic and contained more moisture than the corresponding rind (Table 1). Moisture in the curd varied slightly between cheeses, from 24% to 28%, while moisture in the rind had a larger range between cheeses, from 4% to 27% (Table 1).

To determine the types of bacteria that were present in the cheese communities, we analyzed the 16S rRNA gene using Illumina sequencing of the total extracted DNA from cheese curd and rind, and Sanger sequencing of cultured cheese isolates. There were more organisms identified to the genus level in the culture-independent approach than culture-dependent approach (see "other" category, Figure 1).

Unculturable organisms that were common to the cheese rinds included *Streptococcus* spp. (comprising 24% of total bacterial cells in the Comte rind and 55% of the Maggie's Round rind), *Lactococcus* spp. (39% of all cells in Missouri Truckle rind), *Yaniella* spp. (20% of Stichelton rind), *Prauseria* spp. (18% of Vermont Shepherd rind), *Halomonas* spp. (17% of Stichelton rind and 7% of Alpage Gruyere rind) and *Lactobacillus* spp. (16% of Missouri Truckle rind and 13% of Comte rind). In the curds, prevalent uncultured taxa included *Lactococcus* spp. (91% of Missouri Truckle rind and 89% of Stichelton rind), *Streptococcus* spp. (78% of Maggie's Round rind, 73% of Comte rind, and 26% of Alpage Gruyere rind), and *Lactobacillus* spp. (71% of Alpage Gruyere rind, 35% of Comte rind). In the curd, 43% (Figure 1B), and in the rind, 65% (Figure 1C) of bacteria were not culturable on PCAM agar. Thus, a significant fraction of bacterial species was not recovered on PCAM plates.

THE RIND HARBORS A MORE COMPLEX BACTERIAL COMMUNITY THAN THE CURD

In order to determine the bacterial diversity of the rind and curd, we carried out Illumina sequencing of the 16S rRNA gene. Rarefaction curve analysis, which assesses species richness from samples, showed all samples approached the asymptote and revealed that the overall bacterial diversity was well represented (Figure 2). The curds of Alpage Gruyere and Comte had the fewest unique OTUs (Figure 2A) while the largest number of unique OTUs was found in the rind of Alpage Gruyere cheese, followed by the rinds of Stichelton and Comte cheeses (Figure 2B). The Shannon diversity index, a measurement of overall diversity, of pooled data from the rinds and curds of the seven cheeses indicated more species richness and evenness in the rind communities than the curd (Figure 2C). Together, OTU distribution and richness data demonstrate higher alpha diversity in rind communities.

Through the identification of the organisms present in the cheese samples, we found that, with the exception of the Sonnet cheese, the only phylum represented in the curd communities was *Firmicutes* (Figure 3). In contrast, *Firmicutes*, *Actinobacteria*, and *Proteobacteria* were found in the rind communities. In a similar trend, no more than ten taxonomic units were found in each of the curd communities, while no less than ten genera were identified in each of the rind communities (Figure 3). In general, dominant genera in the rinds and curds were widespread among sampled cheeses. Cheese curds were dominated by lactic acid fermenters including *Lactobacillus*, *Streptococcus*, and *Lactococcus* while the rind communities showed a greater relative abundance of *Brevibacterium* and *Actinomycetaceae* (Figure 3; rind). However, variation was present within both the rind and curd communities. For example, while the Missouri Truckle, Sonnet, and Stichelton curds were almost completely dominated by *Lactococcus*, this organism made up less than 2% of the Maggie's Round, Comte, and Alpage Gruyere cheeses (Figure 3; curd, purple).

We next sought to investigate whether the differences in community composition could be associated with other abiotic factors such as moisture content, pH, or milk type. Principal coordinates of analysis (PCoA) demonstrated that communities were found to cluster by rind or curd (Figure 4), but not by milk type or moisture content (Supplementary Figure 1). A trend towards clustering was seen with pH (Supplementary Figure 1).

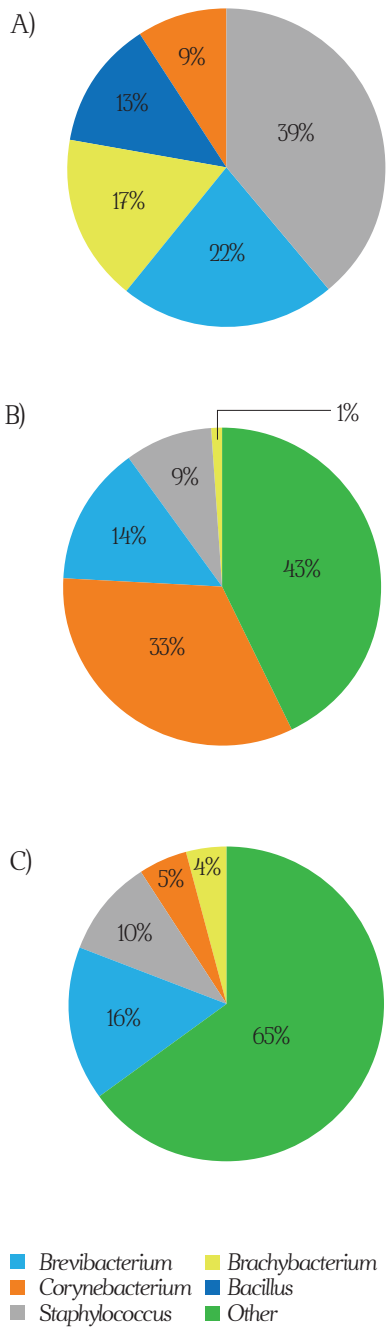


Figure 1. Prevalence (in % operational taxonomic units, OTUs) of cheese microbes successfully identified to the genus level through A) culture-dependent Sanger sequencing of the 16S rRNA gene of organisms isolated from both the rind and the curd, as compared to the prevalence of these organisms found through high-throughput community sequencing in either B) the curd or C) the rind. The green "other" category in Figure 1 represents bacteria identified by Illumina sequencing but not detected by culturing.

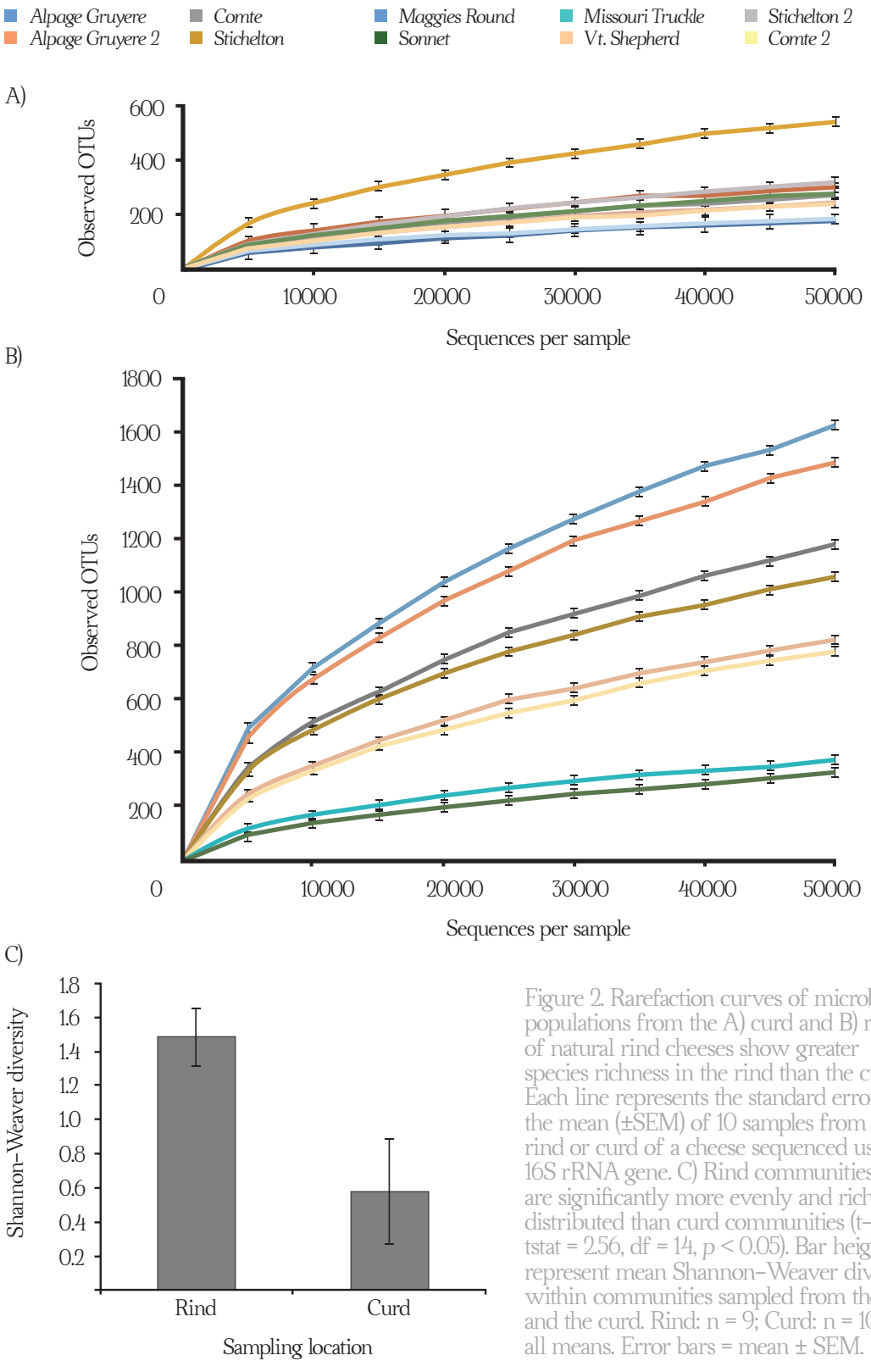


Figure 2. Rarefaction curves of microbial populations from the A) curd and B) rind of natural rind cheeses show greater species richness in the rind than the curd. Each line represents the standard error of the mean (±SEM) of 10 samples from the rind or curd of a cheese sequenced using 16S rRNA gene. C) Rind communities are significantly more evenly and richly distributed than curd communities (t-test, tstat = 2.56, df = 14, $p < 0.05$). Bar heights represent mean Shannon-Weaver diversity within communities sampled from the rind and the curd. Rind: n = 9; Curd: n = 10 for all means. Error bars = mean ± SEM.

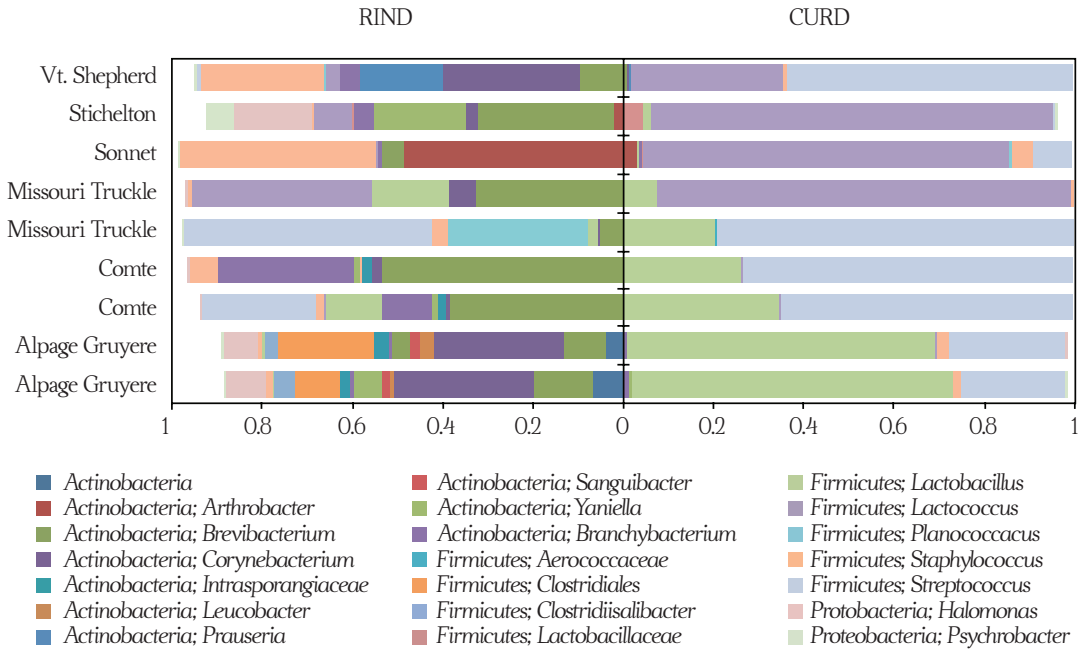


Figure 3. Relative abundance of microbial phyla and genera from the rind and curd of six natural rind cheeses. Horizontal bars represent microbiome samples from six cheeses in the rind and the curd and are colored according to the microbial phyla and genus found in these environments through Illumina sequencing of the 16S rRNA gene. Darker shades represent *Actinobacteria* while lighter shades represent either *Firmicutes* or *Proteobacteria*. Represented organisms were found at ≥ 2% relative abundance.

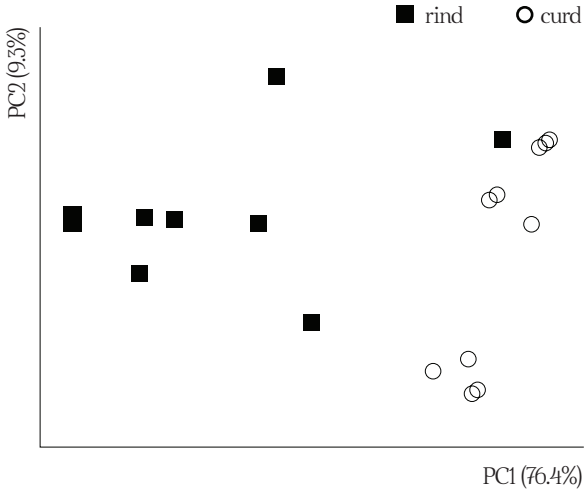
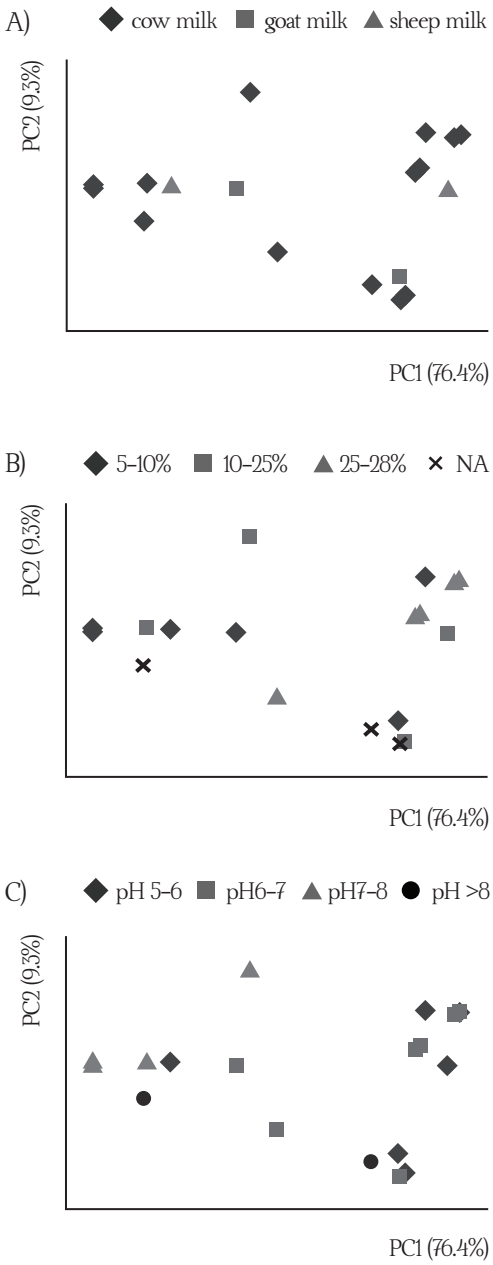


Figure 4. Principal coordinate analysis of rind and curd microbial communities. Clustering is seen with rind samples and curd samples, regardless of the cheese type. A 16S rRNA gene dataset was analyzed with QIIME and R was used to generate the principal coordinate analysis. Each white or black mark represents averaged community composition data of the rind or curd for the cheese sampled.



Supplementary Figure 1. Principal coordinate analysis of weighted Unifrac showing clustering based on other abiotic factors without consideration for rind or curd sampling location. A) Milk type B) Moisture and C) pH. Values range from 5.04 to 8.24.

ISOLATES FROM U.S. CHEESES DISPLAY HIGHER OVERALL SUSCEPTIBILITY TO ANTIBIOTICS THAN EUROPEAN CHEESES

To explore the outcomes of potential interactions between bacteria and fungi in the cheese communities from regionally diverse cheeses, inhibition of bacterial growth by fungal and bacterially derived antimicrobials was examined for cheese isolates. Isolate susceptibility levels were compared between cheeses and their two respective geographical regions of origin: the U.S. and Europe (Figure 5). Thirty-five bacterial isolates sampled from six cheeses were tested for their susceptibility or resistance to six antibiotics on nutrient agar plates: ampicillin (AM10), penicillin (P10), erythromycin (E15), rifampin (RA5), neomycin (N30), and novobiocin (NB30). Among the six cheeses examined, French Comte has the highest percentage of resistant isolates while U.S. Maggie's Round and Sonnet cheeses have the lowest percentage of resistant isolates (Supplementary Figure 2). Between the two geographical regions, fifty-five percent of the total resistant isolates belong to the European cheeses while bacterial isolates from the U.S. cheeses constitute seventy percent of the total susceptible isolates (Figure 5A and 5C). Compared to European cheese isolates, American cheese isolates showed larger percentages of intermediate susceptibility when exposed to ampicillin (AM10), penicillin (P10), and rifampin (RA5) (Figure 5B). Isolates from the three European cheeses exhibited higher percentages of resistant isolates, especially those isolated from French Comte (Supplementary Figure 2A). In general, American cheese isolates revealed higher percentages of susceptible isolates, especially Maggie's Round and Sonnet (Supplementary Figure 2). However, the small and uneven numbers of cultured isolates and cheese types for each of the regions limited the ability to further analyze the correlation between the cheese origin and susceptibility to antibiotics.

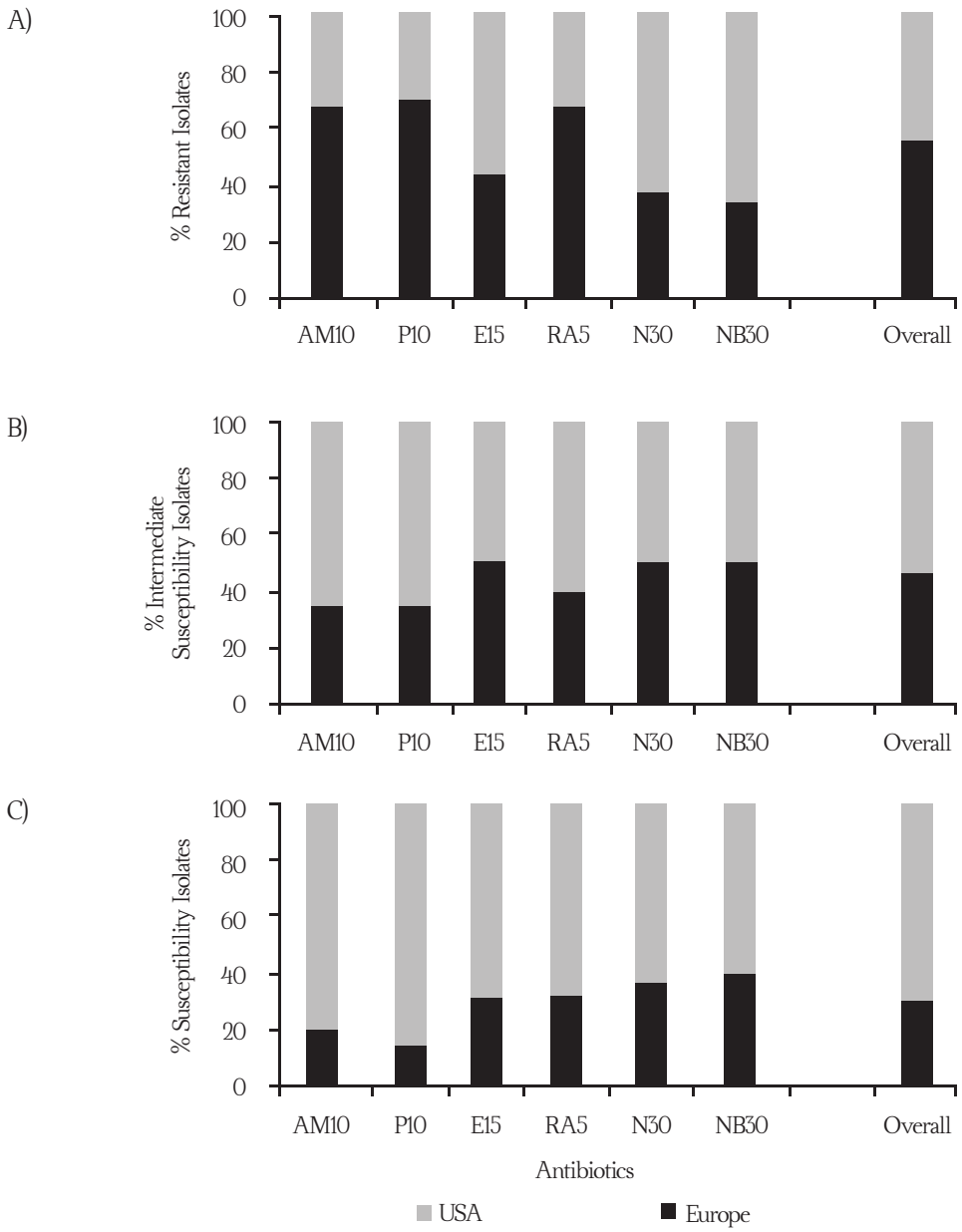
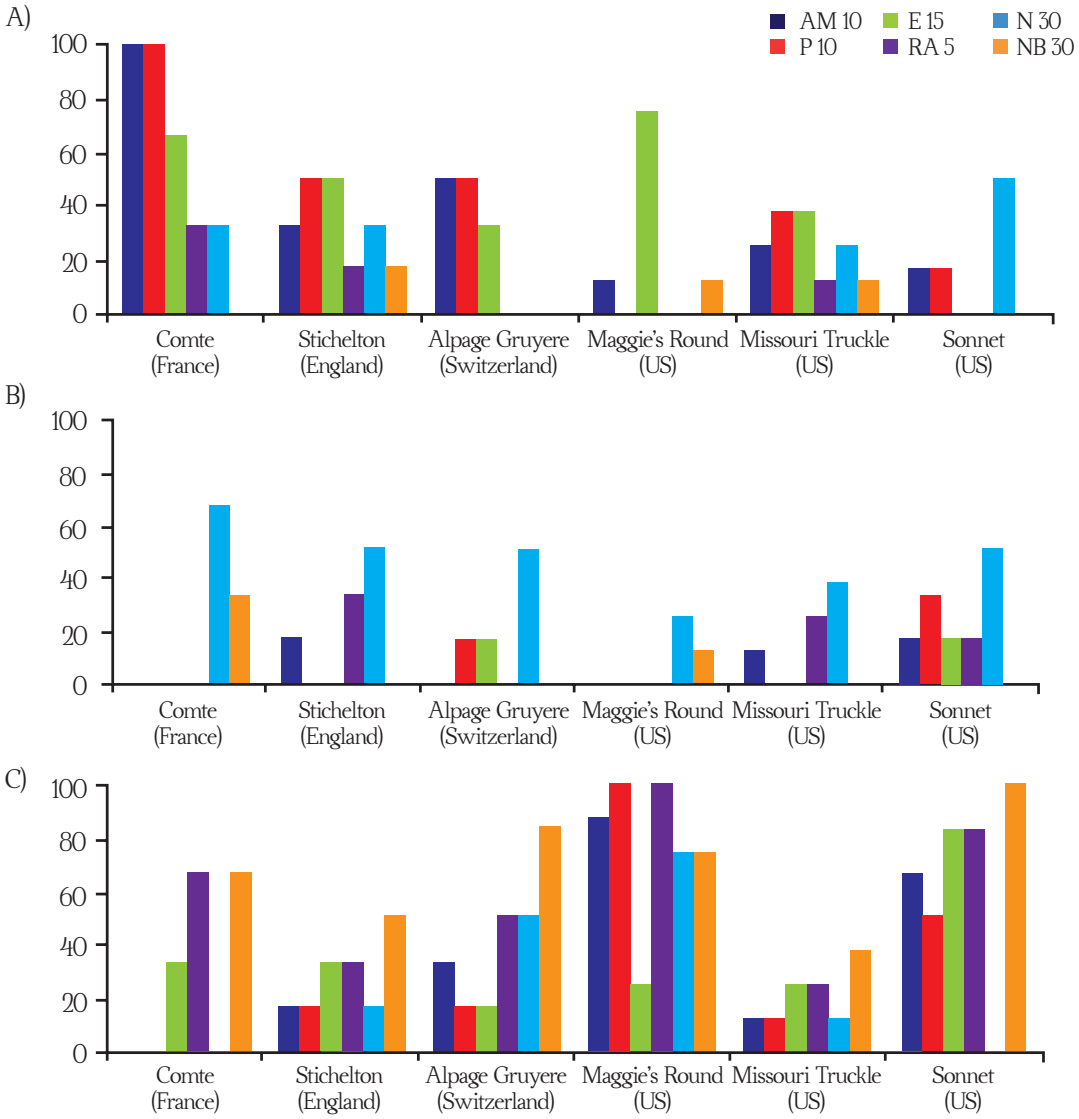


Figure 5. Antibiotics assay reveals U.S. cheese bacterial isolates constitute a larger percentage of the total susceptible cheese isolates than European cheese bacterial isolates. Growth inhibition by six distinct antibiotics was tested among 35 bacterial isolates sampled from 6 cheeses, which represent two geographical regions, Europe (blue) and USA (red). A) Resistant isolates, B) Intermediate susceptible isolates and C) Susceptible isolates. Diameter measurements of the zone of clearance (in mm) were grouped into the following susceptibility categories: a) Resistant (13 mm or less); b) Intermediate Susceptible (14 – 16 mm); and c) Susceptible (17 mm or more). Cheeses: [France] Comte, [England] Stichelton, [Switzerland] Alpage Gruyere, and [USA] Sonnet, Missouri Truckle, and Maggie's Round. AM10 = Ampicillin 10 ug; P10 = Penicillin 10 IU/IE/UI; E15 = Erythromycin 15 ug; RA5 = Rifampin 5 ug; N30 = Neomycin 30 ug; NB30 = Novobiocin 30 ug.



Supplementary Figure 2. Antibiotic assay shows a higher percentage of European cheese bacterial isolates that are resistant to the antibiotics tested than U.S. cheese bacterial isolates. Susceptibility level to six distinct antibiotics was tested among 35 bacterial isolates sampled from 6 cheeses, which represent two geographical regions, Europe: France (Comte n=3 isolates), England (Stichelton n=6 isolates), Switzerland (Alpage Gruyere n=6 isolates), and USA: (Sonnet n=6 isolates, Missouri Truckle n=6 isolates, and Maggie's Round n=8 isolates). Diameter measurements of the zone of clearance (in mm) were grouped into the following susceptibility categories: A) Resistant (13 mm or less); B) Intermediate Susceptible (14 – 16 mm); and C) Susceptible (17 mm or more). Percentages of isolates belonging to one of the three susceptibility levels against each of the six antibiotics examined were plotted for all six cheese types. AM10 (blue) = Ampicillin 10 ug; P10 (red) = Penicillin 10 IU/IE/UI; E15 (green) = Erythromycin 15 ug; RA5 (purple) = Rifampin 5 ug; N30 (cyan) = Neomycin 30 ug; NB30 (orange) = Novobiocin 30 ug.

Community level physiological profiling (CLPP) on both cheese community samples and cultured isolates was performed to examine their metabolic potential and diversity through their utilization of 31 distinct carbon sources (46) (Supplementary Table 2). We expected that 1) cheeses with greater taxonomical diversity would also have community samples that are more metabolically active, with higher numbers of utilized carbon sources, than their counterparts; and 2) cheese community samples would be able to utilize higher numbers of carbon sources than their respective individual isolates within the same cheese. Metabolic diversity (CMD), defined as the number of carbon sources utilized by the sample, increased for all isolates and for whole cheese communities over time (Figure 6; Supplementary Figure 3). Alpage Gruyere community sample was found to be the most metabolically active, with the highest number of utilized carbon sources (23 carbon sources) (Supplementary Table 2). Consistent with our first hypothesis, Alpage Gruyere rind was also the most taxonomically diverse out of all the examined rind communities, containing the most observed OTUs (Figure 2B). However, the second and third most metabolically active cheese community samples, Missouri Truckle (21 utilized carbon sources) and Maggie's Round (19 utilized carbon sources), respectively, contained the least numbers of OTUs in their curd samples (Supplementary Table 2 and Figure 2A). This inconsistency may be attributed to the overall lower taxonomical diversity in the curd communities and that the community samples collected for metabolic analysis were mainly derived from cheese rinds, contributing higher diversity. In addition, with the exception of the Stichelton cheese, cheese microbial community samples utilized more carbon sources than their respective individual isolates at the end of the 7-day sampling period, partially confirming our expectation that, in general, community samples are more metabolically active compared to their respective isolates (Supplementary Figure 3). A closer examination of the top carbon sources utilized by the cheese community samples revealed that there was large diversity of top carbon sources utilized. Cyclodextrin, tween-40 and Alpha-D-Lactose were the top carbon sources most frequently found, though they were the top carbon sources in only two cheeses each. None of the cheeses metabolized the phosphate-activated substrates (glucose-1-phosphate and alpha glycerol phosphate).

DISCUSSION

Cheese is an excellent model system for studying the mechanisms and patterns of microbial diversity because the microbial communities form under controlled and easily manipulated conditions. While the succession of the microbial community during the early curd and rind formation has been well characterized, the differences between the microbial composition of the mature rind and curd are not well understood, especially when compared between different cheeses (1, 10, 16, 20, 21). We characterized the microbial communities present within the mature rinds and curds of seven different natural rind cheeses using culture-independent, high-throughput Illumina sequencing and culture-dependent Sanger sequencing of the 16S rRNA gene. These seven cheeses vary in their geographic region of production and milk type used in the cheese-making process. Additionally, we examined the antibiotic susceptibility and carbon source utilization of the microbial communities in each of the cheeses.

We found that sampling site (curd or rind) was a strong predictor of community structure but milk type (cow, goat, or sheep), geographic origin, moisture content, and pH had little influence on the microbial community structure, in contrast to Wolfe and colleagues, 2014. It was found that rinds of all seven of the cheeses had greater microbial richness, measured as the number of unique OTUs, compared to the respective curds of the seven characterized cheeses (Figure 4B). The only phylum represented within the curds of all seven cheeses, except for that of Sonnet, was *Firmicutes*. *Streptococcus*, *Lactobacillus* and *Lactococcus*, which are genera commonly used as starting cultures during cheese production, were the most abundant genera in the curd and have been previously shown to dominate the curd even in cheese made without starter cultures (1, 6, 16, 17). In contrast, the rinds of these cheeses hosted community members from the *Firmicutes*, *Actinobacteria*, and *Proteobacteria* phyla and, compared to the curd, had a greater relative abundance of the known rind colonizers *Brevibacterium* and Actinomyceteacae (49).

The differences in community composition and complexity between the rind and the curd is likely due to differential exposure to environmental conditions during ripening. While the curd is an anaerobic environment that is largely protected from environmental exposures, the rind is exposed to ambient air and is in direct contact with the surface on which the cheese is aged, providing opportunities for colonization and succession of the rind microbial community by secondary microorganisms from the environment. The colonizing microbes from the environment can influence characteristics of the rind such as pH that can influence further colonization and succession. For example, de-acidification of the rind by certain species of yeast facilitates the colonization and succession of microbes that prefer more alkaline environments such as *Corynebacterium*, *Brevibacterium* and *Brachybacterium*, in keeping with our observation of an increase in relative abundance of *Brevibacterium* in rind samples (27, 37).

ANTIBIOTIC ASSAYS

Another contributing factor to cheese community structure is the interaction between bacteria and fungi. In many cases, interactions among bacteria and yeast may prevent pathogens (27, 29), and opportunistic pathogens like *Staphylococcus aureus* from dominating the rind community and spoiling food (3). A major means of interaction involves the release of antimicrobial chemical compounds from one microbe to the other (23). From the antibiotic susceptibility assays, we observed that isolates taken from microbial communities in the rind and curd of six of the seven cheeses were resistant to a variety of antibiotics. In order to survive on the rind, bacteria most likely develop resistance mechanisms to these antibiotics and mycotoxins produced by species known to inhabit cheese, such as that of *Penicillium nalgiovense* (2, 8, 13, 18, 19, 24, 38).

Our limited analysis suggested that American cheeses overall have a higher susceptibility to antibiotics than do European cheeses. We speculate that the difference we observed could be ascribed to the different mechanisms by which European and American cheeses are aged. European cheeses are more commonly aged on older surfaces than American cheeses, such as wooden shelves or caves constructed centuries ago. Thus, the microorganisms on such surfaces could have had an opportunity to develop resistance over a longer period of time. The documentation of antibiotic resistance within cheese microbiota is a public health concern due to the possibility of a transfer of resistance to pathogenic bacteria in the human colon upon consumption (42). Consequently, an understanding of the stability, diversity, metabolism, and antimicrobial resistance of rind and curd microbiota would advance cheese production and safety.

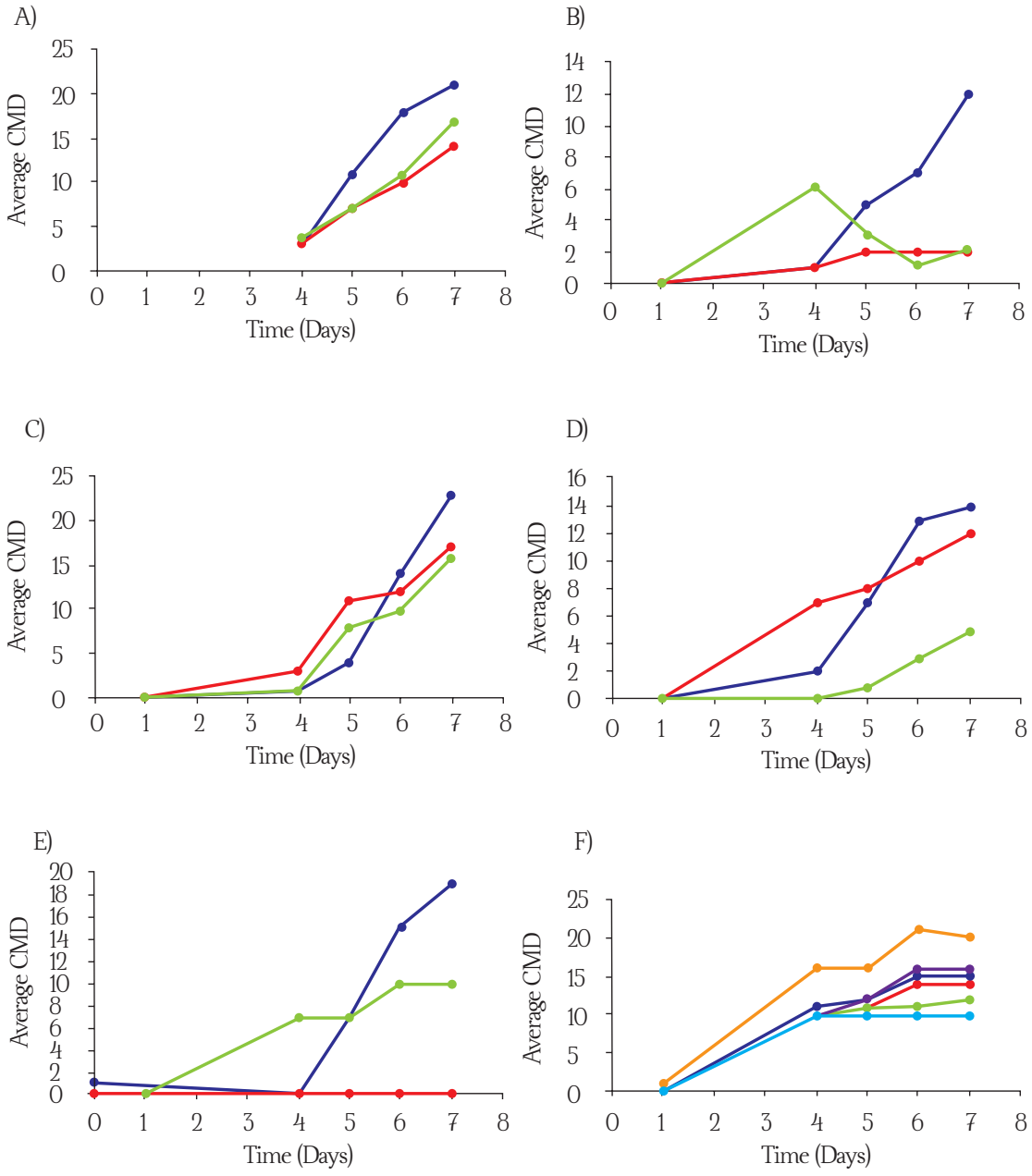
Supplementary Table 2

Cheese Community Sample
Isolate Sample
Curd Community Sample
Utilized Carbon Source

	MISSOURI TRUCKLE	*BRACHYBACTERIUM AIMENTARIUM*	*STAPHYLOCOCCUS WARNERI*	*ALPAGE GRUYERE*	*CORYNEBACTERIUM CASEI*	*BREVIBACTERIUM AURANTIACUM*	*COMTE*	*UNCLEAR*	*UNCLEAR*	*MAGGIE'S ROUNDS*	*BACILLUS MOJAVENSIS*	*UNCLEAR*	*STICHELTON*	*GREEN CURD*	*WHITE CURD*	*UNCLEAR*	*UNCLEAR*	*UNCLEAR*
Pyruvic acid methyl ester	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	0
Tween-40	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Tween-80	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1‡	1‡	0
Cyclodextrin	1	1	1	1	1	1	1	1	1	1	0	1	1*	1	0	0	0	0
Glycogen	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	0
D-Cellobiose	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
alpha-D-Lactose	1*	0	0	1*	0	0	1	1	0	1*	0	0	0	1	0	1	0	0
Beta -Methyl-D-Dlucoside	1	1	1	0	1	1	0	1‡	0	0	1	0	0	0	0	0	0	0
D-Xylose	1	1	1	1	1	1	1*	0	0	1	1	1	0	0	0	0	0	0
I-Erythroitol	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1	1	1
D Mannitol	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1
N-Acetyl-D-Glucosamine	1	1	1	1	1	1	1*	0	0	1	1	1	0	0	0	0	0	0
D-Glucosaminic Acid	1*	0	0	0	0	0	0	1‡	0	0	0	1‡	1	1	1	1	1	1
Glucose-1-Phosphate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D,L-alpha-Glycerol Phosphate	0	0	0	0	0	0	0	1‡	0	0	0	0	0	0	0	0	0	0
D-Galactonic acid, gamma-Lactone	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1
D-Galacturonic Acid	1	1	1	1	1	1	0	0	0	1*	0	0	1	1	1	1	1	1
2-Hydroxy Benzoic Acid	0	0	0	1*	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4-Hydroxy Benzoic Acid	0	0	0	1*	0	0	0	0	0	1*	0	0	1	1	1	1	1	1
Hydroxybutyric Acid	0	0	1‡	1*	0	0	0	1‡	0	0	0	0	0	0	0	0	0	0
Itaconic Acid	0	0	0	1*	0	0	0	0	0	1*	0	0	1	1	1	1	1	1
Alpha-Ketobutyric acid	0	0	0	1*	0	0	0	0	0	1*	0	0	0	0	0	1‡	0	0
D-Malic acid	0	0	0	1	0	1	1*	0	0	1*	0	0	1	1	1	1	1	1
L-arginine	1*	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1
L-asparagine	1	1	1	1	1	1	0	0	0	1	1	0	1	1	1	1	1	1
L-Phenylalanine	1*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-Serine	1	0	1	1	1	0	0	0	0	0	1	1	0	0	0	1‡	1‡	0
L-Threonine	1	0	1	1	1	1	0	0	0	0	1‡	0	0	0	0	1‡	1‡	0
Glycyl-L-Glutamic Acid	1*	0	0	1	1	1	1*	0	0	1	0	1	1	0	0	1‡	1‡	0
Phenylethyl-amine	1*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1‡	0	0
Putrescine	0	1	1‡	1	1	1	1*	0	0	1*	0	0	0	0	0	0	0	0
TOTAL	*21*	*14*	*17*	*23*	*17*	*16*	*14*	*12*	*5*	*19*	*13*	*10*	*15*	*14*	*12*	*20*	*16*	*10*

** Carbon source utlized only in cheese community and/or curd community sample(s)*

‡ Carbon source utilized only in isolate sample(s)



Supplementary Figure 3. Carbon source utilization profiling shows that, in general, cheese community utilizes higher number of carbon sources at the end of the seven day sample period than the individual isolates, with the exception of two isolated from Stichelton. Blue = cheese community sample; Red = first isolate; Green = second isolate; A) Missouri Trucke; B) Vermont Shepherd; C) Alpage Gruyere; D) Comte; E) Maggie's ROund; F) Stichelton: Green Curd (Red), White Curd (Green), Yellow Isolate (Purple), White Isolate (cyan), Orange Isolate (Orange).

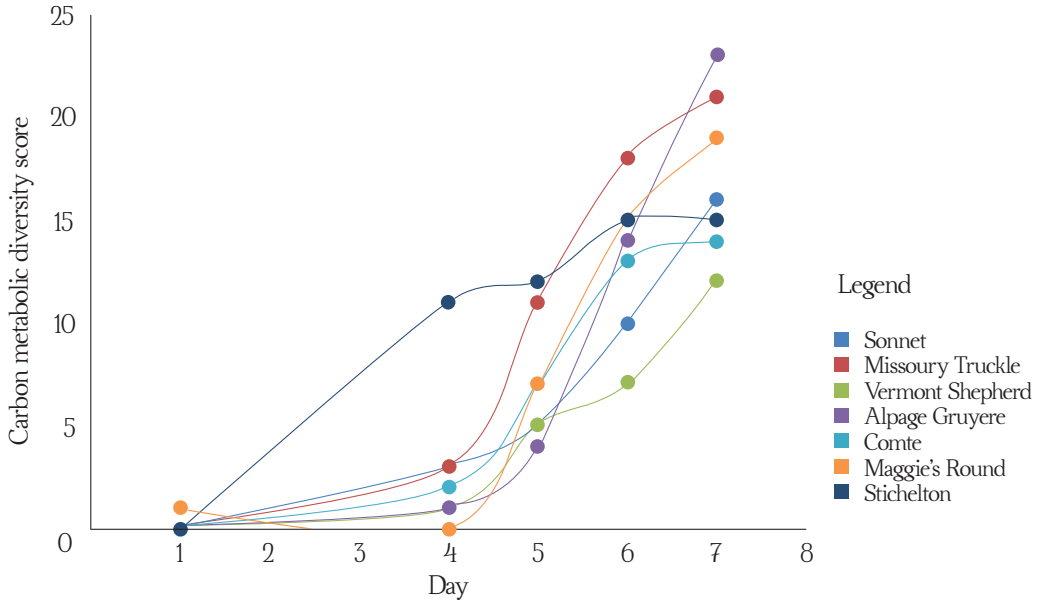


Figure 6. Diversity of carbon metabolism in cheeses over a seven-day period. Utilization of a carbon source was determined by measuring the reduction of tetrazolium salts WTS-1 and WTS-2 to fluorescent purple formazans with the BIOLOG Community-level physiological profiling (CLPP) kit and protocol. For a list of carbon sources see Supplementary Information.

CARBON SOURCE UTILIZATION

An alternative to measuring taxonomic diversity in a microbial community is measuring functional diversity, in this case the composite signature of various microbial metabolic pathways (34, 39). Community-level physiological profiling (CLPP) was conducted on microbiota and isolates from the rind and curd over a seven-day period using the BIOLOG EcoPlate™ assay (46). Sources included detergents, amino acids, and simple and complex sugars, among other compounds. The number of unique carbon sources utilized, here described as metabolic diversity (CMD), increased for all isolates and communities over time, albeit at different rates (Figure 6; Supplementary Figure 3).

It appeared that digestion by some enzymes occurred more quickly than others. Stichelton had the most unique metabolic signature, and was not able to metabolize any of the commonly used polysaccharides (cyclodextrin, xylose and N-acetyl-d-glucosamine). Perhaps this metabolic signature is related to the fact it is a blue cheese and there is contact with the mold inside the cheese. Further study is needed to elucidate a relationship between community composition and metabolic functions including mineralization. Various rind communities have shown to be largely culturable and reproducible (49) suggesting that such study is possible.

Table 2. Top carbon sources utilized by the cheese community samples and their respective isolates for five cheeses: Missouri Truckle, Alpage Gruyère, Comte, Maggie's Round, and Stichelton. Total CMD and Top Carbon Sources refer to that of the whole cheese microbial community samples; CMD in Isolates and Top Carbon Sources per Isolate refer to the individual isolate samples.

Cheese Type	*Isolate*	*Total CMD*	*CMD in isolate*	*Top Carbon Sources*	*Top Carbon Sources per Isolate**
Missouri Truckle	*Brachybacterium*	21	14	Glycogen Alpha-D-Lactose	D-Mannitol D-cellobiose Glycogen
	Staphylococcus	21	17	Glycyl-L-Glutamic	D-cellobiose D-mannitol Glycogen
Alpage Gruyère	*Corynebacterium*	23	17	Alpha-D-lactose Tween-80	D-cellobiose D-Mannitol N-acetyl glucosamine
	Brevibacterium	23	16		D-cellobiose Cyclodextrin Tween-80
Comté	(unclear) #3	14	12	D-cellobiose Cyclodextrin Tween-40	D, L-alpha-glycerol, Phosphate, Beta-methyl-D-glucoside, Cyclodextrin
	(unclear) #9	14	5		Tween-40, Cyclodextrin D-cellobiose
Maggie's Round	Bacillus	19	13	D-mannitol Cyclodextrin	Tween-80, Beta-mthyl-D-glucoside, D-mannitol
	unclear- #10	19	10	Tween-40	Cyclodextrin, Tween-40, N-acetyl-glucosamine
Stichelton	Green curd	15	14	D-Malic acid L-asparagine D-glucosaminic acid	D-malic acid, L-asparagine, D-glucosaminic acid
	Orange isolate Unkown		20		D-glucosaminic acid, D-malic acid, L-asparagine
	White curd		12		D-glucosaminic acid, D-malic acid, L-asparagine
	White isolate Unknown		10		L-asparagine, D-glucosaminic acid, D-malic acid
	Yellow isolate Unknown		16		D-glucosaminic acid, D-galactuonic acid, L-asparagine

Cheese communities maintained higher CMD than isolates (Supplementary Figure 3). It is likely that the purified isolates did not represent the majority of the community. Secondly, many isolates purified from cheese rinds were exposed to substrates typical of milk curds in the EcoPlate™ assay (Table 2). Rind microbes are not selected for in an environment with a predominance of substrates found in raw milk. Strikingly, in the Missouri Truckle cheese, isolates were able to digest two carbon sources that were inaccessible by the community (Supplementary Table 2). This suggests that those microbes that are best suited to aerobic growth on agar plates may not accurately represent the taxonomic or functional makeup of the community. Given that most CLPP of cheese microbes have used exclusively culture-dependent methods (44, 47) our findings suggest that future analyses of cheese community metabolism using CLPP should incorporate culture-independent methods in addition to culture-dependent methods.

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CHARACTERIZATION OF PRODIGININE COMPOUNDS PRODUCED BY A *VIBRIO* SPECIES ISOLATED FROM SALT FLAT SEDIMENT ALONG THE FLORIDA GULF COAST

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- Prodigiosin
- *Vibrio*
- antimicrobial
- biosynthesis
- prodiginine

ABSTRACT

Prodiginines are secondary metabolites produced by several known species of bacteria. These metabolites are known for their bright pigmentation and their potential medicinal uses. Biosynthesis of prodiginine compounds, including the well-studied prodigiosin, has been well characterized in *Serratia marcescens* and other bacterial species, including several marine bacteria. In an effort to isolate and identify natural products from marine organisms, an environmental sample was taken from a salt flat along the Florida Gulf Coast and cultured for bacterial growth. A bacterial species that produces a vibrant pink pigment was isolated and identified as a member of the *Vibrio* genus and was named MI-2. Whole genome sequencing identified a 13-gene operon with homology to the *S. marcescens* prodigiosin biosynthetic operon. The pigment produced by MI-2 was hypothesized to be composed of prodigiosin or related prodiginine compounds and was purified by flash column chromatography and identified by mass spectrometry.

INTRODUCTION

The prodiginine family of bacterial alkaloids includes many vibrantly pigmented compounds, most of which are red, produced as secondary metabolites by a variety of bacterial species. The most notable of these is *Serratia marcescens* from which the best studied prodiginine, prodigiosin, was first isolated in pure form and structurally charcterized (14, 19). Interest in the prodiginines comes not only from their strong red pigment but also from their potential medicinal uses. In addition to the antimalarial activity of prodigiosin itself (4), prodigiosin and other prodiginine derivatives have been shown to have immunosuppressive functions

with novel mechanisms of action (7, 21, 30, 38, 42) and apoptotic effects in human cancer cells (11, 27, 33, 42).

Since the discovery of prodigiosin in *S. marcescens*, prodiginine compounds have been discovered in other bacterial organisms, including *Streptomyces coelicor A3(2)*, *Alteromonas rubra*, *Hahella chejuensis*, and *Vibrio gazogenes* (9, 19). Biosynthesis of prodigiosin has been best studied in *S. marcescens*, *S. coelicor*, *and H. chejuensis*. In *S. marcescens* ATCC 274, the prodigiosin biosynthesis (*pig*) cluster consists of 14 genes abbreviated as *pigA* through *pigN* arranged as an operon that are transcribed as a single 14-gene polycistronic mRNA (17). *H. chejuensis* KCTC 2396 also contains 14 prodigiosin biosynthetic genes, *hapA* through *hapN*, that are similar in their gene layout to that of *S. marcescens* (22). In *S. coelicor* A3(2), the *red* cluster is responsible for prodiginine biosynthesis (5). The genetic arrangement of the *red* cluster is significantly different from *S. marcescens* and *H. chejuensis*, however the presence of 12 homologous genes between it and the *pig* operon suggests the two biosynthetic pathways are similar (17).

Several of the known prodiginine producers, including *H. chejuensis*, *V. gazogenes*, and *A. rubra* are marine microorganisms. The isolation of natural and potential medicinal products from diverse marine microorganisms has been described recently, with several new products originating from microorganisms isolated from marine environments (2, 3, 45). We report here the isolation of a prodiginine-producing *Vibrio* species known as Marine Isolate-2 (MI-2) from a marine salt flat environment along the central Gulf Coast of Florida. Originally isolated as a marine antibiotic-producing bacterium, MI-2 was unique in its ability to produce a distinctly pink prodiginine product under appropriate media conditions. Whole genome sequencing of MI-2 identified a 13-gene prodiginine biosynthetic gene cluster. The analysis of this prodiginine biosynthetic pathway and structural identification of the purified prodiginine compounds are discussed.

MATERIALS AND METHODS

ISOLATION AND GENUS IDENTIFICATION OF MI-2

MI-2 was isolated from a salt flat sediment sample aseptically obtained from Leffis Key in Bradenton, FL. The sediment sample was diluted in 0.5 M NaCl to 10^{-5} grams soil/ml and grown on 0.5 M NaCl potato dextrose agar (PDA) modified with the following contents: tryptone (2 g/l), NaCl (0.5 M), glucose (5 mM), and 10X Neidhardt MOPS salts (0.1X final concentration). The Neidhardt MOPS salt components were prepared as described (32). To inhibit fungal growth, 100 µg/l cycloheximide was added to the initial isolation plates. After initial isolation of MI-2, the organism was maintained on 0.5 M NaCl modified PDA plates and 0.5 M NaCl LB agar plates and stored at 25°C.

Identification of the genus of MI-2 was completed by whole colony PCR amplification of the 16S rDNA. The 16S primers 63F (5'-CAG GCC TAA CAC ATG CAA GTC – 3') and 1387R (5' – GGG CGG WGT GTA CAA GGC – 3') obtained from Integrated DNA Technologies (IDT) were used to amplify the gene using the MyTaqTM mix obtained from Bioline (Taunton, MA). The whole colony PCR reaction conditions were: 1 cycle of 95°C for 10 minutes; 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute; and 1 cycle of 72°C for 5 minutes. Successful PCR amplification was confirmed by agarose gel electrophoresis on a 1% agarose gel in 1X TAE buffer and purified using the Isolate II PCR and Gel Kit from Bioline. Purified PCR products were sequenced at the DNA Analysis Facility on Science Hill at Yale University.

MEDIA AND GROWTH EFFECT ON PIGMENT PRODUCTION

The effect of media composition and NaCl concentration on the pigment production of MI-2 was determined qualitatively by quadrant streaking MI-2 onto modified PDA plates prepared as described previously with supplementation of 2%, 4%, or 6% NaCl and LB plates supplemented with 2%, 4%, or 6% NaCl. The plates were incubated at 30°C for two days before being photographed. The effect was determined quantitatively by inoculating a colony into modified Potato Dextrose Broth (PDB) and LB liquid cultures at 2%, 4%, and 6% NaCl and incubating at 30°C for 48 hours. Absorbance of the sample was taken by wavelength scan from 400 to 700 nm at 5 nm intervals.

The effect of time on pigment production of MI-2 was determined qualitatively by quadrant streaking MI-2 onto modified PDA or LB plates with supplementation of 2% or 4%. The plates were incubated at 30°C for nine days, and photographs were taken after two and nine days. The effect was determined quantitatively by inoculating a colony into PDB and LB liquid cultures at 2% and 4% NaCl and incubating at 30°C for 48 hours. Absorbance of the sample was taken by wavelength scan from 400 to 700 nm at 5 nm intervals.

GENOMIC SEQUENCING AND IDENTIFICATION OF A PUTATIVE PRODIGIOSIN BIOSYNTHESIS GENE CLUSTER

Genomic DNA from MI-2 was extracted from an overnight culture grown in 4% NaCl LB liquid media using the phenol chloroform method (44). The extracted DNA was checked for integrity by agarose gel electrophoresis and quantified using a NanoDrop 2000 (Thermo Scientific). Whole genome sequencing of the extracted DNA was conducted using HiSeq 2500 technology at Purdue University. The resulting genomic sequence was analyzed using the sequence viewer and annotator tool Artemis (35). The genes with homology to the *pig* gene cluster of *S. marcescens* were identified manually using the National Center for Biological Information (NCBI) protein basic local alignment tool (blastp) (15).

COMPARISON OF PUTATIVE PRODIGIOSIN BIOSYNTHESIS GENE CLUSTER FROM MI-2 AND OTHER ORGANISMS

Comparison of prodigiosin biosynthesis genes from MI-2 and other known prodiginine compound producing organisms *Serratia marcescens* ATCC 274, *Hahella chejuensis* KCTC 2396, and *Streptomyces coelicor* A3(2) was done by aligning amino acid sequences using Clustal Omega for each gene within the cluster (37). Gene sequences were acquired using the NCBI GenBank database and the accession numbers as follows: S. *marcescens* – AJB33002, *H. chejuensis* – DQ266254, and S. *coelicor* – AL645882.

PURIFICATION OF PRODIGIOSIN

An overnight culture of MI-2 in 4% NaCl LB liquid media was centrifuged at 6000 Xg for 10 minutes; the supernatant was discarded and the pellet was resuspended in approximately 2 ml of a methanol and 2 N HCl mixture (24:1). The addition of acid was necessary to break down a suspected prodigiosin-associated protein that may sequester the pigment molecule (14, 24). The resuspended pellets were combined in 35 ml scintillation vials wrapped in aluminum foil and left for 12–18 hours on an orbital rotator. Extracts were then centrifuged at 6000 Xg for 10 minutes to remove any cellular residue. The supernatant was collected, and the solvent was evaporated using a rotary evaporator. Hydrophilic impurities were removed by a series of chloroform–water liquid–liquid extractions that were repeated on the organic layer until the water layer no longer appeared cloudy (1). The chloroform was evaporated *via* rotary evaporation, and pigment was redissolved in acetonitrile. Flash column chromatography was performed using silica gel as the stationary phase and acetonitrile as the mobile phase. Fractions from flash chromatography that corresponded to a pigmented smear at 0.82 to 0.63 retardation factor (Rf) regions on silica thin layer chromatography (TLC) plates with acetonitrile as the mobile phase were combined and concentrated by rotary evaporation.

UV-VIS PH ASSAY

The pH of methanol solvents was adjusted to 1.8, 5.0, 7.0, 8.0, 11.1 and 12.0 using solutions of 1 M HCl and 1 M NaOH. One hundred µl of purified prodigiosin pigment extract was suspended in each methanol solvent, and absorbance spectra were measured from 350 nm to 700 nm at 0.25 nm intervals. A baseline correction was performed for all samples.

MASS SPECTROMETRY

Electrospray Ionization (ESI) tandem mass spectrometry (MS/MS or MS2) was performed using Fourier transform mass spectrometry (FTMS) mode on an LTQ-Orbitrap with helium used as the collision gas. Relative collision energy (rCE) ranged from 30–35 in the ion trap component of the instrument. High-resolution mass spectra were obtained with full width half maximum resolving power of 100,000 at 400 m/z in profile mode.

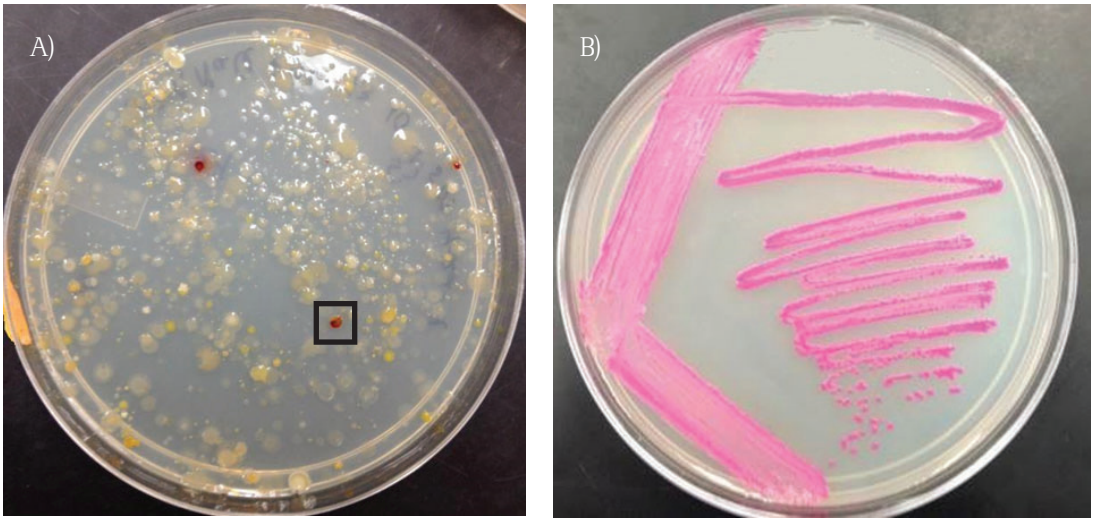


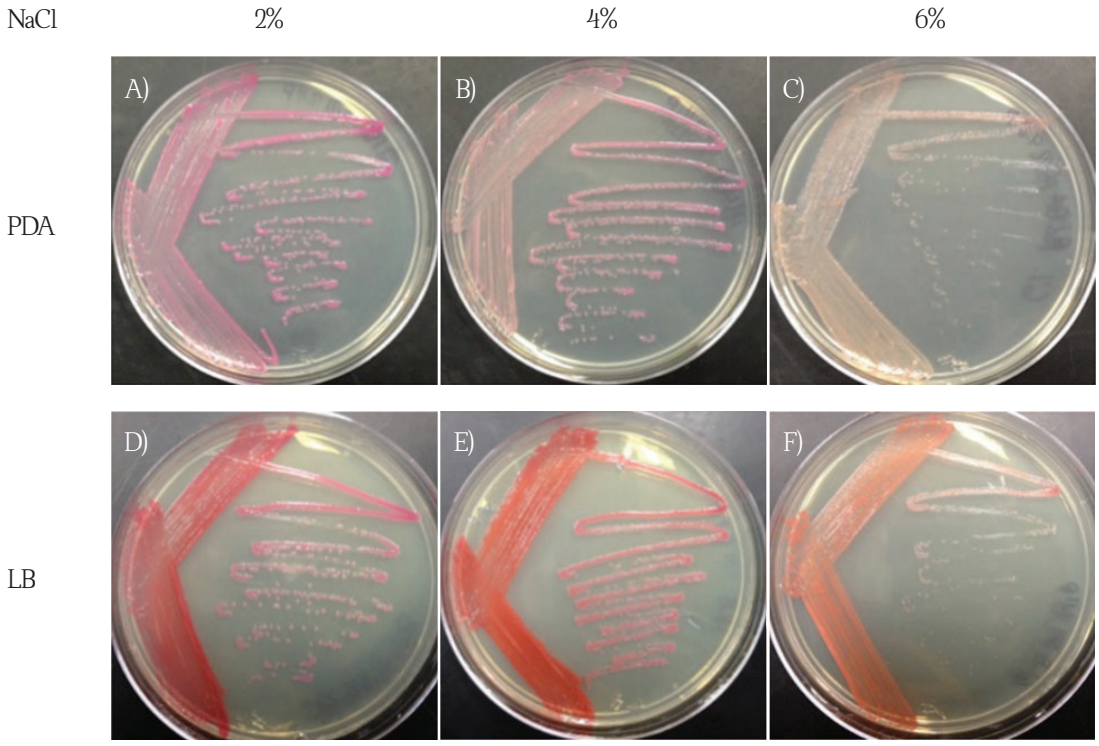
Figure 1. Isolation and Colony Appearance of MI-2
Panel A shows the original isolation of MI-2 from Leffis Key in Bradenton, FL on modified PDA media. The red/pink colony of MI-2 selected for pure culture isolation is shown in the black square. Panel B shows the pure culture and pink pigment production of MI-2 on modified PDA media.

RESULTS

ISOLATION AND GENUS IDENTIFICATION OF MI-2

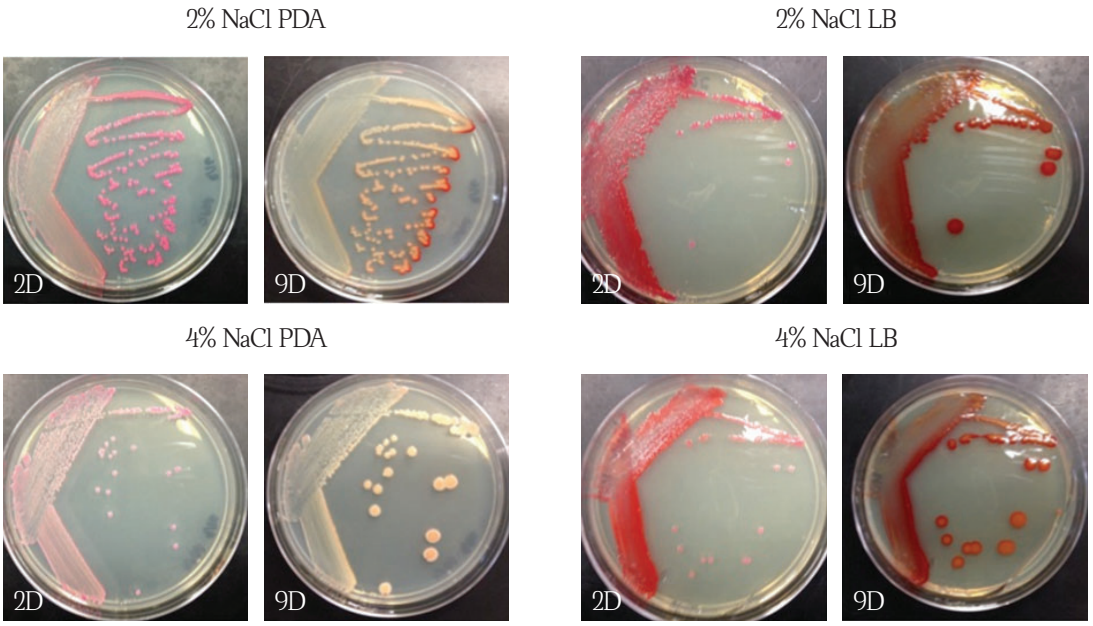
The marine bacterium MI-2 was originally cultured from salt flat sediment obtained at Leffis Key in Bradenton, FL on modified PDA media supplemented with 0.5 M NaCl for marine organisms and tryptone as described in the methods to support growth of fastidious organisms. Figure 1 shows the original marine sediment isolation plate in panel A and the pure culture growth of MI-2 in panel B.

The purpose of this isolation was to identify antibiotic-producing microorganisms from diverse marine environments. MI-2 was identified as an antibiotic-producer (data not shown), but its ability to produce a bright pink pigment when grown on the modified PDA prompted further characterization of this microorganism. Sequencing of the 16S rRNA gene identified the organism as being a member of the *Vibrio* genus with *Vibrio* ruber being the closest related species (data not shown).



Media	*2% NaCl*		*4% NaCl*		*6% NaCl*	
	Max Abs. λ (nm)	*Avg. Abs. (± SE)*	*Max Abs. λ (nm)*	*Avg. Abs. (± SE)*	*Max Abs. λ (nm)*	*Avg. Abs. (± SE)*
PDB	545	1.70 ± 0.14	545	1.87 ± 0.08	540	1.73 ± 0.08
LB	500	0.72 ± 0.02	500	1.12 ± 0.03	500	1.24 ± 0.05

Figure 2. Effect of Media Composition and NaCl on MI-2 Pigment Production
MI-2 was quadrant streaked onto modified PDA and LB plates at the indicated NaCl concentrations and incubated at 30°C for 48 hours. Panels A–C show growth on PDA at 2%, 4%, and 6% NaCl concentrations. Panels D–F show growth on LB at 2%, 4%, and 6% NaCl concentrations. The shade of the pigment is different when grown on PDA compared to LB. This is confirmed by the corresponding table that shows the maximum absorbance wavelength (Max Abs. λ) and the average absorbance value (Avg. Abs.) ± standard error (SE) for three replicates grown in PDB and LB at 30°C for 48 hours.



NaCl Concentration	*PDB*				*LB*			
	Two Days		*Five Days*		*Two Days*		*Five Days*	
	Max Abs. λ (nm)	*Avg. Abs. (± SE)*	*Max Abs. λ (nm)*	*Avg. Abs. (± SE)*	*Max Abs. λ (nm)*	*Avg. Abs. (± SE)*	*Max Abs. λ (nm)*	*Avg. Abs. (± SE)*
2%	545	1.70 ± 0.14	545	2.02 ± 0.30	500	0.72 ± 0.02	450	0.781 ± 0.10
4%	545	1.87 ± 0.08	540	2.30 ± 0.01	500	1.12 ± 0.03	450	1.39 ± 0.20

Figure 3. Effect of Time on MI-2 Pigment Production
MI-2 was quadrant streaked onto LB and modified PDA with 2% and 4% NaCl and incubated at 30°C for nine days. Photographs were taken after two days of growth (2D) and after nine days of growth (9D). The corresponding table shows the Max Abs. λ and Avg. Abs. ± SE for three replicates grown in PDB and LB at 30°C for two days and five days The Max Abs. λ changed dramatically from two days (500 nm) to five days (450 nm) when grown in LB with 2% and 4% NaCl but not in modified PDB at the same salt concentrations. The pigment intensity appeared to be notably decreased after nine days of growth, but this decrease was not reflected quantitatively in the absorbance values.

MEDIA AND GROWTH EFFECTS ON PIGMENT PRODUCTION

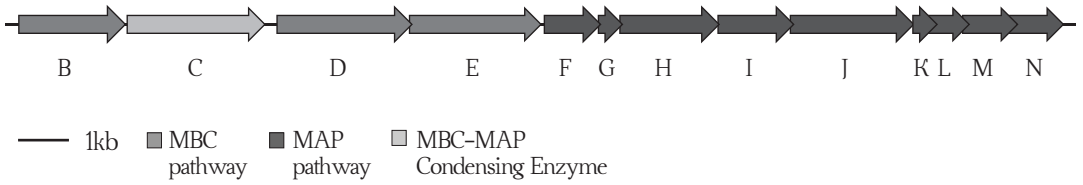
The effects of media (LB vs. PDA) and NaCl concentration (2%, 4%, or 6%) on pigment production of MI-2 are shown in Figure 2. While the pigment produced by MI-2 appeared bright pink on the 2% NaCl PDA plate, it was more red on the 2% NaCl LB plate. This difference was quantitatively confirmed by measuring the absorbance after growth in liquid samples (tabular data in Figure 2). The maximum absorbance wavelength (Max Abs. λ) was 540–545 for PDB and 500 for LB. There was a slight change in the Max Abs. λ for PDB at 4% (545 nm) to 6% (540 nm) which may explain the slight change seen in the pigment color from panels B to C. Pigment production on both media types visually appeared to show a decrease in intensity as the concentration of NaCl in the media increased as seen by comparing panels A to C and D to F, however this decrease was not replicated quantitatively as there is no decreased absorbance seen with higher NaCl concentrations.

The effect of time on pigment production of MI-2 is shown in Figure 3. After nine days of growth, pigment appearance of MI-2 appeared notably altered from where it was after two days of growth. Extensive growth on PDA appeared to result in decreased pigment content, and altered pigment color from red-pink to red-orange was seen after growth on LB. The change in pigment appearance was more dramatic on both media types at 4% NaCl than at 2% NaCl. Quantitative analysis of this (tabular data in Figure 3) confirmed a change in the max. abs. λ from 500 nm to 450 nm when grown in LB while the max. abs. λ of PDB remained relatively unchanged by prolonged growth. Average absorbance values do not show the decreased pigment production that is visible in the figure as values for all samples actually increased from two days to five days.

IDENTIFICATION OF PUTATIVE PRODIGIOSIN BIOSYNTHESIS GENE CLUSTER

In order to better characterize and identify MI-2, its genome was sequenced. After whole genome sequencing and annotation, the putative prodigiosine biosynthesis gene cluster was identified based on homology to the well characterized 14 gene *pig* operon found in *S. marcescens* (17). The MI-2 prodiginine biosynthesis gene cluster is approximately 20.3 kb in length and consists of 13 genes (Figure 4). The overall structure of the MI-2 cluster is highly similar to prodiginine biosynthesis clusters in both the arrangement of protein homologs as well as in the relative sizes of proteins and intergenic spacing. Six of the open reading frames (ORF) overlap with one another, but a significant gap of 177 base pairs was found between the putative *pigC* and *pigD*. This is comparable with the structure of the *pig* cluster in *S. marcescens* as well as the *hap* cluster in *H. chejuensis* (17, 22).

The major difference between the structure of the putative *pig* cluster in MI-2 and *S. marcescens* is the absence of *pigA* in MI-2. A nucleotide alignment of bases preceding *pigB* with the coding nucleotide sequence for *pigA* in *S. marcescens* ATC 2744 showed short regions of homology (data not shown). This suggests that a *pigA* homolog may have been present at one time but has since been lost.



pig Homolog	ORF Size (bp)	Start*	End*
B	2,070	1	2,070
C	2,288	2,440	4,728
D	2,628	4,905	7,533
E	2.556	7,530	10,086
F	1,010	10,170	11,186
G	204	11,197	11,461
H	1,944	11,464	13,408
I	1,467	13,410	14,877
J	2,471	14,821	17,292
K	320	17,302	17,622
L	814	17,563	18,377
M	1,220	18,210	19,430
N	1,094	19,119	20,213

**Nucleotide start and end positions are given relative to the start position of ORF B.*
Table rows highlighted in gray indicate an ORF that overlaps with the ORF preceding it.

Figure 4. Schematic Representation of Putative Prodigiosin Biosynthesis Gene Cluster in MI-2
The cluster is approximately 20.3 kb in length and contains 13 genes. The arrows show the directionality of ORFs. Letters for ORFs correspond to the pigB–N homologs. Different arrow patterns indicate the putative role of the enzyme in the bifurcated prodiginine biosynthesis pathway in which PigC condenses the compound 4-methoxy-2,2-bipyrrole-5-carboxyaldehyde resulting from synthesis of the MBC pathway with the compound 2-methyl-3-n-amyl-pyrrole resulting from the synthesis of the MAP pathway). The corresponding table shows the size of each ORF, the nucleotide start position for each ORF and the nucleotide end position for each ORF.

Table 1. AAI of Prodiginine Biosynthetic Gene Cluster Homologs
The table shows the AAI of MI-2 putative prodigiosin biosynthetic gene cluster homologs compared to known prodiginine biosynthetic proteins from S. marcescens ATCC 274, H. chejuensis KCTC 2396, and S. coelicor A3(2). AAI with MI-2 homologs were calculated using Clustal Omega.

MI-2	*S. marcescens*		*H. cheuensis*		*S. coelicor*	
	Homolog	*% AAI*	*Homolog*	*% AAI*	*Homolog*	*% AAI*
A*	PigA	55	HapA	54	RedW	43
B	PigB	57	HapB	38	RedS	35
C	PigC	69	HapC	54	RedH	40
D	PigD	73	HapD	51	–	–
E	PigE	80	HapE	61	–	–
F	PigF	73	HapF	58	RedI	19
G	PigG	64	HapG	45	RedO	21
H	PigH	72	HapH	58	RedN	59
I	PigI	58	HapI	43	RedM	40
J	PigJ	61	HapJ	33	RedX	
K	PigK	68	HapK	47	RedY	45
L	PigL	17	HapL	19	RedU	19
M	PigM	48	HapM	33	RedV	26
N	PigN	17	HapN	14	RedF	15

PigD and PigE homologs in S. coelicor have not been found.
**PigA homolog is not found within the MI-2 cluster.*

PROTEIN SEQUENCE COMPARISON OF THE PUTATIVE PRODIGIOSIN BIOSYNTHESIS CLUSTER

The amino acid identities (AAI) of the proteins coded for by the putative prodigiosin biosynthesis genes were compared to their homologs of *S. marcescens*, *H. chejuensis*, and *S. coelicor* (Table 1). In addition to the structural homology of the gene cluster, there was also consistent homology between genes in the MI-2 cluster and corresponding genes in the prodiginine biosynthesis operon of the other species. The AAI between MI-2 and Pig homologs typically ranged from 50% to 80%, a comparable range compared with other studies that have shown an AAI of only 23% or more between *Vibrio* and *Serratia* species (16, 20, 39). Exceptions to this can be seen in PigL and PigN homologs, which had very low AAI; however, neither of these two proteins are required for prodigiosin production (43).

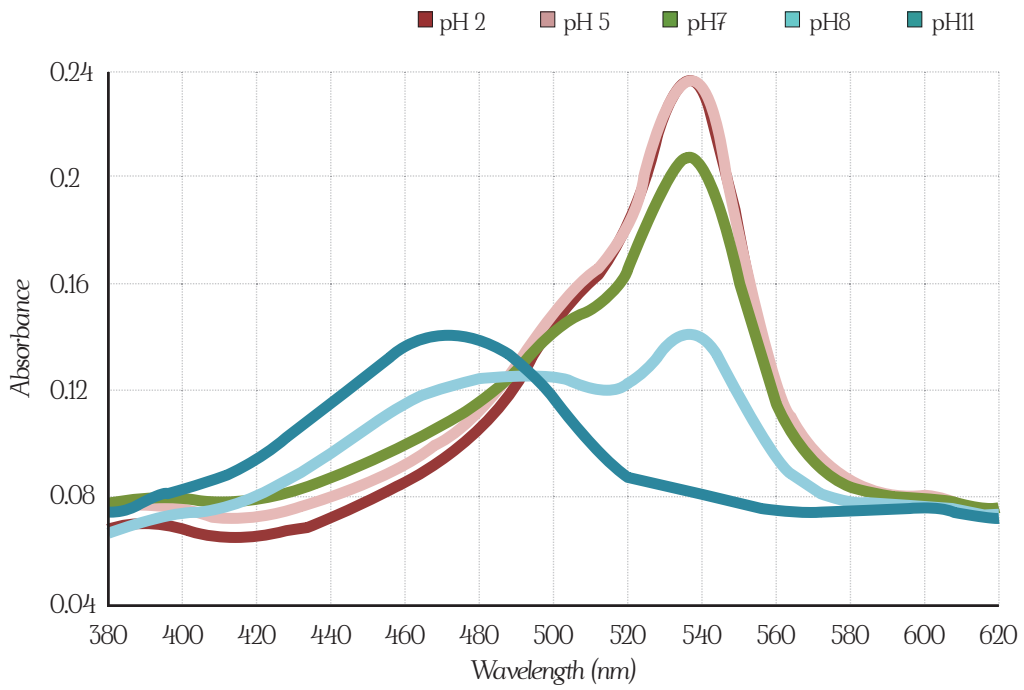


Figure 5. UV-Vis Spectra of MI-2 Pigment Extract at Various pHs
The graph shows the absorbance from wavelengths ranging from 380 nm to 620 nm of MI-2 pigment extract in aqueous methanol solvents at varying pHs. Two maxima can be observed at 470 nm and 535 nm. Absorbance at 535 nm is greater at acidic pH while absorbance at 470 nm is greater at basic pH. An isosbestic point can be observed at 490 nm.

As mentioned previously, there is no homolog for PigA found in the MI-2 putative progidiosin biosynthesis gene cluster. However, a different protein found elsewhere in the MI-2 genome did show homology to PigA. It is the sequence of this putative protein that is used for PigA comparison from MI-2 in Table 1.

PURIFICATION AND UV-VIS SPECTRA OF MI-2 PURIFIED PIGMENT EXTRACT AT DIFFERENT PH

Extraction and purification of the MI-2 pigment was completed by flash column chromatography. The UV-Vis spectra of the purified MI-2 pigment extract resuspended in different pH solutions is shown in Figure 5. The spectra showed two main peaks that depended on the pH of the solvent. This suggests that the compound exists in either a protonated or non-protonated form (34). The peak absorbance for what was presumed to be the protonated form occurred at a wavelength of approximately 535 nm and the non-protonated peak absorbance occurred at approximately 470 nm. These results were consistent with the UV-Vis spectra for prodigiosin (18).

The spectra for the MI-2 pigment extract revealed an isosbestic point at a wavelength of approximately 490 nm. This is the wavelength in which the absorbance remains constant as a conformational change occurs due to the changes in pH of the aqueous methanol. Additionally, the data suggested that the pH at

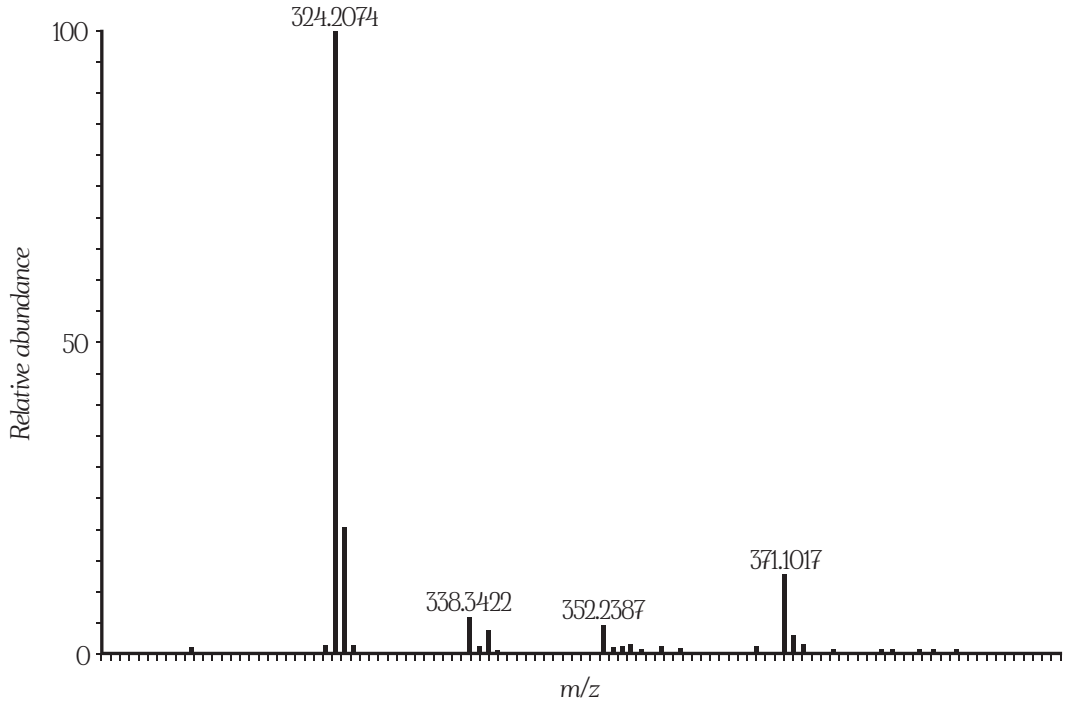


Figure 6. Electrospray Ionization Mass Spectra of MI-2 Pigment Extract
The full mass spectra of the purified MI-2 pigment extract is shown. Peaks represent ions of a particular mass (x-axis) with a relative abundance (y-axis). The peak at 324 is the most prominent while peaks at 338, 352 and 371 appear as minor components.

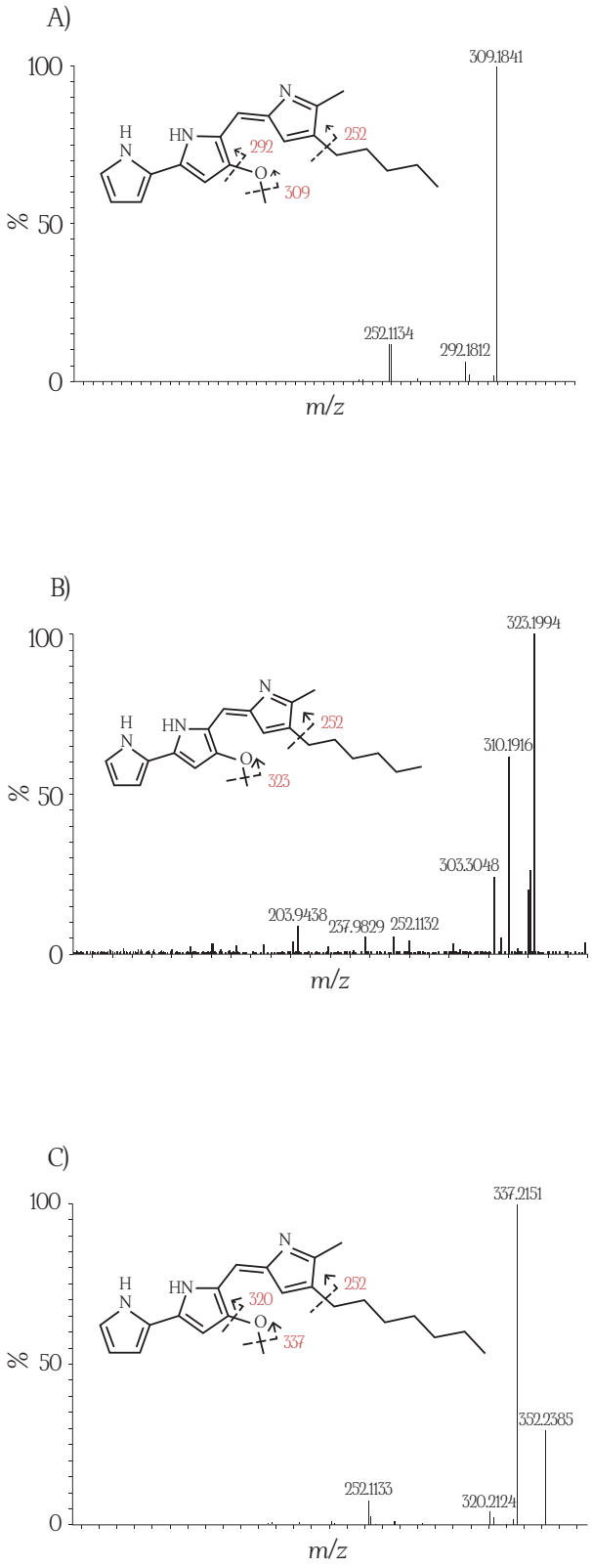
which the absorbance between the two peaks would be equal was just above pH 8, which is consistent with the known pKa value of 8.25 for prodigiosin in acidified ethanol (18).

MASS SPECTRA OF MI-2 PURIFIED PIGMENT EXTRACT

The full mass spectrum of purified pigment extract from MI-2 is shown in Figure 6. This spectrum revealed a mixture of four different compounds in different abundances. A compound with a mass of 324 g/mol was the main component of the mixture, and compounds with masses of 338 g/mol, 352 g/mol and 371 g/mol were present in the extract as minor components.

The tandem mass spectra of each of these four peaks are shown in Figure 7. Tandem mass spectra of compounds with a mass of 324, 338 and 352 exhibited similar fragmentation patterns that were consistent with prodiginine compounds. Specifically, all three contained a peak at 252 m/z, which can be explained by the loss of the alkyl substituent group to yield the 2-methoxy prodiginine core. Additionally, all three showed an ion at a mass consistent with the loss of the methyl group from the methoxy. Compounds 324, 338 and 352 can be represented by compounds with varying lengths of alkyl chains, as there is a mass difference of 14 that can be represented by the addition of a CH_2. The compound with a mass of 271 appeared to be unrelated as it did not contain the prodiginine core 252 peak; however, further analysis would be required to fully elucidate the structure of this compound. The spectrum from this peak is not shown in Figure 7.

Figure 7. Tandem Mass Spectra of Peaks from the Full Mass Spectra
The tandem mass spectra of the relevant prodiginine peaks from the full mass spectra in Figure 6 are shown. Peaks represent ions produced as a result of gas–induced dissociation (GID) of the parent molecule. Panel A shows the tandem mass spectra of a 324 molecular mass molecule with the proposed structure prodigiosin. The most stable ion produced appears at m/z 309 and is the ion produced by cleavage of the methyl on the methoxy group. The peak at 252 represents an ion created as a result of the alkyl chain being cleaved from the molecule and 292 is the loss of the oxygen. These fragmentation patterns are shown with the dashed arrows. Panel B shows the tandem mass spectra of a 338 molecular mass molecule with the proposed structure 2–methyl–3–hexyl prodiginine. The most stable ion produced appears at m/z 323 and is the ion produced by cleavage of the methyl on the methoxy group. The peak at 252 represents an ion created as a result of the alkyl chain being cleaved from the molecule, and the peak at 310 is likely loss of an ethene. Other peaks represented are not readily explainable given the proposed structure. It is possible this sample that is in low abundance is contaminated. Panel C shows the tandem mass spectra of a 352 molecular mass molecule with the proposed structure prodigiosin. The most stable ion produced appears at m/z 337 and is the ion produced by cleavage of the methyl on the methoxy group. The peak at 252 represents an ion created as a result of the alkyl chain being cleaved from the molecule, and 320 is produced after the loss of the oxygen.



DISCUSSION

The biosynthesis of prodigiosin is an interesting metabolic phenomenon as it is a secondary metabolite with no known direct benefit to cellular growth (43). Pure non-pigmented strains of *S. marcescens* show no significant difference in viability from pigmented strains (41). However, in the natural environment, prodigiosin and its derivatives may serve a purpose in bacterial defense as many have antibacterial properties (12, 25).

MI-2 is a prodigiosin-producing marine bacterium of the *Vibrio* genus. Its species designation appears to be closest to *Vibrio ruber*, a known prodigiosin producer (8), though its biochemical test profile differs from *V. ruber* in multiple ways, suggesting MI-2 is a strain of *V. ruber* unique from the wild type (manuscript in preparation). Although prodiginine compounds and the organisms that produce them have been studied for a long time, there is limited research on prodigiosin biosynthesis clusters in different genera. To date, prodigiosin biosynthesis clusters have only been examined in *S. marcescens* ATCC 274, *Serratia* spp. 39006, *H. chejuensis* KCTC 2396, and some *Streptomyces* species (5, 17, 22, 36, 43). The addition of another prodiginine biosynthesis cluster in *Vibrio* species will add more information on the potential catalytic mechanism for each enzyme by revealing key residues in conserved regions of each gene.

The appearance of the prodigiosin pigment production of MI-2 is affected by the media on which it grows and the length of incubation. The pigments produced by growth in LB and modified PDB showed different max absorbances, verifying the different colored apearance seen on the plates in Figure 2. Pigment production on both LB and modified PDA media types appeared to show a decrease in intensity as the concentration of NaCl in the media increased, suggesting an inhibitory effect of excess NaCl on pigment production. This phenomenon could not be validated quantitatively in liquid broth (tabular data of Figure 2), however it is difficult to draw a comparison in intensities from the absorbance values as differences in the concentration of cells in each sample will affect the level of pigment production and therefore the absorbance values reached. These could be further complicated by the regulation of prodigiosin production by quorum sensing (43).

Additionally, increasing the length of incubation from two days to nine days resulted in altered coloration, particularly in LB media, and the appearance of decreased pigment intensity in plates, though this occurrence could also not be reproduced quantitatively in liquid (Figure 3). The qualitative data suggest the pigment produced by MI-2 may not be stable for extended periods of time in the conditions it was grown (at room temperature and exposed to oxygen) as has been shown for other prodigiosin analogs (40). However further testing with a purified sample of the pigment would need to be performed to confirm this. Growth after nine days on PDA with 2% NaCl showed more red coloration on the right hand side of growth in the third quadrant compared to the rest of the plate. This difference is believed to be caused by the pattern of pigmentation fading in the colonies and not an effect of the media itself.

When comparing the structure of the putative prodigiosin biosynthesis cluster in MI-2 to S. *marcescens*, the most notable difference is the absence of *pigA* in MI-2. PigA functions as a flavoprotein desaturase whose catalytic role in the prodigiosin biosynthetic pathway occurs after PigI but before PigJ. An alternative reaction converting the substrate of PigA to the correct product without the use of PigA has been experimentally confirmed (13), perhaps negating the need for PigA in the prodigiosin biosynthetic pathway of MI-2. Additionally, as mentioned in the results, a different protein elsewhere in the genome did show homology to PigA and may be able to function in its place in prodigiosin biosynthesis.

As shown in Table 1, the AAI between proteins encoded by the putative prodigiosin biosynthetic gene cluster and known prodiginine biosynthetic proteins were typically between 50 to 80%. However, AAI comparison is likely not the best method for comparing protein function. Conservation of specific domains may be a better determinant as to whether a group of proteins will serve the same function. Although *H. chejuensis* and S. *marcescens* both produced prodigiosin, the amino acid identity between Pig and Hap homologs generally ranged from only 30% to 55% (23). Despite the seemingly lower amino acid identities between these homologs, these enzymes served the same function in prodiginine biosynthesis (23, 43). A better comparison of protein function can be done by comparing specific amino acid residues that are essential to the function of the protein.

According to the NCBI protein basic local alignment tool, the MI-2 homolog of PigC contained a domain belonging to the pyruvate phosphate dikinase (PPDK) superfamily. PigC performs the final reaction of the prodigiosin biosynthesis pathway, which combines the two precursor molecules 4-methoxy-2,2-bipyrrole-5-carboxyaldehyde (MBC) and 2-methyl-3-n-amyl-pyrrole (MAP) into the final prodigiosin structure (29, 43). The mechanism for PigC is similar to that of PPDK enzymes in that they phosphorylate a carbonyl by facilitating the transfer of a phosphoryl group from ATP using a histidine residue in a phosphoryl transfer domain (PTD) (6). S. *marcescens* strains with a *pigC* knockout completely lost the ability to produce the prodigiosin pigment (43). Selective mutagenesis experiments on PigC in *Serratia* spp. 39006 showed that replacement of His840 with an alanine residue completely eliminated activity. Similarly, residues predicted to be important for ATP binding, Glu281 and Arg295, showed significant reduction in activity when replaced with alanine residues (6). Closer analysis of the amino acid sequence in the MI-2 homolog also revealed residues His841, Glu282 and Arg296 that are analogous to key residues in PigC (data not shown). Given this similarity, it is likely that PigC and the MI-2 protein homolog function identically to one another. Investigations to experimentally confirm the function of the putative prodigiosin biosynthetic proteins in MI-2 are underway.

The prodiginine compounds isolated and identified in this study have been previously recognized and documented in several other species (1, 22, 26). Given the homology between *pig*, *hap*, and MI-2 prodiginine gene clusters it is plausible that MI-2 would produce similar prodiginine derivatives to those found in *Serratia* species and *H. chejuensis*. The mass spectrum of the purified MI-2 pigments contains multiple peaks from prodiginine compounds with different alkyl chain lengths. Prodiginine compounds varying in the length of the alkyl chain substituent group on a pyrrole of the structure are well documented and have been produced as minor byproducts in other species (1, 14, 26). The difference in MI-2 appearance on the modified PDA and the LB plates may be due to the amount of

overall prodiginine production and the ratio of different prodiginine compounds produced on each medium. This ratio has been previously correlated with color change in *V. gazogenes* (1).

Additional pigment spots on the TLC (data not shown) indicated that there may be additional prodiginine compounds produced by MI-2. In other studies, HPLC was used with a reversed-phase C-18 column to purify prodiginines (1, 28). Preparative TLC has also been used to purify different prodiginines (10, 31). Additionally, use of acetonitrile as a solvent for flash chromatography may have impeded separation by not stabilizing a single conformation during separation techniques. Prodigiosins prefer the β conformer in acetonitrile and would likely prefer the α conformer in response to interactions with the silica, which is slightly acidic (34). A similar separation was achieved using ratios of chloroform, methanol and ethanol but was not extensively tested due to the convenience of using a single solvent; however, a better separation might be achieved by experimenting with the solvent ratios further.

Finally, prodiginine compounds have been well documented as possessing antibacterial capabilities against Gram negative and Gram positive organisms (9). The initial antibacterial properties observed in MI-2 (data not shown) may be due to the sole production of prodiginine compounds, production of an unrelated antibacterial molecule, or the combined production of prodiginine and another antibiotic. It is not clear whether another antibiotic is produced by MI-2 or not. Further investigation using techniques such as bioautography on crude MI-2 extracts may show the production of more than one antibiotic.

In summary, a novel pigment and antibiotic-producing marine bacterium named MI-2 was isolated and identified as belonging to the *Vibrio* genus. The coloration and intensity of the pigment is effected by the media type, salt concentration, and time of incubation. After whole genome sequencing, a putative prodigiosin biosynthetic pathway was computationally identified MI-2 by sequence comparison to known prodigiosin producers, suggesting the pigment produced could be a prodigiosin derivative or a member of the prodiginine family. Purification and structural elucidation of the pigment produced by MI-2 demonstrated a mixture of four compounds, three of which were shown to be in the prodiginine chemical family and two of which were prodigiosin. Further research to experimentally verify the function of the 13 genes in the putative prodigiosin biosynthetic pathway and investigate the regulation of their expression is underway.

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METAL SUSCEPTIBILITY OF A HETERO-VANCOMYCIN-INTERMEDIATE METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATE

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- Methicillin-resistance
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- vancomycin
- metals

ABSTRACT

The Gram-positive bacterium *Staphylococcus aureus* is well known for its ability to develop resistance to various antimicrobial substances. Methicillin-resistant S. *aureus* (MRSA), a cause of nosocomial infections worldwide, is becoming increasingly resistant to the glycopeptide antibiotic vancomycin, one of few antibiotics used to treat serious multiple-antibiotic-resistant staphylococcal infections. With the increase in the number of MRSA strains showing reduced sensitivity to vancomycin, it has become important to investigate alternative treatment options. In this study, we examined the effects of five metals: silver, copper, arsenate, zinc, and cadmium on the growth of a clinical MRSA strain MM66 demonstrating heterogeneous intermediate-level resistance to vancomycin. Disc diffusion and gradient plate experiments were used to compare the metal susceptibility levels of strain MM66 to that of the methicillin-resistant S. *aureus* laboratory control strain. MM66 grew less successfully when exposed to metals, and showed an overall increased level of susceptibility to metals compared to the laboratory control strain. Of the metals tested, silver exerted the highest inhibitory effect on the growth of MM66.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial and community-acquired infections worldwide (18, 2, 29, 4). Since its first appearance in the early 1960s (15), MRSA has been the primary cause of nosocomial skin and bone infections as well as bacteremia (24, 6, 19, 13). MRSA infections account for about one-third of all S. *aureus* infections in the United States annually (13). In 2005, MRSA infections caused a higher mortality rate than HIV, killing an estimated 6.3 out of every 100,000 individuals.

Vancomycin was introduced for the treatment of S. *aureus* infections, including MRSA (24, 13). By 1997, more than 50% of

MRSA nosocomial infections responded only to vancomycin (24). However, MRSA strains showing reduced sensitivity to vancomycin have appeared and are now a prevalent problem (24, 2, 19).

Since the isolation of the first vancomycin-resistant *S. aureus* in 1995 (30), isolates with different levels of resistance have been recognized. High-level resistance to vancomycin is attributed to the *vanA* gene, which was detected in all three of the first vancomycin-resistant (VRSA) isolates (30). Intermediate resistance to vancomycin or vancomycin-intermediate *S. aureus* (VISA) is defined by a minimum inhibitory concentration (MIC) of greater than 3 mg/L of vancomycin (Delgado *et al.*, 2007). However, VISA can show an MIC up to 16 mg/L, and vancomycin-resistant *S. aureus* (VRSA) can show an MIC of 32 mg/L or even more (30). Hetero-vancomycin intermediate *S. aureus* (hVISA), a subtype of VISA, initially show lower vancomycin MIC (less than 2 mg/L) but grow successfully when exposed to higher levels of vancomycin, and are likely a precursor to VISA (14).

Antibiotic-resistant *S. aureus* also demonstrate reduced sensitivity to other antimicrobial substances such as household disinfectants (16) and various metals (3). Metals such as zinc, copper, silver, cobalt, and cadmium are well-known for their antimicrobial properties, and have been used in medicine, animal husbandry, and agriculture. Metals such as copper and zinc are common in feed supplements for agricultural animals (3). Cadmium and arsenic are common pollutants in soil and water, exposing environmental bacteria to their oligodynamic action (28, 34). Copper and silver have been used more specifically for their antimicrobial activity. Both are used in making water vessels and food containers to keep the contents disinfected (17, 27, 7). Copper sulfate and other copper salts have also commonly been used as organic biocides and fungicides as well as in the medical setting as astringents (17). Copper salts along with other metals such as mercury salts and tellurium, magnesium, and arsenic oxides have also been used to treat leprosy, tuberculosis, gonorrhea, and syphilis (17). However, many bacteria including *S. aureus* are becoming increasingly resistant to several metals. Resistance of *S. aureus* to various metals has been reported in clinical cases (23), and has been linked with the widespread use of metal-containing compounds such as feed supplements and biocides. A possible link between antibiotic resistance and cross-resistance to metals and biocides has been suggested in several bacteria including *S. aureus* (21, 16). Several studies have suggested that with the development of antibiotic resistance, *S. aureus* strains may also develop decreased sensitivity to metals that were previously toxic at small concentrations (25, 11, 3, 1). Resistance to metals in MRSA has also been associated with methicillin-resistance, being displayed in the same strains (3, 25).

In this study, we examined the susceptibility of a clinical MRSA strain MM66 demonstrating heterogeneous intermediate-level resistance to vancomycin (6) to five metals: silver; copper; arsenic; zinc; and cadmium. Two laboratory control strains of *S. aureus* – a fully antibiotic-sensitive strain ATCC 25923, and a MRSA control strain ATCC 43300 – were used to determine the relative susceptibility of MM66 to the metals tested. Based on the available data from studies involving clinical strains of methicillin-resistant *S. aureus*, we hypothesized that MM66 would demonstrate a comparatively reduced susceptibility than ATCC 44330 to the metals at concentrations previously tested on similar strains. In contrast, this study revealed that MM66 has lower levels of resistance to copper, zinc, arsenic, and cadmium compared to ATCC 43300. Growth of MM66, ATCC 43300 and ATCC 25923 was completely inhibited by silver (2.5 mM).

METHODS AND MATERIALS

STRAINS, GROWTH CONDITIONS, AND REAGENTS

The clinical hVISA strain MM66 was obtained from Prof. John Gustafson's laboratory at Oklahoma State University, Stillwater, Oklahoma. This strain was first isolated in Las Cruces, New Mexico by Delgado *et al.* (2007). Two controls were used in this study; American Type Culture Collection (ATCC) 25923, a fully sensitive laboratory strain of S. *aureus*; and ATCC 43300, a laboratory MRSA strain sensitive to vancomycin. Each strain was streak-plated onto Luria Bertani Agar plate from stock cultures and maintained at 4°C. Metal solutions were prepared from metal salts (zinc chloride, copper sulfate, cadmium acetate, silver nitrate, and sodium arsenate) at a concentration of 200 mg/L and filter-sterilized. Aliquots of 1 mL each were made at 1 mg/mL, 5 mg/mL, and 10 mg/mL to be used for disc preparation. All stock solutions were stored (at 4°C) in the refrigerator. Mueller Hinton Broth (MHB), Mueller Hinton Agar (MHA), Luria Bertani broth (LB) and Luria Bertani Agar (LBA) were prepared per instructions provided on powder containers. Overnight cultures were prepared by inoculating 5mL of LB with one isolated colony of bacteria from the stock plates and incubating at 37°C at 200 rates per minute (rpm) in the shaking incubator (New Brunswick Scientific, I2500 series, Enfield, CT). All experiments were performed in triplicates starting with triplicate overnight cultures in LB. All media used in this study were obtained from BBL Difco (Franklin Lakes, NJ), and all chemicals were obtained from Sigma (Saint Louis, MO) unless indicated otherwise. The strains used in this study are listed in Table 1.

DISC DIFFUSION ASSAY

Metal susceptibility was examined by disc diffusion similarly to the method described by Poston and Saw Hee (1991). For the first trial, a 50 microliter volume of overnight culture was used to inoculate 5 mL of sterile MHB. For the second trial, this amount was decreased to 20 microliters. Tubes were incubated at 37°C, 200 rpm for approximately three to four hours until they visually matched the McFarland turbidity standard of 0.5. Meanwhile, discs were prepared by adding calculated volumes of sterile stock solution of each metal to sterile paper discs of 4 mm diameter. Total liquid volume added to each disc was adjusted to 20 microliters with sterile distilled water to ensure a uniform distribution of the metal salt on the disc.

After incubation, if suspensions were less turbid than the standard, they were placed back in the incubator so that further growth could take place. If they were more turbid, they were diluted with sterile MHB. Once all tubes matched the McFarland standard, culture was inoculated onto the entire agar surface of MHA plates with a sterile cotton swab. Prepared discs were placed on the plates with sterile forceps and gently pressed to ensure that entire disc surface contacted the agar. The plates could sit for one minute before being inverted and incubated at 37°C in a stagnant incubator overnight. Zones of inhibition were measured after 18, 20, 24, 48, and 72 hours.

METAL GRADIENT PLATE ASSAY

Gradient Plate was performed as described by Price *et al.*, 1999. Square plates (90mm x 90mm) were obtained from Fisher Scientific (Pittsburgh, PA) and placed at a 14° angle so that bottom layer of agar would solidify at

Table 1. Strains used in this study.

Strain	*Relevant background*	*Source/reference*
MM66	Clinical hVISA; vancomycin MIC 3.0 μg/ml	Delgado *et al.*, 2007
ATCC 25923	Lab *S. aureus* strain; sensitive to antibiotics	ATCC
ATCC 43300	Lab MRSA strain; sensitive to vancomycin	ATCC

hVISA: hetero-vancomycin-intermediate S. aureus
MRSA: Methicillin-resistant S. aureus

the same angle. To make the bottom layer, 40 mL of freshly prepared MHA was poured and allowed to solidify. The next day, the top layer MHA was prepared by adding calculated volumes of 200 mg/mL stock to the MHA before again pouring 40 mL of MHA, but this time pouring with the plate flat so that the top layer formed a flat surface.

Twenty microliters of overnight culture were transferred to 5 mL of sterile MHB and incubated at 37°C, 200 rpm. After incubation, the cultures were diluted to an optical density (OD) of 0.1 at 600 nm as measured by a spectrophotometer (Thermo Scientific, Waltham, MA). When OD of 0.1 was reached, culture was inoculated onto gradient plate in a line streak from lowest concentration to highest concentration with a sterile cotton swab by streaking the same line three times to ensure equal distribution of the culture along the plate. The plates were then inverted and incubated at 37°C and confluent growth was measured after 18, 20, 24, 48, and 72 hours.

RESULTS

DISC DIFFUSION ASSAY

Exposure to 10, 30, 35, and 40 micrograms of zinc chloride, copper sulfate, and sodium arsenate did not have any effect on the growth of the *S. aureus* isolates. When exposed to 60 micrograms of zinc chloride, ATCC 25923 displayed small, faint zones with visible reduction of growth but ATCC 43300 and MM66 strains remained unaffected. None of the strains showed any zone of inhibition with 60 micrograms of copper sulfate and sodium arsenate.

When exposed to cadmium acetate, the strains showed some zones of decreased growth, herein referred to as 'zones of inhibition'; however, these zones varied in size across the strain types. After 20 hours of incubation around a 40-microgram disc, MM66 displayed the largest zone of effect (35.3 mm in diameter) whereas ATCC 25923 and ATCC 43300 displayed smaller zones of inhibition (12.3 mm and 15 mm, respectively) (Figure 1). MM66 also displayed clear zones of inhibition at lower concentrations of cadmium acetate (30 and 10 micrograms); no zone of inhibition was observed in the control strains at these concentrations.

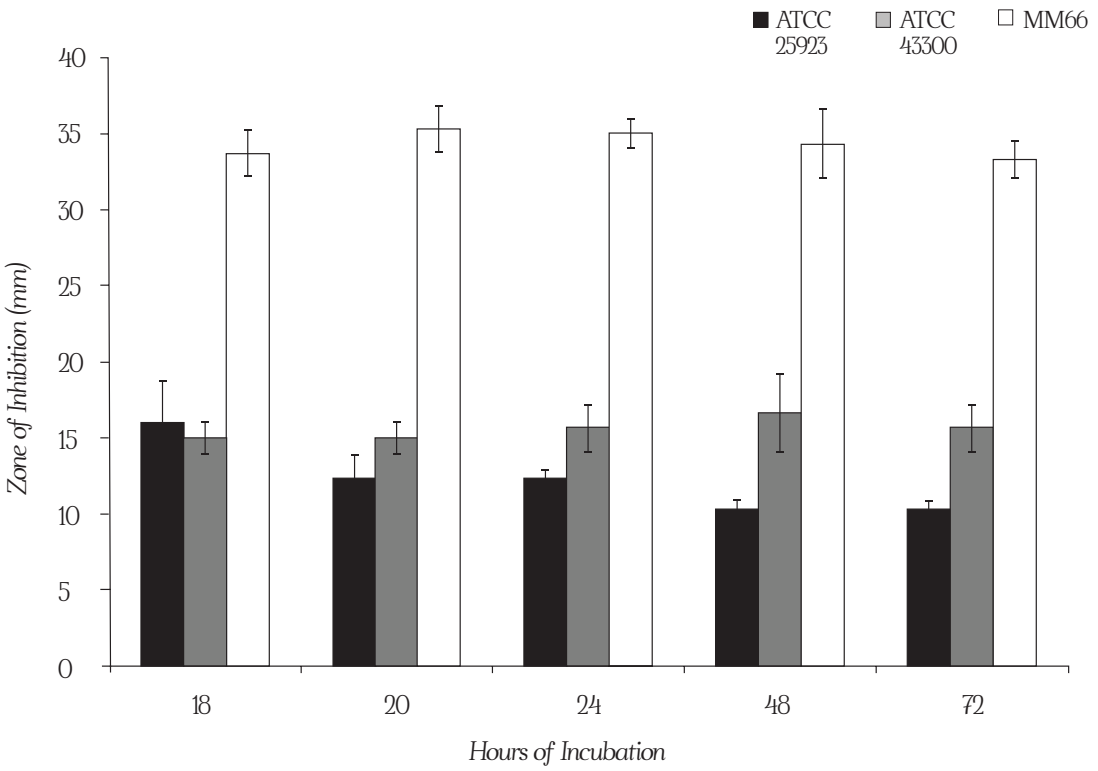

Figure 1. Comparison of the zones of inhibition of 40 µg cadmium acetate disks on S. *aureus* strains ATCC 25923 (black bars), ATCC 43300 (gray bars) and MM66 (white bars). Error bars represent standard deviation (n=3, $p<0.05$). Significantly greater zones of inhibition (connoted by *) were observed in MM66.

Clear zones of inhibition were observed in all three strains at all concentrations of silver nitrate. The zone sizes of all strains were similar (Figure 2). The results were consistent in a second disc diffusion experiment performed on triplicates of each strain.

METAL GRADIENT PLATE ASSAY

On the 5mM copper sulfate gradient plates, MM66 displayed reduced susceptibility compared to ATCC 25923 and ATCC 43300. ATCC 25923 grew up to 83.33±9.87 mm, ATCC 43300 grew with average of 80.667±2.08 mm, and MM66 grew through the entire length of the gradient (90±0 mm) (Table 2). However, when a higher concentration gradient (20 mM) was used, growth of MM66 was more reduced compared to the other strains. ATCC 25923 grew an average of 19.67±1.53 mm, ATCC 43300 grew an average of 16±1 mm, and MM66 grew an average of 6.33±5.01 mm. (Table 3).

At 2.5 mM concentration gradient of sodium arsenate, both ATCC 25923 and ATCC 43300 grew through the length of the plate, measuring an average of 90±0 mm of growth. MM66 grew an average of 39.833±4.26 mm which is significantly less than the growth of the other two

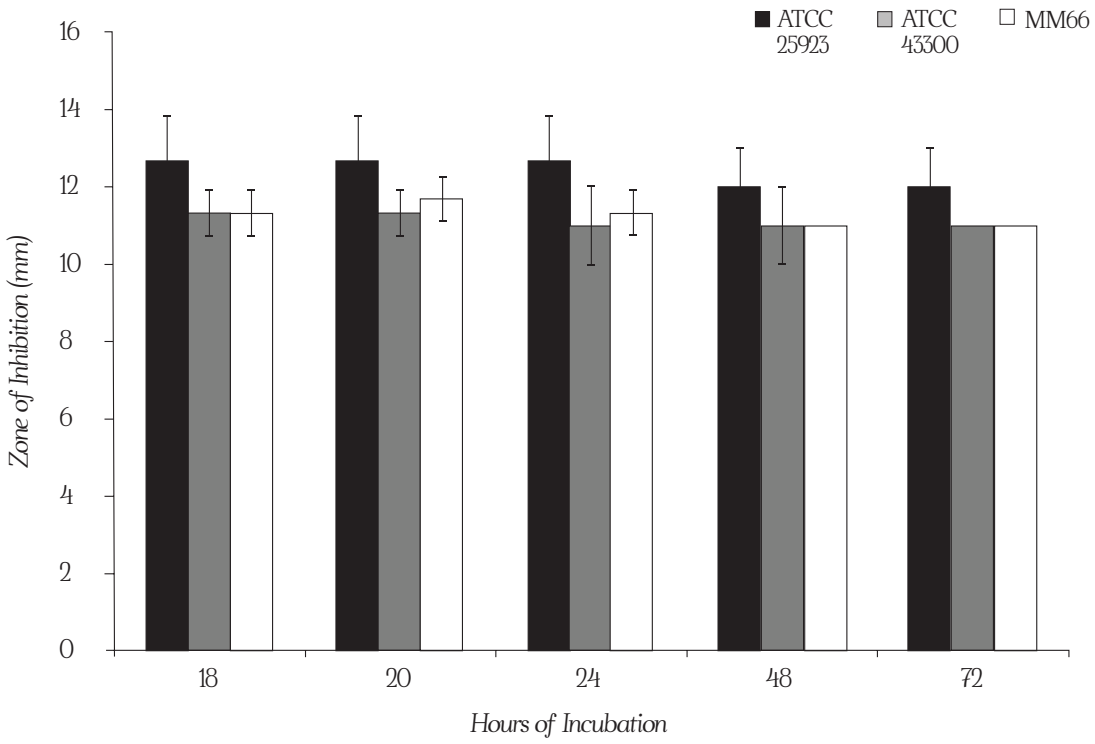

Figure 2. Comparison of the zones of inhibition of 40 µg silver nitrate disks on S. *aureus* strains ATCC 25923 (black bars), ATCC 43300 (gray bars) and MM66 (white bars). Error bars represent standard deviation (n=3, *p*=0.05). No significant differences were observed in zone size.

strains (Table 2). At 5 mM concentration gradient, ATCC 25923 still grew the full length of the gradient, measuring 90±0 mm. However, ATCC 43300 grew an average of 85.33±3.21 mm and MM66 did not grow at all. In a third experiment using 20 mM sodium arsenate gradients, MM66 did not demonstrate any growth, ATCC 25923 grew an average of 40±1 mm and ATCC 43300 grew an average of 23.667±5.68 mm. ATCC 43300 grew significantly less than ATCC 25923 (data not shown in table).

On cadmium acetate, MM66 failed to grow on a gradient of 2.5 mM; ATCC 25923 grew to an average of 30±1 mm at 2.5 mM gradient and failed to grow on 5 mM; and ATCC 43300 grew to an average of 72.667±5.51 mm on a 2.5 mM gradient of cadmium acetate and 16.667±4.51 mm on 5 mM gradient (Tables 2 and 3). The growth of both controls was significantly reduced at a higher gradient of cadmium acetate (10 mM); ATCC 25923 grew to an average of 10.667±9.29 mm and ATCC 43300 grew to an average of 16.333±1.15 mm, and growth of MM66 was completely inhibited.

None of the strains grew on 2.5 mM, 5 mM, and 10 mM gradients of silver nitrate. This result was consistent with all the triplicates. Comparative results of gradient plate assay for all strains are presented in Tables 2 and 3.

Table 2. Metal gradient plate experiment results (at lower concentrations of metals).

Strain	*Metal Gradient*				
	Zn 0→2.5 *mM*	*Cu* 0→5 *mM*	*Cd* 0→2.5 *mM*	*Ag* 0→2.5 *mM*	*Asa* 0→2.5 *mM*
ATCC 25923	32 ± 1.0	83.33±9.8	30±1.0	0	90±0
ATCC 43300	79.3±4.2	80.7±2.1	72.7±5.5	0	90±0
MM66	38.67±2.3*	90±0*	0*	0	49.3±4.5*

Numbers represent mm grown on 90 mm gradient plates and standard deviations (n = 3).
**connotes significant difference compared to MRSA control strain ($p < 0.05$).*

Table 3. Metal gradient plate experiment results (at higher concentrations of metals).

Strain	*Metal Gradient*				
	Zn 0→5 *mM*	*Cu* 0→10 *mM*	*Cd* 0→5 *mM*	*Ag* 0→5 *mM*	*Asa* 0→5 *mM*
ATCC 25923	17 ± 1.7	90±0	0	0	90±0
ATCC 43300	36.3±1.5	90±0	16.7±4.5	0	90±0
MM66	14.5±1.6*	64.2±0*	0*	0	0*

Numbers represent mm grown on 90 mm gradient plates and standard deviations (n = 3).
**connotes significant difference compared to MRSA control strain ($p < 0.05$).*

DISCUSSION

The results of the disc diffusion test suggested a lack of sensitivity in both the laboratory and clinical MRSA strains to zinc, copper, and arsenate. Compared to the laboratory MRSA strain ATCC 43300, MM66 showed increased sensitivity to cadmium acetate. Although the growth of all strains was affected by cadmium acetate, growth of MM66 was the most inhibited; the control strains had smaller zones of inhibition surrounding the cadmium acetate discs. Clear zones of inhibition were only present on MM66 plates, indicating an increased sensitivity of this strain to cadmium. Distinct zones of inhibition were observed around the silver nitrate discs at all concentrations used. This suggested that the MRSA strains are highly susceptible to silver.

Overall, similar effects of the metals on the growth of the strains were observed by both the disc diffusion and gradient plate methods. Therefore, the findings of both experiments supported one another. The gradient plate experiments provided a more comprehensive comparison of susceptibility among the strains. Unlike disc diffusion experiments in which discs with specific concentrations of the metals were used, the gradient plate assays

used continuous concentration gradients of metals. Therefore, bacterial growth starting from the lowest concentration to the highest concentration of the metal could be observed.

In the gradient plate experiments, the hVISA strain MM66 showed reduced growth in presence of zinc compared to the lab MRSA strain ATCC 43300. Zinc is required by the bacteria at a low concentration to carry out various metabolic reactions as catalysts, structural stabilizers, and as coenzymes or cofactors (1). Therefore, growth of the strains at the zero end of the concentration gradient was expected. As the concentration becomes higher, the cells cease to grow due to the toxic effects of zinc. In our experiments, ATCC 43300 could grow at higher levels of zinc compared to MM66, suggesting that the clinical hVISA strain is probably compromised in its ability to resist higher levels of zinc.

The strains showed highly reduced susceptibility to copper sulfate; all three strains grew to almost the entire length of the gradient plates at 5 mM copper sulfate concentration gradients. Strain MM66 displayed the longest distance of growth, suggesting a more reduced susceptibility of this strain to copper compared to the MRSA control strain. Interestingly, in higher concentration gradients of copper (10 mM and 20 mM), MM66 displayed shorter distances of growth compared to its control counterparts. Copper, like zinc, is necessary in trace amounts for the metabolic functions of the bacteria (1). It is possible that MM66 grows more successfully at a lower concentration of copper sulfate, but is affected more rapidly when the concentration gets higher, as seen in the 10 mM and 20 mM gradients. Further research investigating the molecular basis of copper resistance in these strains may provide more information in this aspect.

When exposed to 5 mM arsenate, MM66 grew to almost half the length of the concentration gradient. However, this growth was significantly reduced when compared to the growth of control strains, indicating that MM66 has reduced susceptibility to arsenate compared to the control MRSA strain. This pattern was consistently observed in other concentrations of arsenate (Table 3). Despite the toxicity of arsenic, resistance to high levels of arsenic has been observed in isolates of *S. aureus* (1, 34, 33). Bacteria that are resistant to arsenate compounds contain efflux systems that expel the arsenates out of the cells. Arsenates are analogs of phosphates which are essential for cellular functions, and bacteria may take the arsenates in by phosphate transport mechanisms. Once in the cells, they are converted to arsenites and pumped back out of the cell via efflux pumps (33). The growth of the strains in arsenate gradients indicates that a similar mechanism may be present in these strains. Further research may be necessary to determine the arsenite efflux capabilities of these bacterial strains.

The results of the cadmium gradient plate assay were consistent with the results of the disc diffusion test. In 10 mM gradient of cadmium acetate, the growth of MM66 was significantly reduced compared to that of the control strains. Cadmium is a highly toxic pollutant found in the environment. This may limit its therapeutic use despite its ability to significantly inhibit the growth of MM66. Cadmium kills *S. aureus* by generating oxidative stress and inhibiting thiol metabolism (28). *S. aureus* isolates that are resistant to cadmium possess the *czr*C gene and the *cad* operons that enable them to resist the negative effects of cadmium (3, 5, 8). Methicillin–resistance in *S. aureus* has been linked with cadmium–resistance and the presence of the *czr*C gene in many MRSA isolates (3). The increased susceptibility to cadmium in MM66 indicates the presence of a previously unidentified mechanism in the hVISA strain. This suggests the need of further studies to understand the genetic basis of this phenomenon in MM66.

Silver gradients, like the silver discs, completely inhibited the growth of all three strains of S. *aureus* at the tested concentrations. The oligodynamic action of silver compounds is a known phenomenon, and silver compounds are commonly used as antimicrobial agents, such as 1 % silver nitrate to prevent opthalmia neonatorum and as silver sulfadiazine to treat infected burn wounds (27, 9). Therefore, reduced growth of the S. *aureus* strains in disc diffusion and gradient plate assays was not completely unexpected. What was unexpected was that the growth of all strains, including the clinical MRSA strain, was completely inhibited even at lower concentrations. Even though no differential growth among the strains was observed and hence no data on the relative susceptibility of the MM66 strain could be obtained, it can be said that the clinical hVISA strain MM66 appears to be susceptible to even low concentrations of silver. Silver inactivates bacterial enzymes and damages DNA, and helps in the accumulation of reactive oxygen species in the cells, leading to the death of bacteria (20). Our results are in favor of the proposition that silver compounds could be considered as alternatives to traditional topical antibiotics for the treatment of infections caused by antibiotic-resistant bacteria (27). Since S. *aureus* is implicated in a variety of skin infections, using silver compounds could be a viable option for treating skin infections. Therefore, the possibility of using silver as an effective alternative to antimicrobials in treating MRSA infections should be further investigated.

In conclusion, this research provides interesting new information about the metal susceptibility characteristics of a clinical MRSA strain that was reported as a hetero-vancomycin-intermediate S. *aureus* (6). Despite the high occurrence of cross-resistance to metals in methicillin-resistant S. *aureus* isolates, MM66 does not seem to show these tendencies. Our results suggest an overall reduced metal susceptibility in the clinical hVISA strain, with silver exerting the highest inhibitory effect on growth. The findings of this research underline the need of further investigation on the use of metals as therapeutic options in treating drug-resistant S. *aureus* infections.

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PERSPECTIVE

BECOME A SPOKESPERSON FOR SCIENCE

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THE IMPORTANCE OF OUTREACH: WHY DO IT?

The National Science Education Standards defines scientific literacy to include a "greater knowledge and understanding of science subject matter" and "the role of science in society and personal life (1)." It is important for all members of society to understand the importance of science in our lives as they will be voting on issues, making purchases, and making health choices that involve scientific understanding. Often these decisions directly impact the various industries and organizations we serve as scientists.

There is a public perception that science is difficult and is typically done by Albert Einstein stereotypes in a laboratory with chemicals and strange looking glassware. This has been proven many times in classrooms where students have been asked to draw a scientist, and although there is some variation around the world, they typically depict a male with glasses or goggles and erratic hair (2). It is our responsibility as scientists to help change that perception and broaden the view of what scientists are. We also need to be prepared and able to respond when we see or hear stories that contain scientific inaccuracies being shared between friends, posted to social media or covered by various mainstream media outlets so that we can better stop the spread of misinformation. Let's explore a couple of examples.

Biotechnology, also known as genetically modified organisms (GMOs), has been a target for both media outlets and marketing campaigns using scare tactics to try to influence consumers to purchase organic or non-GMO products. These campaigns and stories do not focus on the science, but rather imply that organic food products are healthier and non-GMO products are safer. Despite the agreement of many esteemed scientists regarding the benefits of GM crops, and 121 Nobel Laureates who signed a letter calling upon governments around the world to not only approve but accelerate the access of farmers to the improved seed technology (3), some consumers at the grocery store continue to be

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KEYWORDS

- outreach
- scientific literacy
- STEM

unwilling to trust the scientific experts when selecting food purchases.

We see the same mistrust of scientists regarding the use of vaccines. The number of parents immunizing their children has decreased to about 60 percent in the U.S. (4). One of the contributing factors to this decline is recent negative media coverage, perpetuating the idea that the vaccines may cause autism or other side-effects. This leaves vulnerable members of the population at higher risk for diseases like measles that were once nearly eradicated. These kinds of stories make headlines but they are harmful to science and contribute to a decline in the general impression and trust the public has regarding science and scientists.

As scientists, we can change the impact of the public's overall impression of science and we can serve as a knowledgeable and reliable resource to answer questions. Trust is an important part of this scenario. As scientists, we know the scientific process continues to ask questions on topics and the continuous testing of theories or hypotheses prove or disprove our current understanding, making the science self-correcting in this respect. We might not have all the answers but given the body of evidence provided we draw logical conclusions about our field of study. But how do we help the public understand this and trust this process?

You can start building trust and breaking down certain stereotypes about scientists by becoming involved in community outreach. If scientists from various backgrounds serve as mentors, we can illustrate the diversity of those working in the sciences and the variety of topics we study. We do not all fit a single mold of a middle-aged male in a lab that is still too commonly thought of today (2). Our research may take us anywhere—from the far reaches of the world to our very own backyard. Showing the public that some scientists study geology in Antarctica, others might collect house fly populations from dairy farms in Australia, while some may take water and soil samples from our local rivers and pastures. Illustrating the range of jobs in the scientific community and sharing stories about how this work leads to technology that can improve people's day-to-day life will help begin to broaden the public's overall understanding of the positive impact the scientific community makes to the world. It's important to be willing to share our stories.

Students are one of the most important groups for scientists to focus on. In my opinion, one of the most rewarding experiences of doing outreach is when a student comes up to you and says you changed the course of their studies or influenced their career choice. As role models for future generations of scientists it's important to note the need for growing the number of students pursuing science degrees. The U.S. Bureau of Labor Statistics projects that there will be 9 million more STEM jobs available between 2012 and 2022 (5). The President's Council of Advisors on Science and Technology (PCAST) states that 1 million more STEM college graduates will be needed over the next decade (6). In addition, PCAST estimates that "fewer than 40% of students who enter college intending to major in a STEM field complete a STEM degree" (6). By being role models for science you can contribute to improving the retention rate of students majoring in STEM degrees.

Giving back to the community helps me see how one person can have such an impact on another. Bringing scientific enrichment opportunities to those who may have none is something that has been a passion of mine. For the last several years, I have worked with the Indianapolis School on Wheels, an organization that provides tutoring and educational enrichment to students experiencing homelessness. At the School on Wheels locations, colleagues and I present a variety of hands-on science demonstrations for this group of students. These hands-on activities were created by Dow AgroSciences' volunteers—known as the Science Ambassadors.

Another personal experience was when I was a graduate student and worked with local educators to provide interactive hands-on activities presented by undergraduate mentors for their classes. Students and classroom supervisors were motivated to learn new subject material and new ways to present or experience science in their classroom. We can all give back to our communities and serve as scientific mentors at any age or level of education.

IMPACT ON DEVELOPING YOUR SKILLS AS A SCIENTIST: WHAT DO YOU GAIN?

One of the best ways to illustrate your competency in a subject is to be able to effectively communicate it to others. Learning to tailor messages to your audience is key. Explaining what you do to a group of third graders is different than explaining your job to a group of college students—despite the general ideas of the subject matter being discussed is the same. Regardless of age, if the person you are trying to reach is outside of your specific scientific field of study, scientific jargon can easily make them feel like an outsider in the discussion. One way to keep people involved is to consider everyone a learner; try to understand what information will be new and how best to present that, explaining critical vocabulary words.

Creativity in delivering your message can also be a way to help engage the audience during your outreach talks and activities, but it is often one of the hardest. I personally find this to be one of the most challenging aspects of science outreach. How can I create an experience for someone during my outreach interactions so they will connect with the subject? I do not merely want them to see the subject demonstrated; I want them to interact with it so that they will care about the subject longer than the one-time presentation. My undergraduate courses in education and communication have helped me in this regard.

Communication can come in a variety of forms: public presentations, written papers, social media, etc. My personal preference is to have face to face interactions, because it is easier to see how my message is coming across and know if I need to further explain topics based on the instant feedback of my audience's body language and engagement—or lack thereof—in conversation. For example, I sometimes use Skype to connect with students located in different parts of the country. Snapchat, Facebook, Twitter, blogs, and other social media channels all have uses in communicating science to the public because this is where the public is easily accessible. The mode of communication you should choose comes down to what you are most comfortable with and good at. Some people are better performing live demonstrations, while others have made a name for themselves doing scientific outreach through blogs and YouTube videos. The main objective is to communicate your science in a relatable way, this helps support all sciences because it helps the public build trust and puts science and scientists in a positive light.

Outreach activities also require you to demonstrate many of the soft skills that

employers desire in job candidates. There is the leadership you show by getting involved and planning an outreach event or activity. Building your organization, creativity, and ability to communicate your science in an understandable way are skills that are important to career success in the scientific world. Scientists volunteering to do outreach activities also develop time management skills as almost all outreach is performed beyond the traditional course curriculum or work hours.

Outreach also forces you to think about your own research differently, from another person's perspective. The confidence you have in yourself and your research hits a high when you see someone else understand your research through outreach. I had a student at a Science Day look at my vinegaroon, (a whip scorpion that I use for entomology discussions), from afar. It was obvious that she was clearly nervous but also curious. I started asking her questions about why she was nervous and assured her that he didn't bite, pinch, or sting and that he had some interesting characteristics. We continued to talk about the fact that the only way he defended himself was to spray acetic acid. I followed that up by telling her that acetic acid is vinegar which she's probably had on her salad. By the end of a few minutes of conversation she had approached the table and was reaching out to pet the creature that she initially feared. She even said he was kind of cute. This small breakthrough for this one person I believe will have an impact the next time she sees a "scary" looking arthropod, which in turn will impact how she sees the study of these creatures.

BUILDING YOUR NETWORK

You've probably already heard how important networking is in the job search and in professional development. Outreach not only allows you to practice talking to people and explaining your work, but it automatically expands your network. Depending on the type of activities you get involved in you will be working with members of your department, across departments within your university, and your field of study across universities. The professors you work with on outreach events can be great resources for future letters of recommendation for graduate school or job applications. Or, if your outreach program takes you across many different universities, you might find a faculty member with which you would like to pursue graduate studies.

If the event is sponsored by an external organization, it is likely you will meet others doing public outreach for other institutions or companies. These contacts can provide mentorship and possibly introduce you to job opportunities. My job at Dow AgroSciences came about this way. I was involved in outreach in graduate school and attended the Entomological Society of America meeting. There was a symposium on outreach that I attended and several the speakers were industry representatives. After the talks, I asked the organizer if she could explain how she as an industry professional was involved in outreach, and why. She explained that it was important for industry scientists to be talking to the community about the work we do in agricultural sciences. During that same conversation, she asked me when I was graduating and I pulled out my CV—noting that I still needed to finish school that spring. The next thing I knew I received a phone call asking me to fly to their global headquarters for an interview, and now I have been an employee at Dow AgroSciences for 8 years.

If you choose to participate in science outreach programs, you will also be interacting and networking with the community participating in your event. This could include students, teachers, parents, the public, media, and scientists in other fields. Community events give you a significant platform to reach a large audience, but it is good to prepare before the event so you can clearly communicate your message and explain why you are there and what you are demonstrating. You want to sound confident and at ease with your subject, not nervous and unsure. The best way to overcome that is to be prepared and practice frequently. Having your answer ready if you get asked will help you sound like the expert that you are.

SCIENCE OUTREACH: I'M INTERESTED BUT HOW DO I DO IT?

The easiest way to get involved is with an event that has already started. For example Earth Day would be a great time to do a special activity in the community. If you're a chemist, the American Chemical Society sponsors National Chemistry Week each year. In the Indianapolis area, there is a large Celebrate Science Indiana event held each October. Investigate what the various clubs and organizations on campus are doing. Many universities offer summer camps for children where you could volunteer. If you're still having trouble finding an event, talk to faculty or others doing science outreach and see what suggestions they have to offer. I'm happy to help you get something started in your local area if you need help.

If you want to take it a step further, demonstrate your leadership and passion for a particular area and start your own event. There's nothing like the feeling of starting an event that becomes so popular that it remains long after you have graduated and moved on. As a graduate student, I had the opportunity to work with faculty at Cornell University to develop the first ever Insectapalooza, a one day open house event in the Entomology Department (*7*). This event is now in its thirteenth year and continues to grow! Insectapalooza highlights the diversity of insects and entomology research. Activities have included everything from insects in the movies to roach races to citizen science projects to insect physiology. The entire department is engaged, which allows undergraduates, graduate students, and faculty to work together to develop the materials and activities for the event. The day of the event everyone interacts with the public. Undergraduates from other disciplines also participate. We've partnered with the art department to showcase insects and have communications students doing interviews for stories in the university newspaper.

Another option is to be a guest speaker for local schools. As an undergraduate I would often go back and talk to students at my former elementary school and high school. Contact someone at your elementary, middle, or high school and see if you can come speak to a class or organize a larger assembly and bring some of your college colleagues to each do activities. If you have moved away from your hometown for college, get to know some of the teachers near your university. Many teachers are eager to have students come in to talk about science. You provide a role model for students to aspire to and help the teacher with knowledge they might not have.

CONCLUSIONS

Scientific outreach is an important endeavor that is often overlooked in the day to day aspect of coursework and research. Work in this area impacts our community's opinions and understanding of science. Personal impacts include a sense of pride in the work you do, giving back to the community, building upon skills, and making connections with others. There's freedom and creativity in expressing your chosen scientific field and connecting that with others. Overall it's rewarding and fulfilling to set aside a day or even an hour to talk about something you have invested your time and talents into studying. If you are looking for ways to get involved in scientific outreach talk to others with a similar interest and start or contribute to an event in your area. As Nike so famously put it— "Just Do It!"

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POST/BIOTICS

Vidhi Mehta
Theresa Schachner

www.post-biotics.com
Find more about Post/Biotics on BBC, Wired, FastCompany & ScienceAlert

What if the next antibiotic is in your backyard? Post/Biotics is a platform that facilitates finding new antibiotics by integrating citizen scientists in the process of identifying natural substances with antimicrobial properties.

Opportunity

Most known antibiotics are developed from natural extracts, implying a potential new antibiotic could be anywhere. Post/Biotics distributes empirical research to the public by creating a platform and the according tools for participating in this process. The pop-up lab allows anyone to discover substances with antimicrobial values by testing locally available plants, vegetables, fruits, fungi, mushrooms & soil. With the help of the corresponding app, the results are stored on an online platform to create a library of antimicrobials. The value of this "microbial fingerprint" of our environment is significantly important for developing new drugs.

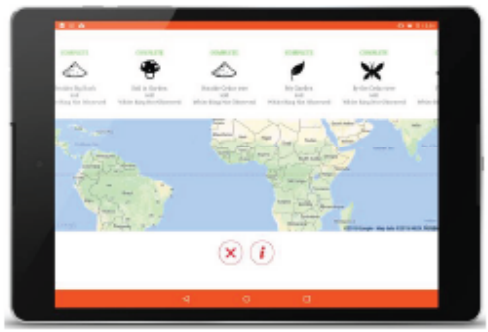
By partnering with universities & pharmaceutical companies, the massive costs for finding new drugs can be diminished when giving access to this platform.

Impact

The coming cost of antimicrobial resistance is 10 million deaths per annum by 2050. This is estimated to cost about 3% of the global GDP. Antibiotic resistance is growing faster than pharmaceutical companies are finding new ones. Corporate Social Responsibility of a pharma company can largely benefit from an educational initiative that promotes growth of young minds in STEM careers in research.

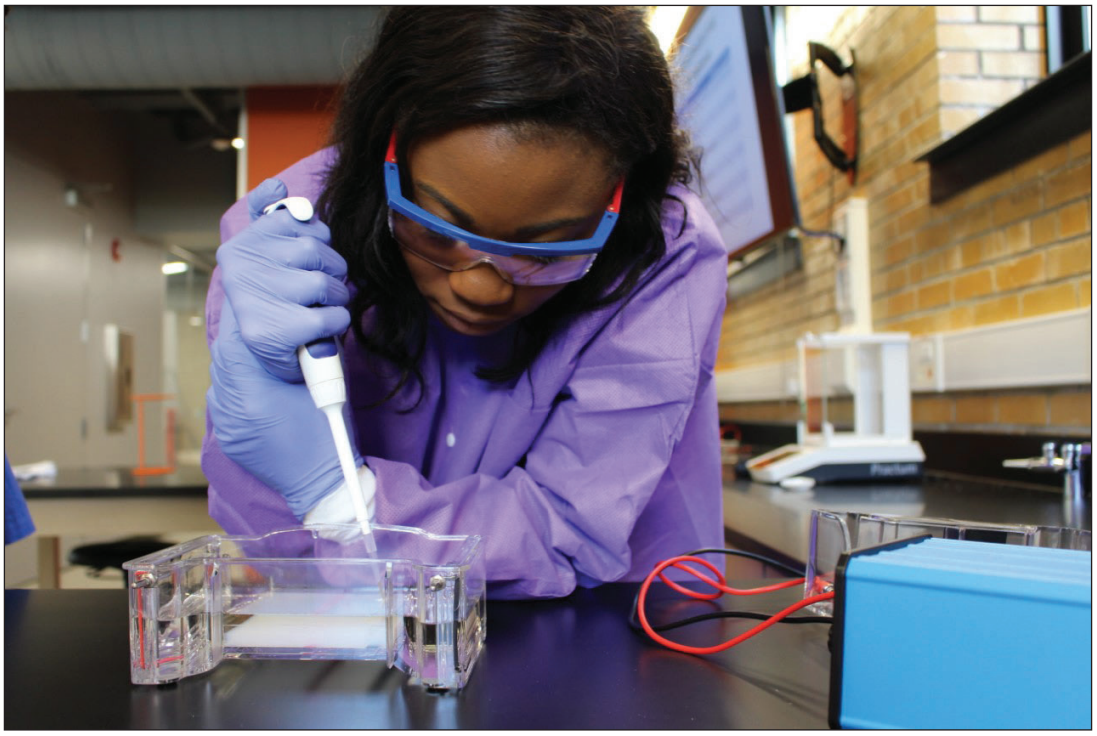
Method

Users test their samples like a crushed bark of a tree in their backyard with the toolkit that contains E.coli bacteria. If the tested tree bark has antimicrobial properties, it will be recognized by the platform and the user is encouraged to submit the original platform. Using gamification, users are incentivized to test different samples, geographical areas, and to use their peers for verifying their findings.



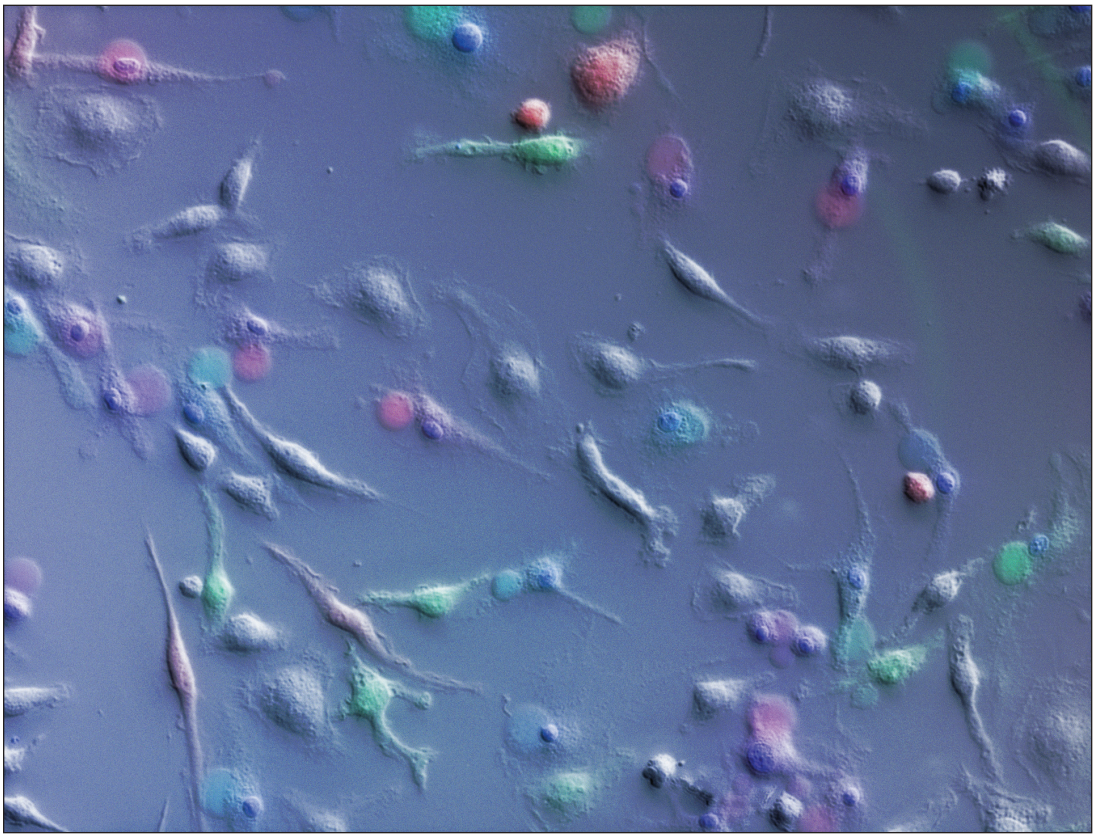
Vision:

Post/Biotics is a vision for revolutionizing drug discovery and raising awareness for the problem of antibiotic resistance.



Arren Simpson works in the laboratory on an undergraduate research project in the biomedical sciences, as part of the ReBUILDetroit initiative. The ReBUILDetroit program is a consortium of Detroit academic institutions funded by a National Institutes of Health (NIH) grant. ReBUILDetroit is fueling an academic renaissance of Detroit by establishing it as the center for biomedical research training for underrepresented undergraduate students. Arren is a Biology/Pre-Med freshman from Rochester Hills, Michigan at University of Detroit Mercy and a ReBUILDetroit scholar. She aspires to receive an MD and perhaps Ph.D. in Human Anatomy or Genetics.

Autumn 2016 *Fine Focus* student team: Front row, left to right: Corinne Webber, Courtney Johnson, Mary York, Kristen Buenconsejo, Hannah Fluhler, Dr. John McKillip; back row, left to right: Roth Lovins, Lexie Crump, Ellen Wagner, Mason Boles, & Gino Carnevale



A mixture of murine bone-marrow derived macrophages undergoing pyroptosis in the presence of glycine. Visible are wild-type cells expressing endogenous tdTomato or GFP fluorophores and non-fluorescent caspase-1/11-deficient controls. Pyroptotic cell nuclei are stained with Sytox Blue. Glycine promotes fluorophore retention in pyroptotic cells. "I wrote software to rapidly analyze changes in fluorescent intensity over time with single-cell resolution, and am applying that to investigating the kinetics of pyroptosis in mouse macrophages."

Photo & caption by Haas Undergraduate Fellow Recipient Lucian DiPeso : University of California – Berkeley, Molecular & Cell Biology. Mentor: Russell Vance, Ph.D. Special Acknowledgement: Molecular Imaging Center at UC Berkeley.

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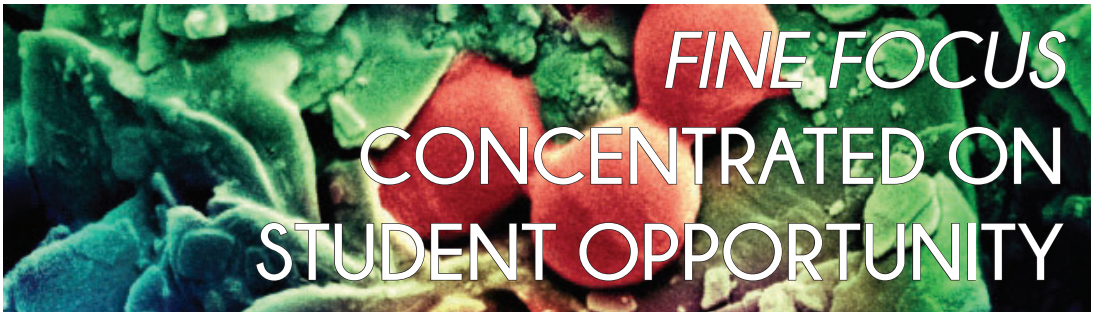

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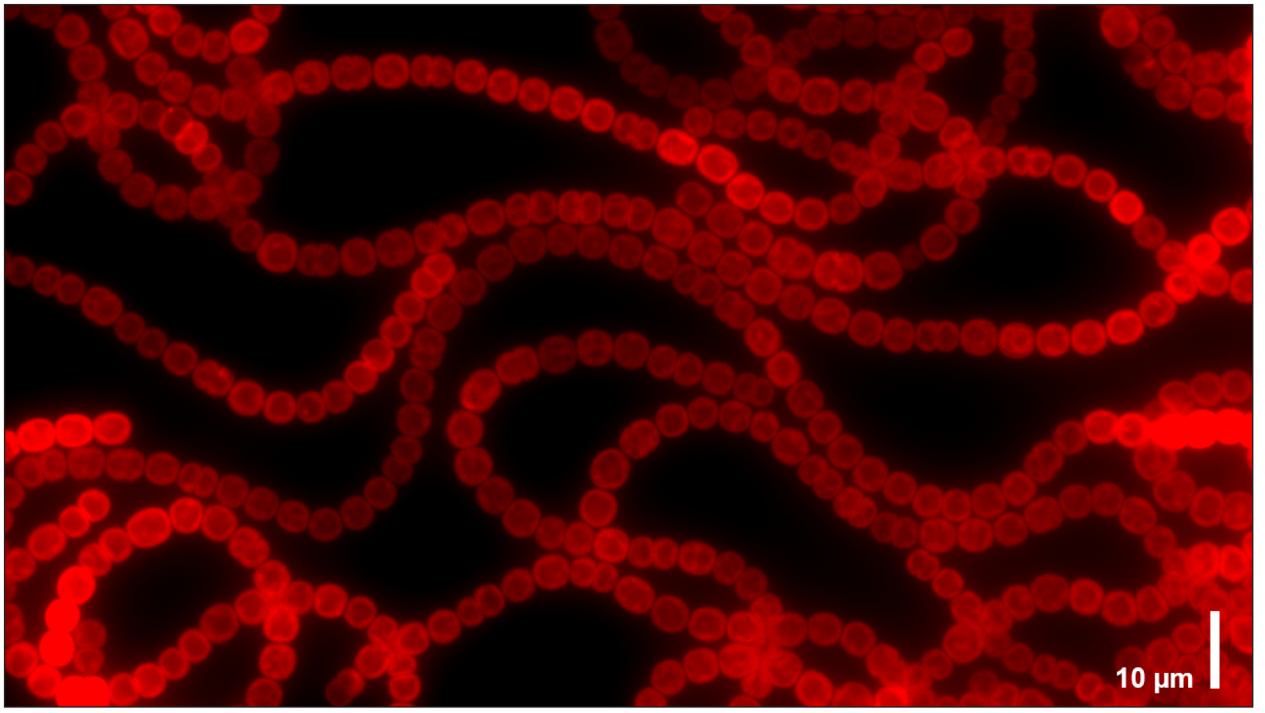

Photo by: Blake Beehler, Illinois Wesleyan University class of 2016

The freshwater filamentous cyanobacterium Anabaena sp. *strain 7120 contains photosynthetic pigments that autofluoresce red. Image taken at 1000x total magnification.*