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CHEMICAL INVESTIGATION OF BACTERIAL
INTERACTIONS INVOLVING PATHOGENS

BY

HILARY JOAN GRANT RANSON

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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UNIVERSITY OF RHODE ISLAND

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DOCTOR OF PHILOSOPHY DISSERTATION

OF

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ABSTRACT

Chemical communication mediates the vast majority of collective bacterial behaviors, orchestrating gene expression and community functions. A fundamental understanding of how pathogens are able to do this underlies the philosophy of natural product chemistry, providing insights into novel mechanisms for controlling pathogenic bacterial infections. To increase the effectiveness of antibiotics, alternative therapeutic approaches can be used that target bacterial phenotypes associated with infections, including swarming motility and quiescence. This dissertation explores molecules that interfere with such phenotypes in two different pathogens: uropathogenic *Escherichia coli* (UPEC) and *Vibrio parahaemolyticus*. Increased understanding of how bacterial behaviors can be modulated in pathogens may significantly improve our ability to tackle these diseases.

The first study, which is reported in chapter two, describes the role of peptidoglycan, a ubiquitous bacterial cell wall component, in facilitating the reversal of quiescence in UPEC. This study uses an *in vitro* model of the quiescent state to test isolated peptidoglycan as a chemical signaling molecule that reverses dormancy. A single purified peptide was isolated from cultures of UPEC using bioassay-guided fractionation and enzyme treatment. The tetrapeptide, Ala-Glu-DAP-Ala was found to be a potent proliferant that reverses quiescence in UPEC. This is the first reported compound to possess this bioactivity.

Chapter three describes an investigation to better understand chemically-mediated bacteria-bacteria interactions between the marine bacteria *Bacillus pumilus* YP001 and *Vibrio parahaemolyticus* PSU5429. The investigational approach used a combination

of experimental methods, emphasizing Matrix-Assisted Laser Desorption Ionization-Time of Flight Imaging Mass Spectrometry (MALDI-TOF IMS), whole genome analysis, and chemical isolations. The main finding showed that multiple compounds produced by the *B. pumilus* may act in concert to effect a lethal outcome on *V. parahaemolyticus* cells. When *B. pumilus* was grown in close proximity to the *V. parahaemolyticus* on an agar surface, secreted secondary metabolites induced swarming motility in the vibrio, chemically attracting toward a lethal zone containing an antibiotic.

Chapter four describes whole genome sequencing and bioinformatics based secondary metabolite profiling of genetically unique bacteria strains isolated from the American lobster *Homarus americanus*. This body of work implicates potentially important genetic pathways for both pathogenic and commensal bacteria. Furthermore, these studies exemplify the use of next generation sequencing and biosynthetic pathways tools to probe the potential for bacteria to produce unique natural products.

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PREFACE

This dissertation has been written in the manuscript format as directed by the guidelines set forth by the graduate school of the University of Rhode Island.

TABLE OF CONTENTS

ABSTRACT.....	ii
ACKNOWLEDGMENTS.....	iv
PREFACE.....	vii
TABLE OF CONTENTS.....	viii
LIST OF TABLES.....	x
LIST OF FIGURES.....	xiii
CHAPTER 1	1
INTRODUCTION.....	1
CHAPTER 2	24
<u>MANUSCRIPT 1:</u> Peptidoglycan sensing prevents quiescence and promotes quorum-dependent growth of uropathogenic <i>Escherichia coli</i>	25
CHAPTER 3	79
<u>MANUSCRIPT 2:</u> Production of lipopeptides and antibiotics by a marine <i>Bacillus pumilus</i> creates a ‘catch and kill’ effect on a <i>Vibrio parahaemolyticus</i>	80
CHAPTER 4	154
<u>MANUSCRIPT 3:</u> Draft genome sequence of the putative marine pathogen <i>Aquimarina</i> sp. I32.4	155
<u>MANUSCRIPT 4:</u> Draft genome sequence of the putative marine pathogen <i>Thalassobius</i> sp. I31.1	162

<u>MANUSCRIPT 5: Draft genome sequence of <i>Loktanella maritima</i> YPC211, a commensal bacterium of the American lobster (<i>Homarus americanus</i>)</u>	178
CONCLUDING REMARKS	174
APPENDICIES	176
<u>APPENDIX A</u>	176
<u>APPENDIX B</u>	186
<u>APPENDIX C</u>	202

LIST OF TABLES

TABLE	PAGE
INTRODUCTION	
<u>Table 1.</u> Known chemical signaling functions of compounds from peptidoglycan	22
MANUSCRIPT 1	
<u>Table 1.</u> Prevalence of quiescence among UPEC ST131 isolates	60
<u>Table S1.</u> <i>E. coli</i> strains and plasmids used for genetic analysis and constructions in this study	68
MANUSCRIPT 2	
<u>Table 1.</u> Genes encoding the surfactin biosynthetic gene cluster in <i>B. pumilus</i> YP001, their proposed function and homology to the biosynthetic genes of <i>B. amyloquelofaciens</i> BGC0000443. Alignments carried out in NCBI-nBLAST	118
<u>Table 2.</u> Genes encoding the amicoumacin biosynthetic gene cluster in <i>B. pumilus</i> YP001, their proposed function and homology to the biosynthetic genes of <i>B. subtilis</i> subsp. Inaquosoum KCTC 13429. Alignments carried out in NCBI-nBLAST	119
<u>Table S1.</u> Amino acid alignments for amicoumacin production from <i>B. pumilus</i> YP001.	128

TABLE	PAGE
<u>Table S2.</u> Amino acid alignments for surfactin production from <i>B. pumilus</i> YP001	119
<u>Table S3.</u> ¹ H and ¹³ C NMR data (δ in ppm, <i>J</i> in Hz) for compound 1 in DMSO- <i>d</i> ₆ .	133
<u>Table S4.</u> NMR Data for amicoumacin B (500 MHz) in DMSO- <i>d</i> ₆	152
<u>Table S5.</u> NMR Data for AI-77-F (500 MHz) in DMSO- <i>d</i> ₆	153
APPENDIX B	
<u>Table 1.</u> Zones of inhibition (mm) from screened lobster isolates (5 μ L spot plates) against <i>Aquimarina</i> sp. strain I32.4, <i>Thalassobius</i> sp. I31.1 and <i>Vibrio anguillarum</i> NB10Sm.	193
<u>Table 2.</u> Optimizing growth conditions for <i>Aquimarina</i> 'homaria'. Symbols correspond with + (Low Clumps), ++ (Clumps), +++ (High Clumps) and + (Low Growth), ++ (Growth), +++ (High Growth)...	194
<u>Table 3.</u> Data obtained from NCBI for all known <i>Aquimarina</i> genomes and analyzed using the antiSMASH cluster analysis database.* <i>Aquimarina pacifica</i> and <i>Aquimarina agarilytica</i> possess no known biosynthetic gene clusters (BGCs)..	195
<u>Table 4.</u> Data obtained from NCBI for all known <i>Thalassobius</i> sp. genomes and analyzed using the antiSMASH cluster analysis database.	196
TABLE	PAGE

Table 5. Monoculture XAD16 extracts activity against *V. anguillarum* NB10Sm. . 198

Table 6. Adult *H. americanus* trial carried out over 12 days. 200

Table 7. Single strain testing of potential probiotics against PL lobsters assessing mortality over 3 days..... 201

Table 8. Ability of probiotic treatment to protect PL lobsters from challenge with the putative pathogen *Thalassobius* I31.1 mortality.. 202

APPENDIX C

Table 1. Data obtained from NCBI for all known genomes of *Loktanella* and analyzed using the antiSMASH cluster analysis database.. 211

LIST OF FIGURES

FIGURE	PAGE
INTRODUCTION	
<u>Figure 1.</u> Model of bacterial chemical communication. Chemical signals in the form of autoinducers and secondary metabolites that act as inter- and intra-species communications to facilitate phenotypic changes.....	20
<u>Figure 2.</u> The structure of peptidoglycan. A) A single mucopeptide disaccharide pentapeptide. B) The structure of peptidoglycan within the cell wall, showing the dibasic amino acid cross-link between strands that forms the structure rigidity.	21
MANUSCRIPT 1	
<u>Figure 1.</u> The quorum-regulated quiescent state of <i>E. coli</i> CFT073 cells on glucose M9 minimal agar is reversible	62
<u>Figure 2.</u> Quiescent CFT073 cells are filamentous at 24 h.....	63
<u>Figure 3.</u> Quiescent CFT073 Δ <i>lysP</i> and Δ <i>argP</i> cells fail to proliferate with L-lysine	64
<u>Figure 4.</u> ST131 strain JJ1886 displays quorum dependent growth	65
<u>Figure 5.</u> Peptidoglycan fragments promote proliferation of quiescent JJ1886 cells .	66
<u>Figure S1.</u> Quiescent CFT cells are filamentous at 48 h.....	69
<u>Figure S2.</u> UPEC strain growth in glucose M9 minimal media.....	71

FIGURE	PAGE
<u>Figure S3</u> . D-lysine and diaminopimelic acid (DAP) fail to stimulate proliferation of CFT073 and JJ1886 cells on glucose M9 minimal agar	72
<u>Figure S4</u> . <i>E. coli</i> , <i>B. subtilis</i> and <i>S. aureus</i> peptidoglycan fragments stimulate proliferation of CFT073 and JJ1886 cells.....	73
<u>Figure S5</u> . Peptidoglycan fragments, including <i>S. aureus</i> pentapeptide, stimulate proliferation of quiescent UPEC cells.....	74
<u>Figure S6</u> . Candidate proliferants tested for reversal of JJ1886 quiescence.....	75
<u>Figure S7</u> . <i>E. coli</i> peptidoglycan fragment analysis by tandem mass spectrometry ...	76
<u>Figure S8</u> . Analysis of quiescence stimulation by PG tetra- and pentapeptides.....	78

MANUSCRIPT 2

<u>Figure 1</u> . Growth inhibition of <i>B. pumilus</i> YP001 against (a) <i>V. harveyi</i> BB120 (b) <i>V. parahaemolyticus</i> PSU5429, (c) <i>V. anguillarum</i> NB10Sm, and (d) <i>V. coralliitlycus</i> RE22 after 24 h incubation on YP30 at 27 °C.	115
<u>Figure 2</u> . Swarming motility assay for <i>B. pumilus</i> YP001 plated in co-culture with <i>V. parahaemolyticus</i> PSU5429 at concentrations of (a) 1×10^5 CFU/mL, (b) 1×10^2 CFU/mL, (c) 1×10^1 CFU/mL, and (d) no dilution. Plate imaged after 24 h incubation at 27 °C.....	116
<u>Figure 3</u> . MALDI-IMS imaging shows the spatial distribution of compounds produced during chemical interaction of <i>B. pumilus</i> YP001 and <i>V. parahaemolyticus</i> PSU5429.	117

FIGURE	PAGE
<u>Figure 4.</u> Chemical structures of compounds 1 and 2	120
<u>Figure 5.</u> ^1H – ^1H COSY and key HMBC correlations for compound 1	121
<u>Figure 6.</u> Swarming bioactivity in response to compound 3 at 10 μg (a), 100 μg (b) and compound 4 10 μg (c), and 100 μg (d) after 24 h incubation at 27 °C on YP30 agar.....	122
<u>Figure 7.</u> Effects of purified surfactin (20 $\mu\text{g}/\text{disk}$) and amicoumacin (20 $\mu\text{g}/\text{disk}$) (a), surfactin (20 $\mu\text{g}/\text{disk}$) only (b), amicoumacin (100 $\mu\text{g}/\text{disk}$) only (c) and <i>B. pumilus</i> YP001 colony (5 μL at 1×10^5 CFU/mL from a broth culture) (d) on <i>V.</i> <i>parahaemolyticus</i> PSU5429 after 24 h incubation at 27 °C.....	123
<u>Figure S1.</u> High resolution TOF-MS for 3 m/z 1034.6790 $[\text{M}-\text{H}]^-$ in negative ion mode. The declustering potential was set to -185 and collision energy to -33.	124
<u>Figure S2.</u> High resolution TOF-MS for 4 m/z 1048.7 $[\text{M}-\text{H}]^-$ in negative ion mode. The declustering potential was set to -200 and collision energy to -50.	125
<u>Figure S3.</u> MADLI-TOF IMS total ion counts (TIC) taken after 48 h incubation indicating the production of pumilacidin B (m/z 1059 $[\text{M}+\text{Na}]^+$), F/G (m/z 1073 $[\text{M}+\text{Na}]^+$), and E (m/z 1087 $[\text{M}+\text{Na}]^+$).....	126
<u>Figure S4.</u> Biosynthesis of amicoumacin by <i>B. pumilus</i> YP001 domain predictions carried out in Geneious	127
<u>Figure S5.</u> Chemical isolation scheme for <i>B. pumilus</i> YP001	132

FIGURE	PAGE
<u>Figure S6.</u> Swarming assay for (a) lipoamide C (100 µg), and (b) lipoamide B2 (100 µg) against <i>V. parahaemolyticus</i> PSU5429.	134
<u>Figure S7.</u> MADLI-TOF IMS ionization of amicoumacin B m/z 425 $[M+H]^+$ and 447 $[M+Na]^+$ plated at 10 mM in methanol onto a YP30 plate.	135
<u>Figure S8.</u> Swarming assay for 100 µg of surfactin D (i) and C-15 (ii) against (a) <i>Vibrio coralliilyticus</i> RE22, (b) <i>Vibrio harveyi</i> BB120, (c) <i>Vibrio anguillarum</i> NB10Sm after 48 h incubation at 27 °C.....	136
<u>Figure S9.</u> 1H -NMR spectrum (500 MHz, DMSO- d_6) of compound 1 from <i>B. pumilus</i> YP001.....	137
<u>Figure 10.</u> ^{13}C -NMR spectrum (500 MHz, DMSO- d_6) of compound 1 from <i>B. pumilus</i> YP001.....	138
<u>Figure S11.</u> : 1H - 1H COSY NMR spectrum (500 MHz, DMSO- d_6) of compound 1 from <i>B. pumilus</i> YP001.....	139
<u>Figure S12.</u> HSQC NMR spectrum (500 MHz, DMSO- d_6) of compound 1 from <i>B. pumilus</i> YP001.....	140
<u>Figure S13.</u> HMBC NMR spectrum (500 MHz, DMSO- d_6) of compound 1 from <i>B. pumilus</i> YP001.....	141
<u>Figure S14.</u> IR spectrum of compound 1	142
<u>Figure S15.</u> HRESIMS of compound 1 measured in positive ion mode (m/z 351.2226 $[M+H]^+$ and 373.2048 $[M+Na]^+$).	143

FIGURE	PAGE
<u>Figure S16.</u> Lipoamide C (2) IR data.....	144
<u>Figure S17.</u> HRESIMS of lipoamide C (2) recorded in positive ion mode (m/z 345.1713 $[M+H]^+$).....	145
<u>Figure S18.</u> ^1H -NMR spectrum (500 MHz, $\text{DMSO-}d_6$) of surfactin D (4) from <i>B. pumilus</i> YP001.....	146
<u>Figure S19.</u> ^{13}C -NMR spectrum (500 MHz, $\text{DMSO-}d_6$) of surfactin D (4) from <i>B. pumilus</i> YP001.....	147
<u>Figure S20.</u> gCOSY-NMR spectrum (500 MHz, $\text{DMSO-}d_6$) of surfactin D (4) from <i>B. pumilus</i> YP001.....	148
<u>Figure S21.</u> TOCSY-NMR spectrum (500 MHz, $\text{DMSO-}d_6$) of surfactin D (4) from <i>B. pumilus</i> YP001.....	149
<u>Figure S22.</u> HSQC-NMR spectrum (500 MHz, $\text{DMSO-}d_6$) of surfactin D (4) from <i>B. pumilus</i> YP001.....	150
<u>Figure S23.</u> ^1H -NMR spectrum (500 MHz, $\text{DMSO-}d_6$) of surfactin C (3) from <i>B. pumilus</i> YP001.....	151
<u>Figure S24.</u> ^1H -NMR spectrum (500 MHz, $\text{DMSO-}d_6$) of of AI-77-F from <i>B. pumilus</i> YP001.....	154

APPENDIX A

<u>Figure 1.</u> Peptidoglycan isolation scheme indicating bioassay guided fractionation of crude peptidoglycan digestion product from <i>E. coli</i> CFT073.....	178
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Figure 2. Isolation methods for peptidoglycan from *E. coli* CTF073 cells to produce ‘crude’ peptidoglycan preparations used for testing in the quiescence bioassay 179

Figure 3. Synthesis, recycling and catabolism pathways for peptidoglycan in the cell. Indicating transporters on the cellular membrane involved in uptake and regulation into the cell. All mutant knockout pathways target a different source of peptidoglycan transport..... 180

Figure 4. High resolution tandem mass spectrometry for disaccharide tetrapeptide (NAG-NAM-Ala-Glu-DAP-Ala) as a potential constituent of crude peptidoglycan preparations. Total ion counts (TIC) for crude peptidoglycan (a), extracted ion counts (XIC) for disaccharide tetrapeptide (b), and MS/MS fragmentation of disaccharide tetrapeptide mass to confirm its structure (c)..... 181

Figure 5. High resolution tandem mass spectrometry for disaccharide tripeptide (NAG-NAM-Ala-Glu-DAP) as a potential constituent of crude peptidoglycan preparations. Total ion counts (TIC) for crude peptidoglycan (a), extracted ion counts (XIC) for disaccharide tripeptide (b), and MS/MS fragmentation of disaccharide tripeptide mass to confirm its structure (c). 182

Figure 6. High resolution tandem mass spectrometry for disaccharide dipeptide (NAG-NAM-Ala-Glu) as a potential constituent of crude peptidoglycan preparations. Total ion counts (TIC) for crude peptidoglycan (a), extracted ion counts (XIC) for disaccharide dipeptide (b), and MS/MS fragmentation of disaccharide dipeptide mass to confirm its structure (c)..... 183

Figure 7. High resolution tandem mass spectrometry for pentapeptide (Ala-Glu-DAP-Ala-Ala) as a potential constituent of crude peptidoglycan preparations. Total ion counts (TIC) for crude peptidoglycan (a), extracted ion counts (XIC) for pentapeptide (b). Ion abundance was too low to accurately assess the structure by MS/MS 184

Figure 8. High resolution tandem mass spectrometry for tetrapeptide (Ala-Glu-DAP-Ala) as a potential constituent of crude peptidoglycan preparations. Total ion counts (TIC) for crude peptidoglycan (a), extracted ion counts (XIC) for tetrapeptide (b), and MS/MS fragmentation of tetrapeptide mass to confirm its structure (c). 185

Figure 9. High resolution tandem mass spectrometry for tripeptide (Ala-Glu-DAP) as a potential constituent of crude peptidoglycan preparations. Total ion counts (TIC) for crude peptidoglycan (a), extracted ion counts (XIC) for tripeptide (b). Ion abundance was too low to accurately assess the structure by MS/MS 186

APPENDIX B

Figure 1. Overlaid chromatograms for SNP spiking experiment at doses 0, 1 and 10 μ M. (A) *B. pumilus* YP001, (B) *Pseudoalteromonas* sp. YPC21, (C) *Pseudoalteromonas* sp. YPC22, (D) *L. maritime* YPC210 (E) *L. maritime* YPC210 198

Figure 2. Assessment of growth for putative probiotics by absorbance of light wavelength (600 nm) as a proxy for cell density over time 199

APPENDIX C

FIGURE	PAGE
<u>Figure 1.</u> NRPS biosynthetic gene cluster analysis and alignment to known gene clusters. Binding pocket sequence analysis was used to predict the substrate specificity	212
<u>Figure 2.</u> Initial chemical screening, 96 well plate fractionation from crude extract to identify the peak responsible for the observed antibiotic activity.	213
<u>Figure 3.</u> Large scale chemical isolation of agrobactin from a co-culture of <i>Loktanella maritima</i> YPC211 and <i>Thalassobius</i> sp. strain I31.1	214
<u>Figure 4.</u> High resolution tandem mass spectrometry intensity from direct injection ESI in positive ion mode (declustering potential 150; collision energy 30) for agrobactin m/z 637.2566 $[M+H]^+$, 659.2373 $[M+Na]^+$, and 681.2185 $[M+Fe]^+$	215
<u>Figure 5.</u> Direct injection TOF MS/MS fragmentation positive ion mode of agrobactin m/z 637.2 $[H+H]^+$ to produce identifiable building blocks and structure confirmation	216
<u>Figure 6.</u> Proton NMR spectrum of agrobactin Varian NMR 500 MHz with DMSO- d_6 as the solvent at 25 °C	217
<u>Figure 7.</u> Analytical HPLC chromatogram of agrobactin as purified product	218

FIGURE	PAGE
<p><u>Figure 8.</u> Disc diffusion assay indicating that only crude extracts from co-cultures of <i>Thalassobius</i> I31.1 and <i>L. maritima</i> YPC211 are able to elicit an antibiotic effect against <i>V. anguillarum</i> NB10Sm. Positive control is ciprofloxacin, negative controls are crude media extract and methanol blank. Testing monoculture extracts of <i>Thalassobius</i> I31.1 and <i>L. maritima</i> YPC211</p>	219
<p><u>Figure 9.</u> MIC assay for agrobactin against MRSA plotted as averages and standard deviation * P<0.05 ** P<0.01 from agrobactin (methanol) and ciprofloxacin (DMSO). Cell density measured by optical density (600 nm absorbance).</p>	220

CHAPTER 1

INTRODUCTION

1.1 Bacterial signaling and infections

Molecular signaling occurs across every known life form ensuring coordinated behaviors for cells and organisms. In eukaryotes, chemical cues may be exchanged in the form of hormones whereas prokaryotes are well known to utilize a phenomenon called quorum sensing. Quorum sensing is a defined mechanism of chemical communication facilitating coordinated behaviors akin to those of multicellular organisms (1).

In bacteria, cell-cell communication facilitates key processes such as bioluminescence, the secretion of virulence factors key for disease states, production of secondary metabolites, reduction of metabolic activity and the formation of biofilms (2-4). All of these are energetically costly and ineffective strategies for an individual cell, but become extremely advantageous when undertaken by a population. How individuals correctly interpret the blend of signals in their vicinity to appropriately regulate their gene expression is crucial to their survival and success.

Chemical relationships between all organisms have been co-evolving over billions of years. As bacteria move through their environments, they also encounter

and respond to complex mixtures of molecules produced by other organisms including competitors. These foreign compounds can also influence gene expression or have toxic consequences. Many of these molecules secreted by bacteria can act on diverse cell types, even altering cell functions of eukaryotic cells (5). These inter-kingdom communications mediate symbiotic and pathogenic relationships between bacteria and their animal and plant hosts (6). Understanding the roles that specific compounds play in natural environments can inform new ways of controlling bacterial populations, especially for infections. This dissertation aims to expand our understanding of chemical signaling to facilitate new treatment strategies for bacterial infections.

1.2 Quorum sensing, *N*-acyl homoserine lactones and quiescence

Quorum sensing is the underlying communication pathway that controls density dependent decision-making in prokaryotes. Individual cells produce and excrete signal molecules that can be detected by their kin. The concentration of these signal molecules, referred to as autoinducers, increases until a receptor response is initiated within the population (2). Hence, this phenomenon relies upon the abundance of extracellular compounds as a proxy for cell abundance, and enables coordinated, multi-cellular behaviors by bacteria.

One of the largest classes of autoinducers are *N*-acyl homoserine lactones (AHLs). These molecules are produced prolifically across phylogenetically-diverse

Gram-negative bacteria and are able to freely diffuse across bacterial membranes making them ideal cell-cell signaling molecules. When AHLs accumulate above the required threshold, the autoinducers bind to a receptor and trigger a signaling cascade that results in population-wide alterations in gene expression (7). Quorum sensing has been shown to control virulence factor production and biofilm formation during infections (2). Virulence factors often trigger the signs of infection in the host organism. While AHLs have been extensively studied in Gram-negative bacteria, the chemical diversity of quorum sensing signals remains unknown. Understanding the identity and roles of autoinducers is important for finding new strategies and treatments for bacterial infections (8).

In Chapter 2 of this dissertation, I explored the use of peptidoglycan fragments as chemical signals to reverse dormant phenotypes in uropathogenic *Escherichia coli* (UPEC). Some pathogens, including *E. coli*, rely upon dormant (i.e. non-proliferating) states to persist within hosts. Growing cells release ~7% peptidoglycan (PG) fragments from their cell wall during exponential growth (9). These fragments have been shown to be key signals for reversing dormancy in *Bacillus* and *Mycobacterium* (2,10). Hence, certain PG fragments may serve as quorum signals to indicate favorable conditions and exit this state, leading to recurrent infections of the host (11).

The mechanisms and requirements for dormancy (i.e. quiescence) in UPEC and the sensing molecules that reverse this phenotype are now becoming better understood. UPEC cells are able to adhere to human bladder epithelial cells, where they are internalized to form intracellular bacteria communities (IBCs). Once internalized, UPEC cells enter into a quiescent state, during which they evade both host immune response and antibiotic treatment (12). The phenomenon of quiescence has been reported for several bacteria including *Bacillus* spp., *Bordetella pertusis*, and *E. coli* (3,12). For certain UPEC, the quiescent state can be reliably simulated through agar based nutrient limitation (12).

The archetypical peptidoglycan structure consists of a repeating disaccharides (*N*-acetylglucosamine and *N*-acetylmuramic acid) backbone covalently bonded to a stem peptide chain containing 2-5 amino acid residues. The stem peptide structure is predominantly L-alanine, D-glutamic acid, meso-diaminopimelic acid (mDAP) and D-alanine. The dibasic amino acid DAP forms crosslinks with adjacent strands forming the structural rigidity of the cell wall (Figure 2). Bacterial peptidoglycan structure variability is extensive, including modifications of both the sugar backbone and amino acid stem peptide chain (13). For example, *Staphylococcus* peptidoglycan exhibits a modification of L-lysine at the third position of the stem peptide chain (14). Most Gram-positive bacteria contain a deacetylase, encoded by the gene *pdgA*, which causes *N*-deacetylation of either one or both of the sugars in the backbone (14). Many studies

have investigated the properties of peptidoglycan fragments; however, very few have been able to deduce the single bioactive components.

Components of peptidoglycan have been investigated for their function in modulating developmental transitions out of non-growing, dormant cell states and for their effects on host cells during infections. Peptidoglycan has been shown to stimulate resuscitation and germination of *Mycobacterium tuberculosis*, *Mycobacterium smegmatis* and *Bacillus subtilis* (10, 15, 16). Hosts cell interactions with peptidoglycan fragments vary from symbiotic to pathogenic. In whooping cough, the anhydrous disaccharide tetrapeptide of peptidoglycan (TCT) causes tracheal cytotoxicity to human ciliated epithelial cells (17, 18). Smaller fragments of the stem peptide structure also elicit similar dose dependent toxicity (17). The same product from *Neisseria gonorrhoeae* is cytotoxic to human fallopian tube epithelial cells (19, 20). Contrastingly, *Vibrio fischeri* utilizes TCT to induce normal cell metamorphosis in the light organ of bobtail squid forming a mutualistic relationship (21, 22).

Peptidoglycan-derived fragments are recognized by host immune systems and promote defense responses against bacterial infections in mammals, invertebrates and plants (5, 17, 23). Thus, peptidoglycan can serve as an inter-kingdom signal molecule, modulating both prokaryotic and eukaryotic cells. Table 1 summarizes the known functions of common peptidoglycan fragments. Interestingly, most studies are unable

to identify the specific PG fragments that are singly responsible for the observed effects.

Although mostly bacterial pathogens have been studied in their ability to form a dormant state, about 60% of the microbial biomass on Earth is thought to be quiescent (11). These organisms may survive in the absence of nutrients for periods of up to years. Creating robust *in vitro* assays to demonstrate and study quiescence are inherently difficult, but essential for understanding the control mechanisms for reversing these states (7). The occurrence and signaling mechanisms that facilitate the behavior into and out of bacterial quiescence are currently understudied and represent an important area of research for tackling recurrent bacterial infections.

1.3 Natural products and bacterial signaling

Natural products have historically served as a powerful source of novel therapeutic agents against bacterial infections. Currently, 59% of all FDA approved drugs to treat bacterial infections originated from natural products produced by microorganisms (24). Traditionally these molecules are isolated and tested as pure compounds in bioassays. However, in natural environments many signals are being produced concurrently and may work cooperatively. This creates a complex interplay that is likely important for microbial competition and disease progression but poorly understood. Replicating the ‘cocktails’ of compounds used in nature represents an

untapped opportunity to design of new therapeutic drugs and drug combinations. This is especially true as new knowledge emerges regarding phenotypes that influence drug resistance and genomic technologies enable more rapid discovery of novel natural products skeletons.

Vibrio spp. are ubiquitous in marine ecosystems and prolific pathogens. For example, *V. parahaemolyticus* is the leading cause of seafood-borne illness in the United States (29), causing gastroenteritis that often requires treatment with antibiotics. Natural products produced by marine bacteria may offer novel ways of tackling the prevalence of this bacterium in seafood. Swarming motility is one phenotype that increases antibiotic resistance and speed of infection on solid or semi-solid surfaces such as tissues (25, 26). Bacterial swarming is induced by quorum sensing, alterations in ion channel fluxes, and iron scarcity (27). Swarming is enabled by molecules that decrease surface tension, thereby allowing coordinated, directional movement of cells (27, 28). Identification of molecules that induce or inhibit swarming motility could lead to unconventional tools for controlling infections.

The ubiquitous Gram-positive marine bacterium *Bacillus pumilus* produces structurally diverse secondary metabolites including lipopeptide, polypeptides, macrolactones, fatty acids, polyketides and isocoumarins (30-32). As explored in Chapter 3 of this dissertation, partnering antimicrobial activity with induction of bacterial motility poses an unconventional and intriguing approach for controlling and

modulating infections. These ecological observations could unlock further research on combinations of secondary metabolites as multifaceted treatment strategies.

1.4 Emerging techniques for understanding the roll of cell signaling molecule bacterial diseases.

Antibiotic resistant bacterial infections pose a world health threat, causing more than two million illnesses in the USA in 2014 (33). The increased prevalence of multi-drug-resistant (MDR) pathogens has caused decreased effectiveness of front line antibiotics. In the United States alone, 23,000 people die annually from drug-resistant infections (34). Despite incentives from the Infectious Disease Society of America (IDSA), approval of new antibiotics has dropped significantly in recent years and only four novel drugs have been approved since 1998 (35). Alternative treatment options are needed to increase the effectiveness of our current antibiotics while avoiding the emergence of drug resistance. Such options could include compounds that modulate crucial bacterial behaviors involved in pathogenesis.

1.4.1 Matrix-assisted laser desorption ionization-time of flight imaging mass spectrometry (MALDI-TOF IMS)

Mass spectrometry is an analytical technique utilized to understand the chemical diversity of the natural world. It is highly sensitive and has allowed significant

advances in the detection of naturally occurring molecules and measurement of their ecologically relevant concentrations. The development of imaging mass spectrometry (IMS) techniques has advanced this approach to include spatial mapping of ionizable molecules on a surface. IMS is now particularly useful for probing the biological effects induced by signaling molecules (36).

Visualizing chemical production *in situ* is fundamental to understanding the direct relationships between signal molecules, allelopathic secondary metabolites and bacterial response. Matrix-Assisted Laser Desorption Ionization-Time of flight Imaging Mass Spectrometry (MALDI-TOF IMS) allows *in situ* data collection for compounds produced by actively growing bacterial colonies on agar surfaces. This technique involves culturing one or more bacteria on thin agar plates and applying a uniform dry coat matrix. The matrix is generally a combination of small organic acids that co-crystallize with the sample and facilitate ionization from laser energy. MALDI-TOF IMS is able to capture the metabolic exchange of information between two bacteria grown in close proximity (37). This powerful technique can be applied to rapidly discriminate bacterial function based on key metabolite production (38). MALDI-TOF IMS was used in Chapter 3 of this dissertation to investigate the chemical interplay between a marine *B. pumilus* and *V. parahaemolyticus*.

1.4.2 Genomic and computational approaches for natural product discovery

Advances in genomics have revealed that the majority of biosynthetic gene clusters (BGCs) are not linked to any previously identified natural products (39). The abundance of ‘silent’ BGCs within even well studied bacteria has identified a bottleneck for natural product discovery (40). Genomic data also provides substantial insights into the pool of secondary metabolites that individual bacteria strains are able to produce. Several powerful online tools now provide structure predictions of biosynthetic products from sequence data (41), allowing targeting of novel natural product scaffolds from genetically unique pathways. These chemical pathways are frequently not activated under standard laboratory conditions but may be induced during co-culturing conditions due to competitive conditions that better mimic the natural environment. Chapter 4 of this dissertation, includes a collection of three manuscripts describing the genomes of marine bacteria and their expected biosynthetic potential.

1.5 Future perspectives for bacterial disease treatment

In nature, diverse microbes are in intimate contact with each other, and cell-cell chemical signals are exchanged within and between phylogenetically distinct populations (42). These signals can range from simple lactones to complex peptides. Each individual compound can alter the expression of vast numbers of genes, affecting biological processes and bacterial infections. Interrupting these signals may block

virulence factors responsible for antibiotic-resistance-increasing phenotypes without increasing MDR, thus offering possible advantages for treating bacterial infections. Alternative treatment options for bacterial infections, including those that increase the effectiveness of our current antibiotics, may someday significantly improve our ability to confront drug resistance. The overall theme of my dissertation research has been to explore how bacterial metabolites influence the behaviors and growth of two problematic pathogens: human UPEC and the marine pathogen *V. parahaemolyticus*. The aim was to reveal possible new mechanisms and tools to tackle these infectious diseases.

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Figure 1: Model of bacterial chemical communication. Chemical signals, in the form of quorum sensing autoinducers and secondary metabolites, act as inter and intra-species communications to facilitate phenotypic changes.

Figure 2: The structure of peptidoglycan. A) A single mucopeptide disaccharide pentapeptide. B) The structure of peptidoglycan within the cell wall showing the dibasic amino acid cross-link between strands that forms the structure rigidity.

Table 1: Known chemical signaling functions of compounds from peptidoglycan.

Peptidoglycan Fragment	Structure	Producing Organism	Function
Disaccharide tripeptide		<i>E. coli</i> and <i>B. subtilis</i>	Exit from dormancy in <i>B. subtilis</i> (3).
Anhydro-disaccharide tetrapeptide		<i>B. pertussis</i> <i>V. fischeri</i> <i>N. gonorrhoeae</i>	Tracheal cytotoxicity to human ciliated cells (17). Signal for morphogenesis in the bobtail squid (21). Cytotoxic to human fallopian tube epithelial cells (19).
Disaccharide pentapeptide		<i>Aeromonas</i> spp.	Beta-lactamase induction in <i>Aeromonas hydrophila</i> (43).
Anhydro-disaccharide pentapeptide		<i>E. coli</i>	Beta-lactamase induction in <i>Enterobacter cloacae</i> (44).
Lac-tetrapeptide		<i>B. pertussis</i>	Tracheal cytotoxicity to human ciliated cells (17).

Tetrapeptide	<div> <div>L-al</div> <div>D-glu</div> <div>mDAP</div> <div>D-ala</div> </div>	<i>B. pertussis</i>	Partial tracheal cytotoxicity to human ciliated cells (17).
Dipeptide	<div> <div>D-glu</div> <div>mDAP/L-lys</div> </div>	<i>B. subtilis</i> /pDML995	Increased beta-lactam resistance in <i>Bacillus licheniformis</i> (45).

CHAPTER 2

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Peptidoglycan sensing prevents quiescence and promotes quorum-independent growth of uropathogenic *Escherichia coli*

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Abstract

Uropathogenic *Escherichia coli* (UPEC) are the leading cause of human urinary tract infections (UTIs) and many patients experience recurrent infection after successful antibiotic treatment. The source of recurrent infections may be persistent bacterial reservoirs *in vivo* that are in a quiescent state and thus are not susceptible to antibiotics. Here, we show that multiple UPEC strains require a quorum to proliferate *in vitro* with glucose as the carbon source. At low cell density, the bacteria remain viable but enter a quiescent, non-proliferative state. Of the clinical UPEC isolates tested to date, 35% (51/145) enter this quiescent state, including isolates from the recently emerged, multidrug-resistant pandemic lineage ST131 (i.e., strain JJ1886) and isolates from the classic endemic lineage ST73 (i.e., strain CFT073). Moreover, quorum-dependent UPEC quiescence is prevented and reversed by small molecule proliferants that stimulate growth, including peptidoglycan (PG) stem tetra- and pentapeptides, and the amino acids L-lysine and L-methionine. Peptidoglycan fragments originate from the peptidoglycan layer that supports the bacterial cell wall but are released as bacteria grow. These fragments are detected by a variety of

organisms, including human cells, other diverse bacteria and, as we show here for the first time, UPEC. Together, these results show that (i) PG stem peptide sensing and uptake of L-lysine by UPEC modulate the quorum-regulated decision to proliferate, and (ii) UPEC quiescence can be prevented by both intra- and interspecies PG fragment signaling.

Introduction

At least 80% of urinary tract infections (UTIs) are caused by uropathogenic *Escherichia coli* (UPEC) strains, and 27% of patients with a UTI experience recurrence within 12 months after successful antibiotic treatment [1-3](#). UPEC appear to enter a quiescent, non-growing state within urothelial transitional cells in the bladder wall that may enable *E. coli*, long after antibiotic treatment has been terminated, to resume division within bladder urine after apoptotic release from those cells [4-5](#).

We recently reported the discovery of a quiescent state of *E. coli* that is common among UPEC strains [6](#). *E. coli* CFT073, a member of the major UPEC lineage sequence-type (ST) 73, and approximately 80% of ST73 strains tested, proliferate on glucose minimal agar plates seeded with 10^6 or more CFU, but become quiescent on glucose minimal agar when plated at a cell density of less than 10^6 CFU [6](#). Moreover, 23% of randomly selected UPEC strains of diverse STs isolated from community-acquired UTIs also become quiescent on glucose minimal plates seeded with less than 10^6 CFU [6](#). Remarkably, these results indicate that growth of many UPEC strains on glucose minimal agar is quorum-dependent [6](#). Alternative sole carbon sources, including acetate, arabinose and *N*-acetylglucosamine (NAG), also lead to cells entering quiescence at low plating density, whereas glycerol, ribose and xylose support proliferation [6](#). We also reported that mini-Tn5 transposon insertions in five central carbon metabolism genes (*gdhA*, *gnd*, *pykF*, *sdhA*, *zwf*) prevent

quiescence, suggesting that metabolic function must remain intact during quiescence even though the cells are non-proliferative. CFT073 quiescence is also prevented when cells are placed near a single colony of actively growing CFT073 or *E. coli* MG1655, a K-12 laboratory strain, suggesting that the actively growing cells release one or more molecules that act as a proliferant [6](#). Indeed, we showed that L-lysine is a proliferant for quiescent CFT073 cells, but only in combination with either L-methionine or L-tyrosine, yet CFT073 is not auxotrophic for lysine, methionine or tyrosine, and the remaining 17 amino acids play no role in preventing CFT073 quiescence [6](#). Quorum-dependent proliferation suggests the ability to sense a cell-to-cell signal(s). In this case the signal includes L-lysine and L-methionine, and signal detection underlies the decision by each individual cell to grow and divide or to enter the quiescent state. This suggests that the transition between the proliferative and quiescent states is controlled by a quorum sensing system [7,8](#).

Here we show that quiescent CFT073 cells are filamentous and that L-lysine import into quiescent CFT073 stimulates proliferation, suggesting that L-lysine synthesis is inhibited during quiescence. In two pathogenic UPEC strains, including CFT073, a prototypic ST73 UPEC strain, and JJ1886, a pandemic ST131 strain that displays increased sensitivity to proliferants, we show that peptidoglycan (PG) stem peptides prevent quiescence. PG is a component of the bacterial cell wall that contains polymers of alternating linked sugars NAG and N-acetyl muramic acid (NAM), with

attached stem peptides containing D-amino acids that crosslink. PG fragments have been reported to regulate development of *Bacillus subtilis* and *Mycobacterium tuberculosis* [9-11](#). PG fragments are also normally secreted by growing *E. coli* [12](#), and here we show for the first time that they act as quorum-signaling molecules in UPEC. In this study we demonstrate that population-sensing and metabolite availability modulate the quiescent state of UPEC, and we define an *in vitro* model system for UPEC quiescence for further study of UPEC physiology.

Results

Quorum-dependent growth of UPEC and prevention of quiescence on glucose minimal agar plates by secreted signals. UPEC strain CFT073 enters a quiescent state when plated at low cell density ($<10^6$ cells per plate) on glucose minimal agar yet grows robustly when plated at high cell density ($>10^6$ cells per plate), demonstrating that growth of CFT073 on glucose minimal agar plates is quorum-dependent (Fig. S1A). As reported previously, quiescent CFT073 cells can be resuscitated by challenge with specific external stimuli, called proliferants, including 5 μ l of a solution containing L-lysine (1 mM) and L-methionine (1 mM), 5 μ l of human urine, or a single colony of actively growing *E. coli* spp. (CFT073 or MG1655), added to the plate [6](#). In a soft agar overlay containing 10^4 quiescent CFT073 cells, we tested the known proliferants and in each case observed the development of a zone of actively

growing colonies surrounding the site of challenge (Fig. 1A). Without challenge, cells failed to grow throughout the course of the experiment (24 h) and remained quiescent. We also tested additional clinical isolates and found that quorum-dependent growth on glucose minimal agar is common among the ST131 UPEC strains in this study (8/33) (Table 1); however, we discovered that the number of cells constituting a quorum varies among strains. For example, the ST131 strain JJ1886 was proliferative on glucose minimal agar at a plating density greater than 10^3 CFU, but quiescent at or below 10^3 CFU (Table 1). Of the 35 strains tested in this study, 10 were reversibly quiescent on glucose minimal agar, and three of those strains were quiescent at or below a plating density of 10^3 CFU, but non-quiescent at 10^5 CFU (Table 1). These results show that sensitivity for quorum detection varies by strain, i.e., is strain-specific.

Next, we monitored the zone of growth of CFT073 cells as they exited quiescence over 72 h. First, we poured soft agar plates containing quiescent CFT073 cells seeded at 10^4 CFU per plate, placed an actively growing colony of CFT073 onto the surface of each plate by toothpick and measured the zones of growth around the sites of inoculation at 24, 48 and 72 h. We observed that the zone of growth expands in diameter until the entire plate is covered by a lawn of actively growing cells (Fig. 1B and 1C). There are two potential scenarios to explain this result. First, the

proliferant(s) that stimulates growth of quiescent cells is continuously released from the toothpicked colony throughout the experiment and induces growth of distal quiescent cells as it diffuses across the plate. Alternatively, the proliferant(s) that stimulates growth of quiescent cells is released from the toothpicked colony but, upon release of the signal, neighboring and formerly quiescent cells begin secreting their own signal, thereby stimulating adjacent quiescent cells to grow. To distinguish between these possibilities, glucose minimal agar plates were seeded with 10^4 quiescent CFT073 cells from a stationary phase liquid glucose minimal medium culture, and a single CFT073 colony was toothpicked onto the center of each plate. The plates were then incubated for 24 h. During that time, a small zone of growth formed on each plate around the central toothpicked colony (Fig. 1B, 1C and 1D). After the zone had formed at 24 h, the toothpicked colony was removed to stop the colony from secreting additional signal(s). Even after removal of the toothpicked colony from the plate, the zone of growth continued to expand, unabated, for an additional 48 h and at a similar rate as the control plate, where the colony had been left intact (Fig. 1C and 1D). These results suggest that the proliferation-promoting signal propagates across the plate from CFT073 cells as they exit quiescence. These results further show that quiescent CFT073 cells at the edge of the plate remain viable and can be stimulated to proliferate for up to 72 h.

Quiescent CFT073 cells are filamentous on glucose minimal agar plates. During infection, UPEC cells from quiescent intracellular reservoirs (QIRs) display several different morphologies, including rods, spheres and filaments [13-15](#). To determine if quiescent cells in our *in vitro* quiescence system also display morphological features that are distinct from actively growing cells, we compared the cell morphologies of CFT073 cells under several culture conditions, including LB agar, and glucose minimal agar supplemented with L-lysine (1 mM) and L-methionine (1 mM), where indicated (Fig. 2A). After 24 h on LB agar or on glucose minimal agar supplemented with L-lysine and L-methionine, colonies were apparent and individual cells collected and analyzed by microscopy appeared as short rods with mean lengths of 1.72 ± 0.59 μm ($n = 200$) and 2.33 ± 0.80 μm ($n = 200$), respectively (Fig. 2A and 2B). By contrast, after 24 h on non-supplemented glucose minimal agar plates, no colonies were detected and quiescent cells harvested from the plate had a broad length distribution, with a mean length of 7.17 ± 4.34 μm ($n = 200$), which is 300% longer than cells grown on glucose minimal agar supplemented with lysine and methionine (Fig. 2A and 2B). Many quiescent cells were longer than 10 μm and 16.5% of them appeared to contain one or more incomplete septa (Fig. 2A and 2B) (Fig. S1B). To assess for changes in cell length with extended incubation, we also measured cell length at 0 h and 48 h after entry into quiescence. At 0 h, cells harvested from a stationary phase culture grown in liquid glucose minimal medium were short rods with

a mean length of $2.14 \pm 0.58 \mu\text{m}$ ($n = 200$). After 48 h on glucose minimal agar plates, quiescent cells were filamentous, with a mean length of $6.84 \pm 3.76 \mu\text{m}$ ($n = 72$), which is similar to the mean length at 24 h (Fig. S1B and S1C). These results suggest that quiescent CFT073 cells elongate during the first 24 h on the plate but fail to complete division.

Lysine import is necessary for lysine to prevent quiescence, but an additional signal that induces proliferation is released from actively growing cells. L-lysine is a proliferant for quiescent CFT073 cells on glucose minimal agar in the presence of L-methionine [6](#). To determine whether L-lysine must be imported into CFT073 cells to prevent quiescence, we constructed a CFT073 *lysP* insertion-deletion mutant by λ -Red recombination (Table S1). Of the three known lysine importers in *E. coli*, LysP is the major importer and a member of the amino acid, polyamine and organocation (APC) family of transporters [16](#)[17](#) [ENREF 9](#). CFT073 Δ *lysP* cells grew well overnight in liquid glucose minimal medium, similar to the wild type CFT073 strain (Fig. S2A) and exhibited quiescence on glucose minimal agar at low cell density (10^4 cells per plate) (Fig. 3); yet, it grew robustly when plated at high density (10^8 cells per plate). Unlike wildtype CFT073, when 5 μl of a solution of L-lysine (1 mM) and L-methionine (1 mM) was added to quiescent CFT073 Δ *lysP* cells plated at 10^4 CFU, no colony growth was observed after 24 h of incubation, suggesting that LysP-dependent

transport is critical for lysine to stimulate proliferation. This also suggests that lysine biosynthesis is inhibited in quiescent cells. CFT073 $\Delta lysP$ was complemented with a plasmid containing *lysP* (pLysP), which restored the ability of the strain to proliferate upon addition of L-lysine in the presence of L-methionine at low cell density (Fig. 3). Although quiescent CFT073 $\Delta lysP$ cells failed to respond to L-lysine, they proliferated after a colony of actively growing CFT073 cells was added to the plate (Fig. 3). Collectively, these results suggest that import of L-lysine is required for L-lysine to stimulate proliferation and override the absence of the quorum, and that one or more L-lysine-independent proliferants that reverse quiescence are secreted from bacterial colonies.

ArgP is a transcriptional regulator of *lysP* [16-18](#). In the absence of lysine, ArgP positively regulates transcription of *lysP*, along with several *E. coli* genes involved in lysine and PG biosynthesis, including *dapB* (dihydropicolinate reductase), *dapD* (tetrahydrodipicolinate succinylase), *lysA* (diaminopimelate decarboxylase) and *lysC* (aspartokinase III) [16-18-20](#). To test whether ArgP plays a regulatory role in quorum-dependent growth or quiescence, we deleted *argP*. Like wild type CFT073, CFT073 $\Delta argP$ cells grew well overnight in liquid glucose minimal medium (Fig. S2A) and were quiescent at low cell density (10^4 CFU per plate) on glucose minimal agar (Fig. 3), indicating that ArgP is not essential for inducing quiescence at low density (Fig. 3).

Similar to the result with CFT073 $\Delta lysP$ cells, quiescent CFT073 $\Delta argP$ cells failed to respond to the addition of L-lysine with L-methionine after 24 h of incubation, but the cells proliferated in response to a colony of CFT073 toothpicked to the center of a glucose minimal agar plate seeded with 10^4 CFU of CFT073 $\Delta argP$ (Fig. 3). Complementation of CFT073 $\Delta argP$ with the wildtype CFT073 *argP* gene on a plasmid (pArgP) restored the ability of the strain to respond to L-lysine in the presence of L-methionine at low cell density (Fig. 3). Since LysP expression has been reported to be repressed 35-fold in *E. coli argP* mutants [20](#), this result both (i) supports the conclusion that external L-lysine must be transported into CFT073 via LysP in order to prevent quiescence and (ii) confirms that expression of LysP is regulated by ArgP. Together, these results also show that there is a molecule(s) other than L-lysine secreted by actively growing cells that stimulates proliferation of quiescent CFT073 cells.

To further clarify why the population threshold that defines a quorum varies between strains, we investigated the ST131 clinical isolate JJ1886, which exhibits quiescence on glucose minimal agar at or below a plating density of 10^3 CFU, but grows robustly on such plates at 10^5 CFU and in liquid glucose minimal medium (Fig. 4A and S2B). We observed that 5 μ l of a solution of L-lysine (1 mM) alone is sufficient to promote growth and prevent quiescence of JJ1886 plated at low density on glucose minimal

agar (10^3 CFU per plate), although growth is more efficient in the presence of L-methionine (1 mM), and L-methionine (1 mM) alone does not stimulate growth (Fig. 4B). Since quiescent JJ1886 cells on glucose minimal agar proliferate in the presence of L-lysine alone, we titrated the L-lysine concentration to determine the threshold for stimulation of growth and compared JJ1886 to CFT073. We determined that a low concentration of L-lysine (5 μ l of 0.1 mM) is sufficient to promote proliferation of quiescent JJ1886 cells (10^3 CFU/plate), whereas quiescent CFT073 cells (10^3 CFU/plate) proliferated at a higher concentration of L-lysine (>0.5 mM) and only in the presence of L-methionine. These results suggest that although JJ1886 behaves phenotypically similar to CFT073 with respect to quiescence, it is more sensitive to detecting or responding to an external signal(s) that prevents quiescence. We then tested if quiescent JJ1886 also responds to the other known CFT073 proliferants, including 5 μ l of urine or a colony of actively growing *E. coli* cells, and observed that both agents were effective for stimulating proliferation of quiescent JJ1886 cells (Fig. 4B).

Since L-lysine stimulates proliferation of both JJ1886 and CFT073, we tested 5 μ l of D-lysine (1 mM) and 5 μ l of the biosynthetic lysine precursor diaminopimelic acid (Dap) (1 mM) for stimulating proliferation of quiescent cells. Both agents failed to stimulate growth of quiescent CFT073 and JJ1886 (Fig. S3A and S3B).

Peptidoglycan fragments prevent UPEC quiescence. Next, we considered PG as a potential signal that would communicate a quorum since PG fragments, including di-, tri-, and tetrapeptides, are normally secreted by actively growing bacterial cells including *E. coli* [10](#)[12](#)[21](#)[22](#). We tested if peptidoglycan fragments from *E. coli* could prevent quiescence and stimulate proliferation of CFT073 and JJ1886. Peptidoglycan was digested with mutanolysin, a mixture of two N-acetylmuramidases and an N-acetylmuramyl-L-alanine amidase, to release a heterogeneous mixture of peptidoglycan fragments, including various muropeptides and stem peptides (Fig. 5A). Addition of the total mixture of digested peptidoglycan fragments from *E. coli* induced proliferation of quiescent JJ1886 cells, plated at 10^3 CFU per plate (Fig. 5A), and quiescent CFT073 cells, plated at 10^4 CFU per plate (Fig. S4A). Titrating the amount of total peptidoglycan added to the agar plate revealed a dose-dependent response, leading to differences in the zones of growth around the site of addition (Fig. S4B). Digested peptidoglycan from *B. subtilis* and *Staphylococcus aureus* also stimulated proliferation of quiescent JJ1886 and CFT073 cells (Fig. S4C and S4D). Furthermore, peptidoglycan fragments were also effective for stimulating proliferation of quiescent CFT073 Δ lysP cells to proliferate, indicating that the peptidoglycan response is independent of lysine import (Fig. S5A). Interestingly, these results show that quiescent *E. coli* cells respond to PG fragments from various bacterial species, which

can differ in the identity of the amino acid at the third position of the stem peptide, usually *meso*-Dap (i.e., *E. coli*) or L-lysine (i.e., *S. aureus*) [10:23:24](#).

Peptidoglycan stem peptides act as signals to promote proliferation of quiescent JJ1886 and CFT073 cells. To identify which fragment(s) of digested PG stimulates proliferation of quiescent cells, we performed high performance liquid chromatography-mass spectrometry (HPLC-MS) on *E. coli* PG fragment mixtures (Fig. 5A) and tested fractions across the elution for the ability to promote proliferation of quiescent JJ1886 cells. We found that early fractions that were collected with retention times up to 10 min were sufficient to stimulate proliferation of quiescent JJ1886 cells (Fig. S5B). Tandem mass spectrometry confirmed that these fractions contain low molecular weight peptidoglycan fragments, consistent with stem peptides, such as the tri- and tetrapeptides. In contrast, later eluting fractions containing muropeptides did not stimulate proliferation of quiescent JJ1886 cells (Fig. S5B).

To further explore the molecular determinants of PG peptides that stimulate proliferation of quiescent cells, we compared several high-purity stem peptide fragments of varying amino acid compositions and lengths. We observed that a tripeptide, representing the minimal unit of an *E. coli* peptidoglycan stem peptide that could be generated from amidase and carboxypeptidase activities in the *E. coli*

periplasm (L-Ala-D-Glu-*m*Dap), failed to stimulate proliferation of quiescent JJ1886 cells (Fig. 5B). However, a peptidoglycan pentapeptide, L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (A-E-K-A-A), similar to the stem pentapeptide present in *S. aureus* peptidoglycan, stimulated proliferation of quiescent JJ1886 (Fig. 5C) and CFT073 (Fig. S5A) cells. We also tested a smaller fragment of the pentapeptide, acetyl-L-Lys-D-Ala-D-Ala (K-A-A) but did not observe stimulatory activity (Fig. S6A). Fragments including the disaccharide moiety alone, containing NAG and NAM (NAG-NAM) (Fig. 5D), and higher molecular weight molecules with the attached stem peptide (i.e., disaccharide tetrapeptide) (Fig. 5E), in various states of crosslinking, eluted later, after 10 min (Fig. S7), and had no proliferant activity. NAG-NAG, NAG and NAM were also tested, but none of these sugars stimulated proliferation of quiescent cells (Fig. S6B).

To determine if a peptidoglycan stem peptide longer than three amino acids and derived from *E. coli* stimulates proliferation of quiescent cells, we generated tetrapeptide in situ by digesting purified disaccharide tetrapeptide (isolated by HPLC) with AmiD, an amidase from *E. coli* [22](#). AmiD cleaves a stem peptide from attached sugars (Fig. S8A). After cleavage to release the tetrapeptide, the reaction products were highly active for stimulating proliferation of both quiescent JJ1886 cells (Fig. 5F) (Fig. S8B) and quiescent CFT073 cells (Fig. S8C). NAG-NAM displays no

activity alone, therefore these results suggest that peptidoglycan stem peptides from *E. coli* (tetrapeptide) and *S. aureus* (pentapeptide) function as proliferants.

Finally, to further clarify if stem peptides exert their function without entering the cytoplasm or if they are imported into a quiescent cell and then enter the peptidoglycan recycling pathway, we constructed JJ1886 strains deleted for the oligopeptide transporter operon, *oppABCDF* [25-29](#), and also the peptidoglycan tripeptide-specific delivery protein *mppA* [27-30-32](#). Both deletion strains grew well in liquid glucose minimal medium and became quiescent when plated at 10^3 CFU per plate, similar to parent strain JJ1886; furthermore, like JJ886, both strains were stimulated to proliferate by addition of stem peptides to the plate (Fig. S8B and S8D), indicating that it is not necessary for the stem peptides to enter the cytoplasm via the Opp transporter in order to act as a signal to overcome quiescence.

Discussion

Here, we show that two virulent UPEC strains, CFT073 and JJ1886, enter a quiescent state when plated on glucose minimal agar, and this state is prevented and reversed by proliferants, including peptidoglycan stem peptides consisting of four (e.g., *E. coli*) or five (e.g., *S. aureus*) amino acids. Quiescence is a unique physiological state described for *E. coli* that is common among UPEC strains (i.e., 51/145 isolates tested) and is

quorum-regulated. The quiescent state is adopted by an entire population of cells under a given growth condition, in contrast to a persister state, which occurs in a small percentage of cells within a population [33-37](#). Adoption of a quiescent, non-proliferative state would require many physiological systems to slow (e.g., transcription, translation) or stop (e.g., DNA replication, division and peptidoglycan synthesis). Accordingly, we observed that quiescent cells adopt a filamentous morphology, failing to successfully divide or proliferate despite extended incubation (Fig. S1B). Finally, different strains respond to known proliferants with varying sensitivities, suggesting that either the sensitivity of detection or the regulatory control over physiological pathways varies among strains. For example, L-lysine is sufficient to induce proliferation of quiescent JJ1886 cells (Fig. 4B), whereas L-lysine must be supplemented with either L-methionine or L-tyrosine to induce proliferation of quiescent CFT073 cells [6](#). While lysine import is one way to overcome quiescence, stimulation by peptidoglycan stem peptides occurs independently of the lysine importer *lysP*, suggesting that there are multiple regulatory inputs that enable reinitiation of the cell cycle. It is also possible that detection of peptidoglycan fragments stimulates lysine biosynthesis or increases intracellular lysine concentration in a coordinated manner, which then induces proliferation.

Muropeptide fragments are known to resuscitate dormant *Mycobacterium smegmatis*

[38](#) and to signal *B. subtilis* endospores to germinate [11](#). Here we showed that in *E. coli*, PG stem tetra- and pentapeptide fragments not only cause cells to reenter the cell cycle, but may also act as a signal to communicate the presence of a quorum, independent from uptake by the Opp import system. Previously, it was shown that an *oppA* mutant is far less infective in a mouse UTI model than the wildtype [39](#), and it was suggested that small oligopeptides, present in high concentration in urine [39](#), are imported and serve as major nutrients for CFT073 growth in urine [39](#). Here, neither the pentapeptide nor the tetrapeptide entered the quiescent cells via Opp, suggesting that they are imported by a second, presently unknown, oligopeptide permease, or act as signals in the periplasm and are recognized by a membrane receptor or signal transduction system that, when activated, promotes cell proliferation. The serine/threonine kinase PknB is a widely conserved PG binding protein that is important for germination of spores in *B. subtilis* [11](#); however, no similar proteins have been found thus far in *E. coli*.

The cause(s) of recurrent UTIs are complex [40](#); however, it appears that UPEC can bind to, enter, and replicate within superficial facet cells in the mouse and human bladder epithelium, resulting in intracellular biofilm-like communities (IBCs) [40-41](#). IBCs escape from infected superficial facet cells within hours of development and several cycles of IBC formation occur, but each successive round is associated with a

reduced replication rate and smaller IBCs [15](#). The infected superficial facet cells exfoliate, exposing underlying transitional epithelial cells that become infected with IBC-derived UPEC progeny. Upon infection of transitional cells, small numbers of UPEC appear to enter a latent or quiescent intracellular state in endosomal vesicles (2 to 12 cells/vesicle), establishing QIRs [42](#) that are resistant to antibiotic treatment and are a possible cause of recurrent UPEC infection. If the physiology of UPEC quiescence *in vitro* is similar to that *in vivo*, understanding how PG stem peptides prevent *in vitro* quiescence at the molecular level may help inform how to prevent recurrent UPEC infections in humans.

Methods

Bacterial strains, plasmids and growth conditions. *E. coli* strains and plasmids used in this study are listed in Table 1. Clinical UPEC isolates were obtained from the collections of J. Johnson (29 strains) and E. Sokurenko (5 strains) (Table 2). Lennox broth (LB) supplemented with agar (LB agar) was used for routine cultivation. Liquid glucose (0.2% or 0.4%, where indicated) M9 minimal medium and 0.2% glucose M9 minimal agar plates were prepared as described previously [6](#). Gene deletions were constructed by λ -Red recombination as described in Datsenko and Wanner and

confirmed by sequencing [43](#). Culture density was monitored by growing cells in liquid 0.2% glucose M9 minimal medium and measuring OD₆₀₀ at the indicated times.

Plasmids for complementation of both CFT073 $\Delta lysP$ and CFT073 $\Delta argP$ gene deletions were constructed using expression vector pBAD24. Both genes were amplified from CFT073 by whole colony PCR (*lysP*, 5'

atcaagaattcacatgggtccgaaactaaaactacagaag 3', 5'

tagataagcttttatttcttatcggtctgcgggaacttc 3'; *argP*, 5'

atcaagaattcacatgcaacccgacacaaaattgtgtcatag 3', 5'

tagataagctttaatcctgacgaagaactttgtgaccataatcg 3'). The resulting PCR products were

digested with EcoRI and HindIII. Digested PCR products were ligated into

EcoRI/HindIII-digested pBad24. Both pLysP and pArgP plasmid constructs were

verified by sequencing and then transformed into either CFT073 $\Delta lysP$ or CFT073

$\Delta argP$ deletion strains by electroporation. To test for complementation, transformants

were grown overnight in 0.4% glucose M9 minimal medium with ampicillin (50 μ g

ml⁻¹) and were assayed for quiescence on 0.2% glucose minimal agar supplemented

with arabinose (0.1%), where indicated.

Quiescence assay. Quiescence assays were performed as described previously [6](#).

CFT073 and JJ1886 wild type and mutant strains were grown on LB agar plates

overnight at 37 °C. An isolated colony was used to inoculate 5 ml of 0.4% glucose M9 minimal medium, incubated at 37 °C with shaking overnight, and then diluted to between 10^3 to 10^8 CFU, as indicated, in 3 ml 0.2% glucose M9 minimal medium containing 0.75% Difco noble agar held at 45 °C. Inoculated soft agar was immediately poured onto plates containing a 12 ml layer of 0.2% glucose M9 minimal medium with 1.5% noble agar or directly into an 8.5 cm² polystyrene culture dish. Putative proliferants, including actively growing cells, amino acids, Dap (2,6-diaminopimelic acid), sterile human urine and peptidoglycan fragments, were added and plates were incubated at 37 °C for 24 to 72 h, where indicated, and then imaged.

Microscopy. Overnight cultures of CFT073 were grown to stationary phase in 0.4% glucose M9 minimal medium. Five μ l of a 3-log diluted culture was spotted onto LB agar or 0.2% glucose M9 minimal medium with 1.5% noble agar and, where indicated, supplemented with L-lysine (1 mM) and L-methionine (1 mM), then allowed to dry for 1 h at room temperature. Plates were incubated at 37 °C for 24 or 48 h. Cells were harvested onto a glass cover slip, resuspended in phosphate-buffered saline (PBS) with ethylenediaminetetraacetic acid (EDTA) (1 mM) and applied to an agar gel pad containing 0.2% glucose M9 minimal medium with 0.5% noble agar. Cells were visualized by differential interference contrast (DIC) microscopy using a Zeiss LSM 700, and images were captured on an AxioCam HRc high-resolution camera with ZEN

2012 software. Images were processed using Adobe Photoshop CC and analyzed using NIH ImageJ software.

Peptidoglycan fragment separation. *E. coli* CFT073 was inoculated in 10 ml of M9 minimal medium containing 0.4% glucose and incubated at 37 °C shaking (200 rpm) for 24 h. Cultures were subsequently inoculated into one liter of M9 minimal medium containing 0.4% glucose and incubated for 24 h shaking (175 rpm). Methods used for PG isolation were adapted from Desmarais *et al.*, 2014 [44](#). Cells were harvested at 5,000 x g for 10 min at room temperature and pellets were resuspended in 3 ml of M9 minimal medium. Cell suspensions were transferred into 6 ml of 6% sodium dodecyl sulfate (SDS) and boiled for 3 h with stirring. After 3 h, heat was discontinued, and cell suspensions were stirred overnight at room temperature. Cell suspensions were centrifuged at 262,000 x g for 30 min, pellets were resuspended in liquid chromatography mass spectrometry (LCMS) grade water and further centrifugation and wash steps occurred until no SDS remained. The final pellet was resuspended in 900 µl of 10 mM Tris-HCl with 0.06% w/v NaCl and 100 µg of Pronase E was added to the cell suspension and then incubated for 2 h at 60 °C. After incubation, 200 µl of 6% SDS was added and the mixture was boiled at 100 °C for 30 min. Samples were centrifuged at 262,000 x g for 30 min and washed 4 times with LCMS grade water. The final pellet was resuspended in 200 µl of PBS and incubated with 200 U of

mutanolysin (Sigma-Aldrich) at 37 °C overnight. Samples were centrifuged at 16,000 x g for 10 min to remove cellular debris, PG supernatant was collected for further testing. Mutanolysin was removed by ultrafiltration using a polyethersulfone filter with a 3-kDa molecular weight cut-off.

Analytical high-performance liquid chromatography (HPLC) was used to purify the PG extract. HPLC experiments were performed on a Shimadzu prominence-I LC-2030C equipped with a PDA detector model LC-2030/2040, pump LC-2030, and autosampler LC-2030. Preparative HPLC experiments were completed with a Waters XBridge Analytical C18 5 μ m, 4.6 $\times$ 250 mm column at 30 °C using a solvent gradient of 10-100% B (5 mM ammonium acetate with 15% acetonitrile pH 4.89) run over 16 min against solvent A (5 mM ammonium acetate pH 4.37). Solvent B was held at 10% for 4 min prior to the gradient and 100% B was held for a subsequent 4 min at the conclusion of the method. Individual peaks were collected according to 205 nm absorbance values at 205 nm. Initial chemical characterization was carried out by liquid chromatography mass spectrometry (LCMS) on a Thermo Fisher ISQ EC coupled to a Shimadzu Prominence-i LC-2030 system. Chemical separation utilized the same mobile phase gradient as previously with a reduced flow rate of 0.6 ml min⁻¹. Chemical characterization of pure compounds was undertaken using electrospray

ionization mass spectrometry (ESIMS) using an AB Sciex TripleTOF 4600 spectrometer and LCMS/MS experiments.

Methods of LCMS/MS were performed on a AB Sciex TripleTOF 5600 spectrometer coupled to a Waters H class UPLC. Methods of LC mirrored that of initial screening with an adjusted flow rate of 0.5 ml min⁻¹ on an XBridge Analytical C18 5 µm, 4.6 × 250 mm column. Acquisition (SWATH) was performed in positive ionization mode. The method specific parameters were as follows: gas 1 (GS1) 55 psi, gas 2 (GS2) 60 psi, curtain gas (CUR) 25 psi. The source-specific parameters were: temperature (TEM) 500 °C, ion spray voltage floating (ISVF) 5500 V, declustering potential (DP): 100, collision energy (CE) 10 and collision energy spread (CES) 15. An initial time-of-flight (TOF) scan was collected from *m/z* 100 to 1500 and SWATH data was acquired (*m/z* 200-1100) over 36 SWATH windows per cycles with a window size of *m/z* 25 and a *m/z* 1 overlap between windows.

Peptidoglycan from *B. subtilis* (Sigma-Aldrich) or *S. aureus* (Sigma-Aldrich) (10 mg ml⁻¹) was incubated with 200 U of mutanolysin at 37 °C overnight. Peptidoglycan-derived stem peptide fragments including ‘A-E-K-A-A’ pentapeptide (L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (Sigma-Aldrich), ‘K-A-A’ tripeptide (acetyl-L-Lys-D-Ala-D-Ala)

(Sigma-Aldrich) and 'A-E-Dap' tripeptide (L-Ala-D-Glu-*m*Dap) (AnaSpec), were each tested at 5 µl (2 mM) against strains JJ1886, JJ1886 $\Delta oppABCDF$ and JJ1886 $\Delta mppA$ (10^3) after growth on M9 minimal agar with 0.2% glucose for 24 h at 37 °C.

Amidase cleavage of disaccharide tetrapeptide. The *amiD* gene sequence coding for residues 18 through 276 was amplified from *E. coli* MG1655, cloned into pET-24b using NdeI/HindIII restriction sites to incorporate a C-terminal hexahistidine tag (5' actgacatatggcaggcgaaaaaggcat 3', 5' gcagtaagcttatcctgcccgatttc 3') and expressed in BL21 (λ de3). This resulted in removal of the N-terminal signal sequence and lipid modification site. The cells were grown in LB supplemented with 50 µg ml⁻¹ kanamycin at 37 °C to an OD₆₀₀ of 0.8. Expression was induced with isopropyl-β-D-thiogalactoside (1 mM) for 3 h at 30 °C. Cells were harvested by centrifugation at 6,000 x g for 30 min at 4 °C and resuspended in 25 ml of 25 mM Tris-HCl (pH 7.8) buffer containing 150 mM NaCl, 1 mM MgCl₂ and 10% glycerol. Cells were lysed by French press. Soluble cell extracts were collected by centrifugation at 30,000 x g for 30 min at 4 °C, then applied to TALON Superflow resin (GE Healthcare), washed with 10 column volumes of lysis buffer and eluted with an imidazole gradient. Buffer was exchanged by PD-10 desalting column to remove imidazole. Isolated *E. coli* PG disaccharide tetrapeptide was digested with 315 ng of AmiD in 8.5 mM Tris-HCl (pH

7.8), 50 mM NaCl, 0.3 mM MgCl₂ and 3% glycerol for 26 h at 37 °C. Cleavage was confirmed by LCMS analysis as described above.

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Author contributions

E.C.D. designed and performed the experiments. E.C.D., P.S.C. and J.L.C. conceptualized the study and analyzed the results. H.J.R. performed, and H.J.R. and D.C.R. designed and analyzed the HPLC and mass spectrometry. J.R.J. designed and coordinated the comparative analysis of clinical isolates. E.C.D. and J.L.C. wrote the manuscript. J.L.C. and D.C.R. obtained the funding for the study. J.L.C. supervised the study and managed the project. All authors reviewed, edited and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper.

(Table S1, Figure S1, S2, S3, S4, S5, S6, S7, S8)

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TABLE 1. Prevalence of quiescence among UPEC ST131 isolates

Strain	Sequence type	ST131 Clade	Quiescent at 10 ³ CFU and reversed by Lys and Met ^a	Source, reference
CFT073	ST73	n/a	+ ^b	6
Nissle 1917	ST73	n/a	+ ^b	6
JJ1886	ST131	<i>H30Rx/</i> C2	+ ^c	J. Johnson
JJ1887	ST131	<i>H30Rx/</i> C2	-	J. Johnson
JJ2547	ST131	<i>H30Rx/</i> C2	+ ^c	J. Johnson
JJ2050	ST131		-	J. Johnson
JJ2528	ST131	<i>H30R1/</i> C1	-	J. Johnson
MVAST0036	ST131	<i>H30R1/</i> C1	-	J. Johnson
MVAST014	ST131	<i>H30R1/</i> C1	+ ^c	J. Johnson
H17	ST131	<i>H22/</i> B0	-	J. Johnson
JJ1901	ST131		-	J. Johnson
JJ2087	ST131	<i>H22/</i> B	-	J. Johnson
JJ1999	ST131	<i>H22/</i> B	-	J. Johnson
MVAST167	ST131	<i>H41/</i> A	-	J. Johnson
MVAST020	ST131	<i>H41/</i> A	-	J. Johnson
JJ2134	ST131	<i>H30Rx/</i> C2	+ ^b	J. Johnson
JJ2183	ST131	<i>H30Rx/</i> C2	-	J. Johnson
JJ2489	ST131	<i>H30Rx/</i> C2	-	J. Johnson
QUC02	ST131		-	J. Johnson
JJ2118	ST131	<i>H30R1/</i> C1	-	J. Johnson
JJ2193	ST131	<i>H30R1/</i> C1	-	J. Johnson
JJ2210	ST131	<i>H30R1/</i> C1	-	J. Johnson
JJ2578	ST131	<i>H30R1/</i> C1	-	J. Johnson
JJ2608	ST131	<i>H30R1/</i> C1	-	J. Johnson
MVAST038	ST131	<i>H30R1/</i> C1	-	J. Johnson
MVAST046	ST131	<i>H30R1/</i> C1	+	J. Johnson
MVAST077	ST131	<i>H30R1/</i> C1	-	J. Johnson
MVAST084	ST131	<i>H30R1/</i> C1	-	J. Johnson
MVAST131	ST131	<i>H30R1/</i> C1	-	J. Johnson
MVAST158	ST131	<i>H30R1/</i> C1	-	J. Johnson
MVAST179	ST131	<i>H30R1/</i> C1	+ ^b	J. Johnson
JJ2546	ST131		-	E. Sokurenko
JJ2548	ST131		+ ^b	E. Sokurenko
JJ2963	ST131		-	E. Sokurenko
JJ2974	ST131		+ ^b	E. Sokurenko

^a Strains were seeded at 10³ CFU and 10⁵ CFU on 0.2% glucose M9 minimal agar plates and incubated at 37 °C for 24 h, (+) represents quiescent, (-) represents non-quiescent.

^b Denotes strains that are quiescent when seeded at both 10³ CFU and 10⁵ CFU on 0.2% glucose M9 minimal agar and grown at 37 °C for 24 h.

^c Denotes strains that are quiescent when seeded at 10^3 CFU, but non-quiescent when seeded at 10^5 CFU on 0.2% glucose M9 minimal agar plates and grown at 37 °C for 24 h.

Figures

Fig. 1. The quorum-regulated quiescent state of *E. coli* CFT073 cells on glucose M9 minimal agar is reversible. (A) CFT073 cells (10^4 CFU) were plated onto M9 minimal agar with 0.2% glucose. A 5 μ l solution of L-lysine (1 mM) and L-methionine (1 mM), sterile human urine, or an actively growing colony of CFT073 was transferred to the center of the plate, cells were incubated for 24 h at 37 °C, and the zone of growth surrounding the challenge site was monitored. (B, C) Zones of growth expand with time after addition of an actively growing colony of CFT073 up to 72 h. (D) Removal of the actively growing colony at 24 h has no effect on zonal expansion. White arrows indicate position of stimulant addition, brackets indicate the zone of colony growth and scale bars are 1 cm in length.

Fig. 2. Quiescent CFT073 cells are filamentous at 24 h. (A) CFT073 cells were grown on LB agar or 0.2% glucose minimal agar at 37 °C with and without L-lysine (1 mM) and L-methionine (1 mM), harvested at 24 h and visualized by DIC microscopy. Scale bars are 5 μm in length. (B) Violin plot representing cell length distribution at 24 h for 200 cells grown under conditions described in (A).

Fig. 3. Quiescent CFT073 $\Delta lysP$ and $\Delta argP$ cells fail to proliferate with L-lysine.

All strains, including CFT073, CFT073 $\Delta lysP$, CFT073 $\Delta argP$, were plated on M9 minimal agar with 0.2% glucose at 10^4 CFU and then challenged with a 5 μ l solution of L-lysine (1 mM) and L-methionine (1 mM), sterile human urine or an actively growing colony of MG1655 transferred to the center of the plate. CFT073 $\Delta lysP$ cells containing pLysP or pBad24, where indicated, were supplemented with 50 μ g ml⁻¹ ampicillin and 0.1% arabinose. CFT073 $\Delta argP$ cells containing pArgP or pBad24, where indicated, were supplemented with 50 μ g ml⁻¹ ampicillin. Plates were incubated at 37°C for 24 h.

Fig. 4. ST131 strain JJ1886 displays quorum dependent growth. (A) JJ1886 cells were plated onto 0.2% glucose M9 minimal agar with and LB agar at 10⁵ and 10³ CFU. JJ1886 displays quorum dependent growth at 10³ CFU on glucose M9 minimal agar. (B) JJ1886 (10³ CFU) were plated onto 0.2% glucose M9 minimal agar. A 5 μ l solution of L-lysine (1 mM) and L-methionine (1 mM), L-lysine (1 mM), L-methionine (1 mM), sterile human urine, or an actively growing colony of CFT073 was transferred onto a JJ1886-seeded plate and plates were incubated for 24 h at 37 °C.

Fig. 5. Peptidoglycan fragments promote proliferation of quiescent JJ1886 cells.

High resolution LCMS total ion counts (TIC) including (A) mutanolysin-digested crude *E. coli* PG, which was tested for stimulating proliferation of quiescent JJ1886 cells plated at 10^3 CFU on 0.2% glucose M9 minimal agar, inset; (B) elution of a PG

tripeptide (L-Ala-D-Glu-*m*DAP), $[M+H]^+$ 391.632 (m/z), similar to an *E. coli* fragment and corresponding test for quiescence reversal, inset; (C) elution of a PG pentapeptide (L-Ala-D-Glu-L-Lys-D-Ala-D-Ala), $[M+H]^+$ 489.380 (m/z), identical to a *S. aureus* PG pentapeptide and corresponding test for quiescence reversal, inset; (D) elution of a NAG-NAM disaccharide, $[M+K]^+$ 535.128 (m/z), and corresponding test for quiescence reversal, inset; and (E) elution of a disaccharide tetrapeptide, $[M+H]^+$ 940.573 (m/z), isolated from crude *E. coli* PG and corresponding test for quiescence reversal, inset; (F) products of the enzymatic reaction of *E. coli*-derived disaccharide tetrapeptide isolated from HPLC and then digested with AmiD to produce tetrapeptide (L-Ala-D-Glu-*m*DAP-D-Ala), $[M+H]^+$ 462.198 (m/z), and corresponding test of reaction products for quiescence reversal, inset. Candidate proliferants were collected and tested in the quiescence assay as described in *Methods* and plates were incubated at 37 °C for up to 24 h. Where shown, white arrows indicate position of proliferant addition. Scale bar is 1 cm in length.

TABLE S1. *E. coli* strains and plasmids used for genetic analyses and constructions in this study

<i>E. coli</i> Strain or Plasmid	Genotype	Identifier	Source, reference or construction
Strains			
CFT073 Str ^r	Spontaneous streptomycin-resistant mutant of CFT073	CFT073	6
MG1655	<i>LAM-rph-1</i>	MG1655	45
BL21 (λde3)	F- <i>ompT gal dcm lon hsdSB(rB-mB-)</i> λ(de3[<i>lacI lacUV5-T7 gene 1 ind1 sam7 nin5</i>])		EMD Millipore, USA
ED0052	CFT073 Str ^r Δ <i>lysP::parE-kan</i>	CFT073 Δ <i>lysP</i>	CFT073; λred
ED0070	CFT073 Str ^r Δ <i>argP::cat</i>	CFT073 Δ <i>argP</i>	CFT073; λred
JJ1886		JJ1886	46
ED0118	JJ1886 Δ <i>mppA::cat</i>	JJ1886 Δ <i>mppA</i>	JJ1886; λred
ED0131	JJ1886 Δ <i>oppABCDF::cat</i>	JJ1886 Δ <i>oppAB CDF</i>	JJ1886; λred
Plasmids			
pKD46	<i>amp</i>		43
pKD3	<i>cat</i>		43
pKD267	<i>kan</i>		B. Wanner ^a
pKD119	<i>tet</i>		43
pBAD24			47
pLysP	<i>amp</i> (expression vector)	pLysP	This study
pArgP		pArgP	This study
pet-AmiD	<i>amp</i> P _{ara} :: <i>lysP</i> <i>amp</i> P _{ara} :: <i>argP</i> <i>kan</i> , (Δ <i>N17</i>) <i>amiD-His</i> ₆		This study

^a J. Teramoto, K. A. Datsenko, and B. L. Wanner, unpublished

Supplementary Figures

Fig. S1. Quiescent CFT073 cells are filamentous at 48 h. (A) CFT073 cells were seeded on 0.2% glucose M9 minimal agar plates and incubated for 24 h at 37 °C at low density (10^4 CFU), which promotes quiescence, and at high density (10^8 CFU), which prevents quiescence and promotes proliferation, leading to a lawn of colony growth on the agar plate. (B) CFT073 cells were grown on 0.2% glucose M9 minimal agar plates at 37 °C, harvested at 0 and 48 h and visualized by DIC microscopy. Scale bars are 5 μm in length. Arrows indicate position of failed septa. (C) Violin plot

representing cell length distribution at 0 h ($n = 200$) and 48 h ($n = 72$), grown under conditions described in (B).

Fig. S2. UPEC strain growth in glucose M9 minimal medium. (A) CFT073, CFT073 $\Delta lysP$, CFT073 $\Delta argP$ and (B) JJ1886 were grown in 0.2% glucose M9 medium at 37 °C for 24 h with OD₆₀₀ measured every 2 h from 0 to 12 h and 22 to 24 h. All strains grow robustly overnight in glucose M9 minimal medium.

Fig. S3. D-lysine and diaminopimelic acid (Dap) fail to stimulate proliferation of CFT073 and JJ1886 cells on glucose M9 minimal agar. (A) CFT073 (10⁴ CFU) and (B) JJ1886 (10³ CFU) were seeded on 0.2% glucose M9 minimal agar and challenged with a solution of either (A) L-lysine (1 mM) and L-methionine (1 mM), D-lysine (1 mM) and L-methionine (1 mM), Dap (1 mM) and L-methionine (1 mM), (B) L-lysine (1mM), D-lysine (1 mM) or Dap (1 mM). Plates were incubated for 24 h at 37 °C and imaged. Arrows indicate position of stimulant addition. Scale bars are 1 cm in length.

Fig. S4. *E. coli*, *B. subtilis* and *S. aureus* peptidoglycan fragments stimulate proliferation of CFT073 and JJ1886 cells. (A, C) CFT073 (10⁴ CFU) and (D) JJ1886 (10³ CFU) cells were plated onto 0.2% glucose M9 minimal agar plates and challenged with a mixture of digested (A) CFT073 PG, (C, D) *B. subtilis* or *S. aureus* PG or (A) PBS. Plates were incubated (D) 24 h at 37 °C and (A, C) 48 h at 37 °C. (B) Digested *E. coli* PG was titrated (0 to 10 µg) on JJ1886 seeded plates and CFU per zone was calculated. White arrows indicate position of stimulant addition. Scale bars are 1 cm in length.

Fig. S5. Peptidoglycan fragments, including a *S. aureus* pentapeptide, stimulate proliferation of quiescent UPEC cells. (A) CFT073 (10^4 CFU), CFT073 Δ *lysP* and (B) JJ1886 (10^3 CFU) cells were plated onto 0.2% glucose M9 minimal agar and challenged with a mixture of digested *B. subtilis* peptidoglycan (50 μ g), *S. aureus* pentapeptide (A-E-K-A-A) (5 μ g) or water. Plates were incubated for 48 to 72 h at 37 °C in (A). (B) Quiescent JJ1886 cells were challenged with early (4 to 10 min) or late (10 to 20 min) fractions of mutanolysin digested *E. coli* peptidoglycan fractionated by HPLC. Plates were incubated for 24 h at 37 °C. Arrows indicate position of proliferant addition and scale bars are 1 cm in length.

Fig. S6. Candidate proliferants tested for reversal of JJ1886 quiescence. LCMS profiles of total ion counts (TIC) for a tripeptide (acetyl-L-Lys-D-Ala-D-Ala), 331.25 [M+H]⁺ (*m/z*) (A), similar to an *S. aureus* PG fragment, which was tested for stimulating proliferation of quiescent JJ1886 cells plated at 10³ CFU per plate on 0.2% glucose M9 minimal agar, inset; (B) elution of a PG pentapeptide identical to a *S. aureus* fragment and corresponding test for quiescence reversal, inset; (C) Candidate proliferants including NAG (0.4 mM), NAM (0.4 mM) and NAG-NAG (1 mM) were tested for their ability to stimulate quiescent JJ1886 cells (10³ CFU per plate) on 0.2% glucose M9 minimal agar plates and plates were incubated for 24 h at 37 °C. White arrows indicate position of proliferant addition and scale bars are 1 cm in length.

Fig S7. *E. coli* peptidoglycan fragment analysis by tandem mass spectrometry.

Tandem mass spectrometry profiles showing total ion counts (TIC) for (A) crude peptidoglycan extracted from *E. coli* and extracted ion counts (XIC) for fragments from digested *E. coli* peptidoglycan and including LCMS/MS fragmentation for (B, E) disaccharide tetrapeptide, (C, F) disaccharide tripeptide and (D, G) disaccharide dipeptide.

Fig. S8. Analysis of quiescence stimulation by PG tetra- and pentapeptides. *E. coli*

peptidoglycan tetrapeptide was generated in situ by digestion of disaccharide tetrapeptide with purified AmiD. (A) AmiD as purified by IMAC and analyzed by SDS-PAGE. (B) AmiD, disaccharide tetrapeptide and AmiD-digested disaccharide tetrapeptide, which released NAG-NAM and tetrapeptide, were tested for the ability to stimulate proliferation of quiescent JJ1886, JJ1886 $\Delta oppABCDF$ and JJ1886 $\Delta mppA$ cells plated at 10^3 CFU on M9 minimal agar with 0.2% glucose or (C) quiescent CFT073 cells plated at 10^4 CFU onto M9 minimal agar with 0.2% glucose and incubated for 48 h. (D) *S. aureus* PG stem pentapeptide (L-Ala-D-Glu-L-Lys-D-Ala-D-Ala) (5 μ g) was tested for the ability to stimulate proliferation of quiescent JJ1886, JJ1886 $\Delta oppABCDF$ and JJ1886 $\Delta mppA$ cells plated at 10^3 CFU on M9 minimal agar with 0.2% glucose and incubated for 24 h at 37 °C. Where indicated, brackets

highlight areas of stimulated growth and white arrows show position of stimulant addition. Scale bar is 1 cm in length.

CHAPTER 3

MANUSCRIPT 2: This manuscript has been written for publication in mSystems. The formatting follows the guidelines set forth by the journal.

Production of lipopeptides and antibiotics by a marine *Bacillus pumilus* creates a
'catch and kill' effect on a *Vibrio parahaemolyticus*

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mass spectrometry

Author Contributions

H.J.R, V.Z.P., A.G.S., and Q.W. designed and performed the experiments. V.Z.P., and A.G.S carried out the MADLI-TOF IMS experiments. H.J.R, and Q.W. completed the isolation and structure elucidations. H.J.R carried out the genome sequencing and analysis. H.J.R and D.C.R wrote the manuscript. L.M.S and D.C.R. supervised the study and managed the project.

Abstract

Bacteria produce a wide range of secondary metabolites that influence the health and behaviors of neighboring cells. These chemically mediated interactions are difficult to decipher since bacteria are both secreting and reacting to suites of compounds at any given time. Visualization of these metabolic exchanges on agar surfaces has been aided by Matrix-Assisted Laser Desorption Ionization-Time of Flight Imaging Mass Spectrometry (MALDI-TOF IMS). In this study, we investigated chemical interactions between colonies of two marine bacteria, *Vibrio parahaemolyticus* PSU5429 and *Bacillus pumilus* YP001. When colonies of the two bacteria were grown in close proximity on an agar surface, we observed commencement of swarming motility by the *V. parahaemolyticus*. As the vibrios moved closer to the *Bacillus*, a zone of bacteria lysis appeared around the *B. pumilus* colony. MALDI-TOF IMS suggested that lipopeptides secreted by the *Bacillus* were responsible for

inducing swarming motility. Genomic and chemical investigation of YP001 verified the production of lipopeptides and amicoumacin antibiotics, and purified compounds recapitulated the induction of swarming and antibiosis, respectively, when tested against *V. parahaemolyticus*. In this scenario, lipopeptide production induces the vibrios to swarm towards a lysis zone, resulting in a possible ‘catch and kill’ effect. These results demonstrate the complexity of behaviors and outcomes by microbes under the simultaneous influence of different allelochemicals and suggest possible interplays between antibiotics and compounds that induce motility.

Importance

Bacterial secondary metabolism provides a wealth of chemically diverse molecules possessing wide-ranging biological activities. Many of these molecules are expected to mediate interactions with other organisms, especially other microbes. However, deciphering the possible ecological roles of individual compounds is difficult due to small spatial scales and low compound abundance. In this study, we used a combination of MALDI-TOF imaging mass spectrometry, biosynthetic gene cluster analysis, and secondary metabolite identification to investigate chemical interactions between the marine bacteria *Vibrio parahaemolyticus* and *Bacillus pumilus*. Our results imply an interplay between lipopeptides produced by *B. pumilus* that induce swarming motility in the vibrio, causing the latter to move toward an antibiotic lysis

zone. These observations highlight the complexity of chemical interactions between microorganisms when multiple compounds are simultaneously secreted and suggest that multiple secondary metabolites could work in concert to influence outcomes.

Introduction

Microbial communities use chemical communication for synchronizing the behaviors of cells, coordinating key processes such as movement, secretion of secondary metabolites, bioluminescence, virulence factor production, and the formation of biofilms (1). Bacteria also secrete molecules for defensive purposes, iron acquisition, and to enhance motility, such as surfactants that modify surfaces to enable coordinated movements such as swarming (2). These biosynthetic processes are energetically expensive and ineffective strategies for individual cells but become advantageous when undertaken collectively in a population.

Deciphering the intended ecological functions of microbial secondary metabolites is challenging due to the small scales on which these interactions occur and the complexity of unraveling simultaneous responses to multiple signals. Recent advances in imaging mass spectrometry (IMS) techniques have helped enable the visualization of chemically mediated microbial interactions (3). IMS methods, such as Matrix-Assisted Laser Desorption Ionization-Time of Flight Imaging Mass Spectrometry (MALDI-TOF IMS), provide a spatial map of ionizable molecules on a

surface as they diffuse away from a source such as a microbial colony. Visualizing the metabolic exchanges between groups of cells can provide clues to the ecological functions of secondary metabolites and aid in the formulation of hypotheses for how compounds influence competition outcomes.

Bacteria belonging to the genus *Bacillus* are recognized as prolific producers of allelochemicals, especially biologically active peptides. The antibiotics polymyxin B and colistin are examples of medically useful peptide drugs derived from *Bacillus* species (4,5). Surfactins are cyclic lipopeptides that lower surface tension and enable coordinated swarming, a form of social motility, observed with many *Bacillus* species (6). Surfactins have also been implicated in antagonism toward other microbes, initiation of biofilm production, surface attachment, and as chemoattractants (7-12). MALDI-TOF IMS is particularly well suited to detection and characterization of secreted peptides and has proven useful in prior studies of chemically-mediated microbial interactions between *Bacillus* and other bacteria (12,13).

Our interest in bacteria interactions in marine aquaculture settings led us to investigate the chemical interplay between *Bacillus pumilus* YP001 and *V. parahaemolyticus* PSU5429, a prevalent marine pathogen in aquaculture. When co-cultured on an agar surface, the vibrio colony was observed to commence swarming motility in the direction of the *Bacillus* colony. As the vibrios approached the *Bacillus* colony, a lysis zone became visible, suggesting that both chemoattraction and

antibiosis were acting upon the motile vibrio cells. To better understand how metabolic exchanges drive this microbial interaction, we combined MALDI-TOF IMS, genome analysis, and studies of purified compounds. Here we demonstrate how multiple compounds may act in concert to combine chemoattractant and antibiosis effects on a community of *V. parahaemolyticus*.

Results

***B. pumilus* YP001 induces *V. parahaemolyticus* to swarm toward a lysis zone.**

B. pumilus YP001 was isolated from an egg bearing female American lobster *Homarus americanus* in Narragansett Bay, RI. As part of a binary screening effort to identify marine bacteria displaying antibiosis toward *Vibrio parahaemolyticus*, we observed that YP001 colonies created zones of inhibition when grown on a lawn of *V. parahaemolyticus* PSU5429, suggesting the production of a compound(s) with growth inhibitory properties (Figure 1b). To explore this interaction further, we next spotted colonies of YP001 and PSU5429 in close proximity on YP30 agar plates. Surprisingly, after incubation at 27 °C for 24 hours, the *V. parahaemolyticus* was observed to commence swarming motility in the direction of the *Bacillus* colony (Figure 2). However, at a distance of approximately 8 mm, the motile PSU5429 appeared to be impeded from closer approach to the YP001, further consistent with the production of an antibiotic compound.

MALDI-TOF IMS reveals metabolic exchanges between bacteria colonies and suggests the role of lipopeptides in swarming by *V. parahaemolyticus*. We hypothesized that bacterial secondary metabolites were responsible for the induction of swarming motility by the vibrio and the lysis zone around the *B. pumilus* colony. A MALDI-TOF IMS approach was implemented to visualize the presence of secondary metabolites secreted by the two bacteria colonies as they interacted on a thin agar plates. *Bacillus* are known to be versatile producers of bioactive peptides (14,15) and MALDI-TOF is well suited for the detection of such molecules. This approach has been previously applied to IMS investigations of secondary metabolism by *Bacillus* growing on agar surfaces (12).

Colonies of YP001 and PSU5429 were cultured in close proximities (1, 8 and 13 mm) on thin agar (10 mL) YP30 plates in triplicate for 24, 48, and 96 h. Swarming motility by PSU5429 and antibiosis were observed in these experiments after 24 hours and continued up to 96 hours. Plates from each time point were prepared for MALDI-TOF IMS by fixing the thin agar onto a steel MALDI plate with matrix (50:50 α -cyano-hydroxycinnamic acid and dihydroxybenzoic acid) and drying under desiccation.

MALDI-TOF-IMS conducted in the positive ion mode revealed a 2-D spatial map of chemical interactions between the two bacteria colonies (Figure 3). Prominent

features included the secretion of compounds with m/z 1058 $[M+Na]^+$ and 1072 $[M+Na]^+$ surrounding the *B. pumilus* colony and extending to the area where *V. parahaemolyticus* was swarming (Figure 3). The predicted size of these compounds suggested that they could be lipopeptides. For example, the *Bacillus* secondary metabolites surfactin C-15 and D (Figure S1 and S2) have molecular weights of 1035.68 and 1049.70, respectively, and therefore could account for the IMS observed features. IMS showed that these molecules diffused all the way to the vibrio colony, consistent with influencing of the observed swarming motility. Consistent with this theory, surfactants are reported to modify surfaces to promote swarming motility of bacteria such as observed for *B. subtilis*, *Pseudomonas* spp., *Proteus mirabilis*, *V. parahaemolyticus*, and *Serratia* spp. (16-20). Additional signals with m/z 1050, 1058, 1059, 1072, 1073, 1086 and 1087 were also observed to diffuse away from the *Bacillus* colony, and might indicate production of the related lipopeptides pumilacidin A-C, and E-G respectively (Figure 3 and S3) (21). Compounds corresponding to these ions were observed to diffuse across the agar surface in a manner similar to the surfactins, further suggesting the production of pumilicidins as possible contributors to the induction of swarming motility in the vibrios.

Other IMS features included ions localized within the zone of inhibition, suggesting a possible role in the observed antibiosis (Figure 3). Prominent ions included those with m/z 445, 467, 672, 823, and 899. Based on a review of antibiotic

compounds produced by *Bacillus* species, the ions at m/z 445 and 467 were consistent with the expected $[M+H]^+$ and $[M+Na]^+$, respectively, of the molecule amicoumacin B, an antibacterial compound produced by a hybrid PKS-NRPS biosynthetic pathway in *Bacillus* sp. (22).

Genome analysis of *B. pumilus* YP001 supports the production of lipopeptides and amicoumacin antibiotics. IMS suggested roles of different compounds for induction of swarming motility and antibiosis. Whole genome sequencing was performed to investigate the biosynthetic potential of *B. pumilus* YP001 and possibly identify secondary metabolites consistent with the IMS results. A draft genome from YP001 was assembled and comprised 14 contigs, averaging 3,640,419 bp in size, with an average GC content of 53.5%. The genome was annotated with the Rapid Annotation Subsystem Technology (RAST) server, which identified 3,797 open reading frames (23). The genome was next analyzed by antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) (24) to identify biosynthetic gene clusters (BSGs) involved in secondary metabolite production. Overall, 12% of the genome (i.e. 427,1038 nt) was broadly characterized as being involved in secondary metabolite biosynthesis. The genome of *B. pumilus* YP001 encoded eleven major secondary metabolism BGCs, including three non-ribosomal peptide synthase (NRPS) clusters, two terpene biosynthesis pathways, type 1 polyketide synthase genes and one hybrid

NRPS cluster. Alignments to previously published biosynthetic pathways were carried out in Geneious (Figure S4).

Analysis of a NRPS cluster indicated that YP001 was capable of surfactin production. This biosynthetic gene cluster showed 53.4% pairwise identity to the surfactin producing proteins srfAA-D from *Bacillus amyloliquefaciens* BGC0000443 (Geneious Global alignment, 70% similarity cost matrix). Individual gene alignments were carried out using NCBI-nBLAST, revealing strong similarities to the surfactin synthase subunits srfAA (100%), srfAB (82%), srfAC (66%), and srfAD (64%) (Table 1).

The hybrid NRPS pathway was found to possess high pairwise identity (64.1%) for the biosynthetic gene cluster (BGC) in *Bacillus subtilis* subsp. inaquosorum KCTC 13429 that produces amicoumacin antibiotics (Geneious Global alignment, 70% similarity cost matrix). Alignments of biosynthetic genes AmiA-M from the KCTC 13429 strain showed high homology between these two biosynthetic gene clusters (Table 2) (25). Amino acid binding site analysis was carried out using Geneious to predict monomer substrate binding in adenylation and ketosynthase domains (Table S1 and S2), further establishing a potential biosynthesis pathway for amicoumacin production.

Isolation and structure identification of lipopeptides, lipoamides and amicoumacins produced by *B. pumilus* YP001. The results from the MALDI-TOF-IMS experiments and genome analysis encouraged the isolation and structure characterization of bioactive compounds produced by *B. pumilus* YP001 in order to validate their identities and bioactivities. *B. pumilus* YP001 was cultured in 9 x 1L of artificial seawater supplemented with yeast (1 g/L) and peptone (5 g/L) in the presence of XAD-16 resin (Figure S5). XAD resin adsorbs secondary metabolites secreted into the media during bacteria growth and has been shown to increase the recovery of secondary metabolites (26). After seven days, the resin was recovered by filtration, extracted with organic solvents, and the resulting crude extract was partitioned between ethyl acetate and water. The organic soluble compounds were fractionated by C18 reversed-phase column chromatography and HPLC and guided by bioassays against *V. parahaemolyticus* PSU5429. The bioassays indicated that separate compounds were responsible for induction of swarming motility and antibiotic activity. Specifically, the PSU5429 swarming motility was induced by fractions that appeared enriched for lipopeptides by ¹H NMR spectroscopy. In contrast, the antibiotic activity, observed as zones of growth inhibition in disc diffusion assays, tracked with fractions containing a compound with a *m/z* of 447 [M+Na]⁺.

Four compounds (**1-4**) were isolated from the culture extracts that promoted swarming motility by PSU5429. Lipoamide B2 (**1**) was obtained as an optically active

colorless oil $\{[\alpha]_D^{21.8} -7.0 (c\ 0.1, \text{MeOH})\}$. High-resolution electrospray ionization mass spectrometry (HRESIMS) analysis established the molecular formula of **1** as $\text{C}_{17}\text{H}_{32}\text{N}_2\text{O}_4$ (m/z 351.2226 $[\text{M}+\text{Na}]^+$, calcd. 351.2254), requiring three degrees of unsaturation. The IR spectrum of **1** displayed characteristic absorptions indicative of amide bond (ν_{max} 1663 cm^{-1}) (Figure 4). Its ^{13}C NMR and DEPT spectra (Table S3), combined with HSQC data, disclosed 17 carbon resonances, including two methyls, ten sp^3 methylenes, two sp^3 methines, and three sp^2 quaternary carbons (three carbonyl carbons at δ_{C} 173.0, 172.3 and 171.9, two of which were assigned as amide carbonyl carbons based on the MS and IR spectrum of **1**. The above functional groups accounted for all three degrees of unsaturation, implying a linear structure in **1**.

The existence of an asparagine unit (unit A, Figure 5) was determined by the clear ^1H -COSY correlations of H-1' (δ_{H} 7.94, d, $J=7.5$ Hz)/H-2' (δ_{H} 4.45 m)/H₂-3' (δ_{H} 2.42, m; δ_{H} 2.56, m) and key HMBC correlations from these protons to C-6' (δ_{C} 173.0) and C-4' (δ_{C} 171.9), along with the identical HMBC correlations from H-1' to C-2' (δ_{C} 48.9) and from H₂-5' (δ_{H} 6.85, s; δ_{H} 7.37, s) to C-4'. Two methyl signals at δ_{H} 0.83 (d, $J=6.6$ Hz) and δ_{H} 0.80 (t, $J=7.5$ Hz) and the broad signal at δ_{H} 1.23 strongly indicated the presence of *anteiso*-methyl-branched fatty acid chain which was then verified by the detailed analysis of COSY correlations of H₃-12/H-10 (δ_{H} 1.32, m) and H₂-11 (δ_{H} 1.11, m; δ_{H} 1.32, m), H₃-13/H₂-9 (δ_{H} 1.07, m; δ_{H} 1.27, m) and H-10, as well as the HMBC correlations from H₃-12/H₃-13 to C-10 and H-10 to C-9. Furthermore,

the HMBC correlation of H₂-2 (δ_{H} 2.07, t, J = 7.2 Hz) and C-1 (δ_{C} 172.3), bearing in mind the MS of **1**, determined the presence of a 12-membered side chain with a C-1 acyl group and a methyl branch at the antepenultimate carbon (unit B, Figure 5). Finally, the connection between two units (units A and B) was established by the correlations from H-1'/H-2'/H₂-2 to C-1, allowing the planar structure construction of **1**. The NMR spectroscopic data of **2** indicated that its structure was closely related to **1** and represents a structural isomer of the previously published lipopeptide C (27).

Parent masses are insufficient to differentiate lipopeptides produced by *Bacillus* sp. therefore the identities of compounds **3** and **4** were determined by extensive investigations of spectroscopic data. Pumilacidin and surfactin differ by one amino acid residue at the fourth position, corresponding to leucine and valine, respectively. Compound **3** was isolated from *B. pumilus* YP001 broth monoculture as a white powder; $[\alpha]_{\text{D}}^{21.8}$ -2.22 (c 0.2, MeOH). Prominent features in the ¹H NMR spectrum were consistent with lipopeptide structures, and ¹H-¹H COSY correlations, identified spin systems corresponding to glutamic acid, leucine, and valine amino acids (Figures S17, S19). The presence of leucine points strongly towards isolated compounds **3** and **4** belonging to the family of surfactins known to be produced by *Bacillus pumilus*. HRESIMS data confirmed the molecular formula of compound **3** to be C₅₃H₉₃N₇O₁₃ (m/z 1034.6790 [M-H]⁻, calcd 1034.6753) corresponding with the known surfactin C-15 (Figure S1).

Compound **4** was isolated as a white powder; $[\alpha]_D^{21.8}$ -4.07 (c 0.2, MeOH) from broth monoculture and the molecular formula of compound **4** was established as $C_{54}H_{95}N_7O_{13}$ based on HRESIMS (m/z 1048.7000 $[M-H]^-$, calcd. 1048.6910) (Figure S2). Based on this data in combination with one- and two dimensional NMR data, compound **4** was identified as surfactin D. Comparison of the optical rotations of **3** and **4** against published values were consistent with these assignments of surfactin C-15 and D (28). Taken in combination with BGC analysis, this evidence was confirmed the structures of compounds **3** and **4**.

Amicoumacin B was obtained as a yellow oil. Based on HRESIMS (m/z 447.1648 $[M+Na]^+$) data we established a molecular formula as $C_{20}H_{32}N_2NaO_4$. The 1H NMR spectra exhibited three aromatic proton singlets for a tri-substituted aromatic unit at δ_H 6.81, 7.47, and 6.84 (H-5, H-6, and H-7), three oxymethines at δ_H 4.69, 3.95, and 3.72 (H-3, H-8' and H-9'), two nitrogen-bonded methines δ_H 4.20 and 3.31 (H-5' and H-10'), and two methyl resonances at δ_H 0.85 and 0.89 (H-1' and H-2') consistent with a isocoumarin ring structure (Table S4), and matching the published values for amicoumacin B (insert ref containing published NMR data for amicoumacin B). MALDI-TOF IMS was used to visualize the isolated amicoumacin B as it diffused through agar from a 10 mM spot on a thin agar MALDI plate (Figure S6). Amicoumacin B ionized readily by MALDI-TOF IMS, further confirming its identity in the co-colony IMS experiments.

***V. parahaemolyticus* swarms toward a source of highly purified surfactins or lipoamides.** A swarming motility assay was used to investigate the effects of specific compounds on initiation of swarming by *V. parahaemolyticus* PSU5429. Close proximity of PSU5429 to either *B. pumilus* YP001 or discs loaded with lipopeptide compounds **1-4** stimulated swarming behavior (Figure 6). Isolated surfactins and lipoamides stimulated swarming motility in PSU5439 with variable effects. Surfactins C-15 and D induced strong directional swarming when loaded onto discs at 10 and 100 µg/disc (Figure 6). In comparison, lipoamides **1** and **2** caused weak swarming response at 200 µg (Figure S6). No swarming was observed in response to discs loaded with amicoumacin B (data not shown).

The motility bioassays were repeated against three other marine *Vibrio* sp. to test if YP001 compounds could stimulate swarming motility in other species. Swarming motility was induced by surfactin C-15 and D for in the oyster pathogen *V. coralliilyticus* RE22, but not for *V. harveyi* BB120 or *V. anguillarum* NB10Sm. Neither *V. parahaemolyticus* PSU5429 nor *V. coralliilyticus* RE22 were observed to swarm in control experiments conducted in the absence of compounds from *B. pumilus* YP001 (Figure S8). All four *Vibrio* sp. were susceptible to the antibiotic effects of amicoumacin B (Figure 1b-d).

Discussion

The IMS results showed that the *Vibrio parahaemolyticus* colony was subjected to a barrage of secondary metabolites secreted by *B. pumilus* YP001, five of which were identified using a combination of genome analysis and compound isolation. Four molecules were found to promote swarming motility in *V. parahaemolyticus*, namely surfactins C-15 and D and lipoamides B2 and C. In addition, the isocoumarin antibiotic amicoumacin B demonstrated killing effects and likely contributes to an observed zone of growth inhibition surrounding the *Bacillus* colony.

MALDI-TOF IMS also revealed ions belonging to compounds that were not isolated from broth cultures of YP001, and these might further contribute to the microbial interactions. For example, ions observed by IMS suggest the production of pumilacidins A, B, C, E, and G by *B. pumilus* YP001 (Figure 3 and S3). Other molecules corresponding to ions concentrated in the antibiotic zone were not identified as known *Bacillus* compounds or isolated from liquid monocultures of YP001. Taken together, this shows that the vibrio cells experience a complex chemical environment when in proximity to *B. pumilus* that influences both their phenotypes and survival.

One interpretation of these combined effects on the vibrio colony is that the *Bacillus* colony promotes swarming motility toward a zone of growth inhibition, resulting in lysis of nearby vibrio cells and providing additional nutrients to support

colony growth. The closely related bacterium *Bacillus subtilis* has previously been shown to engage in bacteria cannibalism, whereby a subpopulation secretes peptide toxins to lyse sibling cells in order to harvest their nutrients (29). *Bacillus subtilis* produces the proteins Slf and bacteriocin-like toxins which lyse nearby cells in a form of bacterial cannibalism (30,31). The dual secretion of motility promoters and antibiotics might serve a similar purpose in interspecies interactions. It was recently shown that the bacterium *Paenibacillus dendritiformis* swarms toward a source of surfactins, consistent with these molecules serving as interspecies recruitment factors (12). The results here extend this possibility to more distantly related bacteria species, since both *V. parahaemolyticus* and *V. coralliilyticus* commenced swarming motility in response to the *Bacillus* lipopeptides. In contrast, the surfactins displayed no growth alterations against *V. harveyi* and *V. anguillarum* NB10 (Figure S8), although these bacteria do not possess the *laf* gene cluster most often conserved for the production of lateral swarming flagellar (32,33).

An alternative hypothesis is that the *V. parahaemolyticus* benefits by coopting surfactin production to enable its swarming motility. This could enable more rapid colonization of the surrounding environment. Motility can increase antibiotic resistance, up regulate virulence factor production and increase the speed of infection on solid or semi solid surfaces such as tissues (34,35). While some bacteria species are sensitive to the antimicrobial effects of lipopeptides, including clinically relevant

Gram-positive and Gram-negative strain (36), others are clearly resistant and might therefore capitalize on their surface modifying properties. In this instance, the *Bacillus* might be producing antibiotics to fend off the approaching swarm of vibrios.

Motility in *V. parahaemolyticus* differs from other swarming bacteria because it possesses two distinct flagellar systems. A single polar flagellum is utilized for swimming while peritrichous lateral flagella are utilized during swarming (37). In contrast to this study, lipopeptides were previously reported as inhibitors of swarming motility in *Vibrio alginolyticus* (38). Surfactins act to decrease surface tension, allowing directional movement of cells. Lipopeptides have also been shown to cause ion channel formation in lipid bilayer membranes, creating imbalances in trans-membrane ion fluxes (20,39,40). This proposed pore formation has been indicated as a mechanism of action of surfactins as antimicrobial and antifungal agents (41-44). The production of surfactin as a competitive interspecies chemical signaling strategy has been shown for *Salmonella*, *Escherichia*, *Bacillus* and *Staphylococcus* species (45,46), and now for *Vibrio*. Surfactins are also important mediators of intraspecies communication and phenotypes, including promoting motility on surfaces, attachment, and biofilm production (7-12). Hence, these molecules serve multiple purposes for mediating both intra- and interspecies interactions.

Genome sequencing was employed to probe secondary metabolite biosynthetic gene clusters and dereplicate IMS signals. Eleven biosynthetic gene clusters,

accounting for 12% of the genome, were identified for YP001, suggesting the impressive biosynthetic capabilities of this bacterium. This is comparable to *Bacillus subtilis* which dedicates ~10% of its genome to secondary metabolism (36). Previous bioinformatic investigation of *Bacillus* sp. found that strains contain 1 to 16 BGCs of which some are highly conserved (NRPS, bacteriocin, and terpene) across all strains whereas other BGCs (PKS, lanthipeptide and thiopeptide) are highly variable (47). Combining genome mining with highly sensitive IMS techniques provided evidence for the compounds involved in the observed microbial interactions, but chemical isolations further verified several of the molecules. The bioassay guided approach also helped identify lipoamides as inducers of swarming motility in *V. parahaemolyticus*.

Isocoumarin antibiotics such as the amicoumacins are commonly produced by various species of *Bacillus* (48) as well as some actinomycetes (49). The amicoumacins inhibit protein synthesis (50,51) and possess antibacterial properties against clinically relevant Gram-positive bacteria (25). In this study we demonstrate that the antibacterial activity of amicoumacin B against *V. parahaemolyticus* (Figure 7). Although purified amicoumacin B readily ionized on agar by MALDI-TOF IMS and produced a ZOI against *V. parahaemolyticus*, production of amicoumacin B from *B. pumilus* colonies was initially somewhat challenging to measure by IMS. Differences could indicate that antimicrobial activities are predominantly cell-bound or that the presence of the extracellular polysaccharide (EPS) matrix and other surface

structures hinder the ionization of these molecules (13). Other high intensity ions were present in the lysis zone surrounding the *B. pumilus* colony which were not identified, suggesting that other anti-vibrio compounds might also contribute to the observed antibiosis.

Bacillus and *Vibrio* species are ubiquitous in most marine environments and are likely competitors for resources such as nutrients and space. The results from this study suggest that these relationships are mediated by complex chemical interactions where multiple secondary metabolites may work in concert to achieve advantageous outcomes. Understanding the roles of specific molecules and their combinations could help inform new ways to influence bacteria behaviors in aquaculture systems and treat infections.

Materials and Methods

General experimental procedures: NMR experiments were conducted using an Agilent Inova NMR 500 MHz spectrometer in DMSO-*d*₆ (99.99%, Sigma-Aldrich, St. Louis, MO, USA) at 25 °C (referenced to residual DMSO at δ_{H} 2.54 and δ_{C} 39.5). High Resolution Electrospray Ionization mass spectra (HR-ESI-MS) and tandem mass spectra were acquired using a TripleTOF 4600 spectrometer (AB Sciex) operating in negative ion mode. Flash chromatography was completed using an Agilent 971-FP flash purification system (Agilent Technologies, Santa Clara, CA, USA) with Biotage

SNAP KP-C18-HS 120g cartridges (Biotage, Charlotte, NC, USA). HPLC experiments were performed on a Shimadzu Prominence-I LC-2030C equipped with a PDA detector model LC-2030/2040, pump LC-2030, and autosampler LC-2030. Optical rotations were acquired on a Jasco P-2000 polarimeter and UV was taken on Beckman Coulter DU 800. IR experiments were carried out on Thermo Nicolet 380 FT-IR. MALDI-TOF IMS was carried out on a Bruker Autoflex Speed instrument.

Isolation, sequencing and computational genome analysis of *Bacillus pumilus*

YP001. Strain YP001 was isolated from the egg mass of a female American lobster, *Homarus americanus*, collected in Narragansett Bay (12/16/15; Fort Weatherall, Jamestown, RI, USA). The eggs were gently brushed with a sterile cotton swab, which was then stored in a sterile whirl-pak™ sampling bag for transportation to the lab. The cotton tip was transferred into 1.5 mL of sterile artificial seawater (Instant Ocean™; Spectrum Brands) under aseptic conditions and then vortexed. A 100 µL aliquot was plated on artificial seawater agar plates supplemented with yeast (1 g/L) and peptone (5 g/L) (YP30). YP001 was picked from the plate as a single colony, streaked to purity (twice on YP30 plates) and cryogenically stored at -80 °C in glycerol stocks.

Total DNA was extracted from overnight cultures of *B. pumilus* YP001 grown in liquid YP30 using the Promega Wizard genomic DNA purification kit (Promega, USA). The DNA was resuspended in 2 mM Tris-HCL buffer (Bio Basic). DNA was

quantified using a NanoDrop 1000 spectrophotometer (ND-1000) and checked for quality on a 1% agarose gel stained with ethidium bromide. The resulting DNA was sequenced on an Illumina MiSeq sequencer at the Genomics and Sequencing Center at the University of Rhode Island. Total genomic DNA was sheared by sonication (Covaris S220), and paired end libraries were prepared using the SMARTer PrepX DNA Library Kit on a SMARTer Apollo system (Takara Bio, USA). Reads were trimmed using the CLC Genomic Workbench (version 9.5.3, Qiagen, Hilden, Germany) for quality, ambiguous base pairs, adaptors, duplicates and size with default parameters. A draft genome was assembled using the SPAdes assembler (Algorithmic Biology Lab, St. Petersburg Academic University of the Russian Academy of Sciences). Coverage was determined by mapping reads to a draft genome of *Bacillus pumilus* (CP016784.1). The genome was annotated using the Rapid Annotation Subsystem Technology (RAST) server and analyzed by antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) for secondary metabolite biosynthesis gene clusters. The draft genome assembly has been deposited in DDBJ/EMBL/GenBank under accession number PRJNA434728. Sequence alignments for amicoumacin biosynthetic cassettes were analyzed for identity through Geneious and NCBI-delta-blastp.

Isolation and Structure Elucidation of YP001 secondary metabolites. *B. pumilus*

YP001 was grown in 9 x 1 L of YP30 broth for 7 days at 25 °C and 150 rpm on a New Brunswick C10 rotary shaker table in the presence of Amberlite™ XAD-16 resin (10 g/L; Sigma Aldrich). Following cultivation, the resin was collected by filtering through two layers of cheesecloth, washed three times with DI water (4 L total) to remove salts, and then extracted with 500 mL of 1:1 CH₂Cl₂ and CH₃OH, followed by 1 L of CH₃OH, and finally 200 mL EtOAc. The combined solvents were removed by rotary evaporation and the resulting extract was partitioned between 100 mL H₂O and three washes of 500 mL ethyl acetate. The combined organic layers were concentrated *in vacuo* to yield 0.8 g of extract.

The organic fraction was separated by flash chromatography using a Combiflash Rf200 equipped with a 50 g C18 RediSepRf High Performance Gold column (Teledyne ISCO). A linear solvent gradient of 5-100% CH₃OH in H₂O (0.1% formic acid) was run over 20 min at a flow rate of 40 mL/min, then the mobile phase was held at 100% CH₃OH for 5 min. Individual peaks were collected by monitoring UV absorbance at 254 nm, yielding 6 fractions (QW1-144-1 to QW1-144-6). HPLC separations were completed with a Waters XBridge preparative C18 5 µm, 10 × 250 mm column at 4 mL/min a linear solvent gradient of 5-100% B (MeOH) run over 16 minutes against solvent A (water + 0.1% formic acid). Solvent B was held at 5% for 4 min prior to starting the gradient and 100% B was held for a subsequent 4 min

following the gradient. Pure lipoamide B2, and C and were isolated by preparative HPLC following the same solvent gradient yielding 11.8 mg and 11.9 mg respectively. Analytical HPLC experiments were carried out on a Waters XBridge Analytical C18 5 μm , 4.6×250 mm column at 1 mL/min using the same mobile phase methods. Purification of surfactin C15 and D followed a solvent gradient of 80-100% B (MeOH) run over 16 min against solvent A (water + 0.1% formic acid). Solvent B was held at 80% for 4 min prior to gradient beginning and 100% B was held for a subsequent 4 min. Amicoumacin B was isolated to purity through flash chromatography collection. In total, amicoumacin B, Surfactin C15, and surfactin D in yields of 11.6 mg, 2.6 mg, and 6.3 mg, respectively.

Antimicrobial Activity Assays. *B. pumilus* YP001 was tested for antimicrobial activity against the pathogens *Vibrio anguillarum* NB10Sm, *Vibrio harveyi* BB120, *Vibrio coralliilyticus* RE22 and *Vibrio parahaemolyticus* PSU5429 through a modified diffusion assay (52). *V. parahaemolyticus* PSU5429 was grown for 24 h in YP30, diluted 10^{-3} fold, and 100 μL of the diluted culture was spread on 15 mL YP30 agar. A 5 μL spot of *B. pumilus* YP001, grown overnight in 10 mL YP30, was added in triplicate and incubated at 25 °C. Zones of inhibition were imaged with a BioRad gel doc universal hood after 24-48 h at 25 °C.

Swarming Motility Assay. YP001 colonies, extracts and purified compounds were tested for induction of swarming motility by *V. parahaemolyticus* PSU5429. Sterile discs were loaded with 20 or 100 µg of test compounds and placed onto the surface of a YP30 agar plate. Alternatively, a 5 µL spot of YP001 grown for 24 h in 5 mL of YP001 broth was spotted onto a 15 mL YP30 agar plate. PSU5429 was grown for 24 h in YP30 broth, diluted to 1×10^5 CFU/mL in sterile media, and spotted at a distance of 3, 8 or 13 mm from the disc or colony. Plates were incubated for 24 h at 27 °C and then imaged with a BioRad gel doc universal hood. This assay is qualitative for the induction of swarming motility.

MALDI-TOF IMS Imaging. MALDI-TOF IMS experiments were carried out on 10 mL YP30 plates. Strains were plated in triplicate and incubated for up to 96 h. One assay from each time point (24, 48 and 96 h) was wet mounted onto a steel MALDI-TOF plate, coated with 50:50 α -cyano-hydroxycinnamic acid (CHCA) and dihydroxybenzoic acid (DHB) matrix and dried in a desiccator for 4 h at 37 °C and analyzed by MALDI-TOF IMS. Samples were analyzed by MALDI-TOF IMS at 65% laser power, 8.6X gain 500 µm raster in positive ion reflectron mode on a Bruker Autoflex Speed instrument. Data was normalized using total ion count (TIC) or root mean square (RMS) as indicated.

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Figures

Figure 1: Growth inhibition of *B. pumilus* YP001 against (a) *V. harveyi* BB120 (b) *V. parahaemolyticus* PSU5429, (c) *V. anguillarum* NB10Sm, and (d) *V. coralliitlyticus* RE22 after 24 h incubation on YP30 at 27 °C.

Figure 2: Swarming motility assay for *B. pumilus* YP001 plated in co-culture with *V. parahaemolyticus* PSU5429 at concentrations of (a) 1×10^5 CFU/mL, (b) 1×10^2 CFU/mL, (c) 1×10^1 CFU/mL, and (d) no dilution. Plate was imaged after 24 h incubation at 27 °C.

Figure 3: MALDI-IMS imaging shows the spatial distribution of compounds produced during the chemical interaction of *B. pumilus* YP001 and *V. parahaemolyticus* PSU5429.

Table 1: Genes encoding the surfactin biosynthetic gene cluster in *B. pumilus* YP001, their proposed functions and homology to the biosynthetic genes of *B. amyloliquefaciens* BGC0000443. Alignments were carried out in NCBI-nBLAST.

Protein	Size (bp)	Predicted Function	<i>B. pumilus</i> YP001	
			Identity (%)	E value
srfAA	10,755	NRPS	100%	0.0
srfAB	10,762	NRPS	82.0%	1e-07
srfAC	3,837	NRPS	65.7%	7e-69
srfAD	732	Thioesterase	64.3%	9e-13
ycxD	1,311	Aminotransferase	100%	0.14

Table 2: Genes encoding the amicoumacin biosynthetic gene cluster in *B. pumilus*

YP001, their proposed functions and homology to the biosynthetic genes of *B. subtilis* subsp. *inaquosorum* KCTC 13429. Alignments were carried out in NCBI-nBLAST.

Protein	Size (bp)	Predicted Function	<i>B. subtilis</i> YP001	
			Identity (%)	E value
AmiA	4,497	NRPS	65.1%	6e-84
AmiB	1,506	Peptidase	66.3%	2e-21
AmiD	702	Thioesterase	70.6%	5e-54
AmiE	345	Dehydrogenase	74.4%	9e-62
AmiF	213	Acyl carrier protein	78.9%	2e-23
Ami-1	9,096	NRPS-PKS	65.6%	0.0
Ami-2	2,670	NRPS	64.1%	1e-49
AmiJ	4,527	PKS	69.8%	0.0
AmiK	7,554	NRPS-PKS	71.9%	0.0
AmiL-M	6,429	NRPS-PKS	66.7%	0.0

Figure 4: Chemical structure of compounds **1** and **2**.

Figure 5: ^1H – ^1H COSY and key HMBC correlations for compound **1**.

Figure 6: Swarming bioactivity of compound **3** at 10 µg (a), 100 µg (b) and compound **4** at 10 µg (c), and 100 µg (d) after 24 h incubation at 27 °C on YP30 agar.

Figure 7: Effects of purified surfactin (20 $\mu\text{g}/\text{disk}$) and ampicoumacin (20 $\mu\text{g}/\text{disk}$) (a), surfactin (20 $\mu\text{g}/\text{disk}$) only (b), ampicoumacin (100 $\mu\text{g}/\text{disk}$) only (c) and *B. pumilus* YP001 colony (5 μL at 1×10^5 CFU/mL from a broth culture) (d) on *V. parahaemolyticus* PSU5429 after 24 h incubation at 27 $^{\circ}\text{C}$.

Supplementary Figures

Figure S1: High resolution TOF-MS for **3** m/z 1034.6790 $[M-H]^-$ in negative ion mode. The declustering potential was set to -185 and collision energy to -33.

Figure S2: High resolution TOF-MS for **4** m/z 1048.7 $[M-H]^-$ in negative ion mode.

The declustering potential was set to -200 and collision energy to -50.

Figure S3: MADLI-TOF IMS total ion counts (TIC) taken after 48 h incubation indicating the production of pumilacidin B (m/z 1,059 $[M+Na]^+$), F/G (m/z 1,073 $[M+Na]^+$), and E (m/z 1087 $[M+Na]^+$).

Figure S4: Biosynthesis of amicoumacin by *B. pumilus* YP001 domain predictions carried out in Geneious.

Table S1: Amino acid sequence alignment for amicoumacin production from *B.*

pumilus YP001.

Protein	Size (aa)	Proposed function	Identity (%)	Accession No.	Proposed Protein Alignment
2163	1413	Aminotransferase, class III [<i>B. pumilus</i>]	99	DW21908.1	OrfI
2144	4416	Non-ribosomal peptide synthetase [<i>B. pumilus</i>]	100	WP_080984813.1	AmiA
2143	1542	β -lactamase containing hypothetical protein [<i>B. pumilus</i>]	100	WP_050945729.1	AmiB
2142	981	Peptidase containing hypothetical protein [<i>B. pumilus</i>]	100	WP_050945730.1	AmiC
2141	711	Thioesterase [<i>B. pumilus</i>]	100	WP_050945731.1	AmiD
2140	858	3-hydroxybutyryl-CoA dehydrogenase [<i>B. pumilus</i>]	100	WP_003214220.1	AmiE
2139	1056	Hypothetical protein [<i>B. pumilus</i>]	100	WP_050945732.1	AmiF
2138	270	Acyl carrier protein [<i>B. pumilus</i>]	100	WP_050945733.1	AmiG
2137	1143	Acyl-CoA dehydrogenase [<i>B. pumilus</i>]	100	WP_050945734.1	AmiH
2136	9090	Hybrid NRPS/T1PKS [<i>B. pumilus</i>]	100	WP_050945735.1	AmiI
2135	2670	Non-ribosomal peptide synthetase [<i>B. pumilus</i>]	100	WP_050945736.1	AmiJ
2134	4506	Polyketide synthase <i>B. pumilus</i>]	100	WP_050945737.1	AmiK

2133	7233	Polyketide synthase <i>B. pumilus</i>]	100	MY19195.1	AmiL
2132	6411	Polyketide synthase <i>B. pumilus</i>]	100	WP_050945739.1	AmiM
2130	1407	Alkaline Phosphatase [<i>B. pumilus</i>]	100	WP_080984808.1	AmiN

Table S2: Amino acid sequence alignment for surfactin production from *B. pumilus*

YP001.

Protein	Size (aa)	Proposed function	Identity/ Similarity (%)	Accession No.
3473	1239	Hypothetical Protein [<i>B. pumilus</i>]	100	WP_050943243.1
3472	1299	Arsenical efflux pump membrane protein ArsB [<i>B. pumilus</i>]	100	WP_050943242.1
3471	678	Amino acid transporter permease [<i>B. pumilus</i>]	100	WP_003214368.1
3470	873	Amino acid ABC transporter substrate binding protein [<i>B. pumilus</i>]	100	WP_050943239.1
3469	333	DUF2680 domain-containing protein [<i>B. pumilus</i>]	100	WP_050943461.1
3468	765	MBL fold metallo-hydrolase [<i>B. pumilus</i>]	100	WP_050943237.1
3467	417	DNA-entry nuclease [<i>B. pumilus</i>]	100	WP_003214307.1
3466	807	AAC(3) family N-acetyltransferase [<i>B. pumilus</i>]	100	WP_050943235.1
3465	387	DNA-entry nuclease [<i>B. pumilus</i>]	100	WP_050943233.1
3464	1698	Methy;-accepting chemotaxis protein [<i>B. pumilus</i>]	100	WP_050943231.1
3463	429	DNA-entry nuclease [<i>B. pumilus</i>]	100	WP_003214307.1
3462	2070	Hydantoin utilization protein A [<i>B. pumilus</i>]	100	WP_050943229.1
3461	1986	Hydantoin utilization protein B [<i>B. pumilus</i>]	100	WP_050943227.1
3460	1086	ABC transporter ATP-binding protein [<i>B. pumilus</i>]	100	WP_050943226.1
3459	1092	Polyamine ABC transporter ATP-binding protein [<i>B. pumilus</i>]	100	WP_050943225.1

3458	870	ABC transporter protein permease [<i>B. pumilus</i>]	100	WP_003214329.1
3457	804	ABC transporter protein permease [<i>B. pumilus</i>]	100	WP_003214190.1
3456	10719	Non-ribosomal peptide synthetase [<i>B. pumilus</i>]	92	WP_003214217.1
3455	3834	Non-ribosomal peptide synthetase [<i>B. pumilus</i>]	100	WP_050945478.1
3454	10136	Non-ribosomal peptide synthetase [<i>B. pumilus</i>]	100	WP_050945479.1
3453	8196	Non-ribosomal peptide synthetase [<i>B. pumilus</i>]	100	WP_050945480.1
3452	741	Thioesterase [<i>B. pumilus</i>]	100	WP_050945481.1
3451	912	EamA family transporter [<i>B. pumilus</i>]	100	WP_050945482.1
3450	1338	PLP-dependant aminotransferase family protein [<i>B. pumilus</i>]	100	WP_050945483.1

Figure S5: Chemical isolation scheme from *B. pumilus* YP001.

Table S3: ^1H and ^{13}C NMR data (δ in ppm, J in Hz) for compound **1** in DMSO- d_6 .

Position	Type	δ_{C}	δ_{H} (J in Hz)
1	qC	172.3	—
2	CH ₂	35.1	2.07 t (7.2)
3	CH ₂	25.2	1.45 m
4	CH ₂	28.5	1.23 m
5	CH ₂	28.9	1.23 m
6	CH ₂	28.9	1.23 m
7	CH ₂	28.9	1.22 m
8	CH ₂	26.5	1.27 m
9	CH ₂	36.0	1.07 m, 1.27 m
10	CH	33.8	1.32 m
11	CH ₂	29.4	1.11 m, 1.32 m
12	CH ₃	11.7	0.80 t (7.5)
13	CH ₃	19.1	0.83 d (6.6)
1'	NH	—	7.94 d (7.5)
2'	CH	48.9	4.45 m
3'	CH ₂	37.0	2.42 m, 2.56 m
4'	qC	171.9	—
5'	NH ₂	—	6.85 s, 7.37 s
6'	qC	173.0	—

Recorded at 300 MHz for ^1H NMR in DMSO- d_6 , chemical shifts (ppm) referred to DMSO (δ_{H} 2.50). Recorded at 125 MHz for ^{13}C NMR in DMSO- d_6 , chemical shifts (ppm) referred to DMSO (δ_{C} 39.5). Assignments were deduced by analysis of 1D and 2D NMR spectra.

Figure S6: Swarming assay for (a) lipoamide C (100 μ g), and (b) lipoamide B2 (100 μ g) against *V. parahaemolyticus* PSU5429 after 24 h incubation at 27 °C.

Figure S7: MADLI-TOF IMS ionization of amicoumacin B m/z 425 $[M+H]^+$ and 447 $[M+Na]^+$ plated at 10 mM in methanol onto a YP30 plate.

Figure S8: Swarming assay for 100 µg of surfactin D (i) and C-15 (ii) against (a) *Vibrio coralliilyticus* RE22, (b) *Vibrio harveyi* BB120, (c) *Vibrio anguillarum* NB10Sm after 48 h incubation at 27 °C.

Figure S9: ¹H-NMR spectrum (500 MHz, DMSO-*d*₆) of compound **1** from *B. pumilus* YP001.

Figure S10: ^{13}C -NMR spectrum (500 MHz, $\text{DMSO}-d_6$) of compound **1** from *B. pumilus* YP001.

Figure S11: ^1H - ^1H COSY NMR spectrum (500 MHz, $\text{DMSO}-d_6$) of compound **1** from *B. pumilus* YP001.

Figure S12: HSQC NMR spectrum (500 MHz, DMSO-*d*₆) of compound **1** from *B. pumilus* YP001.

Figure S13: HMBC NMR spectrum (500 MHz, DMSO-*d*₆) of compound **1** from *B. pumilus* YP001.

Figure S14: IR spectrum of compound **1**.

Figure S15: HRESIMS of compound **1** measured in positive ion mode (m/z 351.2226 $[M+H]^+$ and 373.2048 $[M+Na]^+$).

Figure S16: Lipoamide C (**2**) IR data.

Figure S17: HRESIMS of lipoamide C (**2**) recorded in positive ion mode (m/z 345.1713 $[M+H]^+$).

Figure S18: ^1H -NMR spectrum (500 MHz, $\text{DMSO}-d_6$) of surfactin D (**4**) from *B. pumilus* YP001.

Figure S19: ^{13}C -NMR spectrum (500 MHz, $\text{DMSO-}d_6$) of surfactin D (**4**) from *B. pumilus* YP001.

Figure S20: gCOSY-NMR spectrum (500 MHz, DMSO- d_6) of surfactin D (**4**) from *B. pumilus* YP001.

Figure S21: TOCSY-NMR spectrum (500 MHz, DMSO- d_6) of surfactin D (**4**) from *B. pumilus* YP001.

Figure S22: HSQC-NMR spectrum (500 MHz, DMSO- d_6) of surfactin D (**4**) from *B. pumilus* YP001.

Figure S23: ^1H -NMR spectrum (500 MHz, $\text{DMSO}-d_6$) of surfactin C15 (**3**) from *B. pumilus* YP001.

Amicoumacin B

Table S4. NMR Data for Amicoumacin B (500 MHz) in DMSO-*d*₆.

Position	Type	δ C	δ H
1	CO	169.47	-
2	O	-	-
3	CH	81.45	4.69
4	CH ₂	29.47	2.85, 3.05
5	CH	118.94	6.81
6	CH	136.71	7.47
7	CH	115.67	6.84
8	C	161.32	-
9	C	108.79	-
10	C	141.15	-
1'	CH ₃	21.99	0.85
2'	CH ₃	23.79	0.89
3'	CH	24.44	1.65
4'	CH ₂	39.49	1.33, 1.67
5'	CH	48.54	4.20
6'	NH	-	7.90
7'	CO	173.08	-
8'	CH	72.04	3.95
9'	CH	72.24	3.72
10'	CH	50.58	3.31
11'	CH ₂	33.85	2.18, 2.33
12'	CO	174.18	-

AI-77-F

Table S5. NMR Data for AI-77-F (500 MHz) in DMSO-*d*₆.

Position	Type	δ H
1	CO	-
2	O	-
3	CH	4.69
4	CH ₂	2.97
5	CH	6.84
6	CH	7.49
7	CH	6.87
8	C	-
9	C	-
10	C	-
1'	CH ₃	0.85
2'	CH ₃	0.91
3'	CH	1.65
4'	CH ₂	1.23, 1.68
5'	CH	4.20
6'	NH	7.70
7'	CO	-
8'	CH	
9'	CH	
10'	CH	
11'	CH	
12'	CO	-

Figure S24: ^1H -NMR spectrum (500 MHz, $\text{DMSO}-d_6$) of AI-77-F from *B. pumilus* YP001.

CHAPTER 4

MANUSCRIPT 3: This manuscript was published in 2018 in the American Society for Microbiology Genome Announcements 6 (17) e00313-18. The formatting follows the guidelines set forth by the journal.

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Draft Genome Sequence of the Putative Marine Pathogen *Aquimarina* sp. I32.4

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Running Head: Draft Genome of *Aquimarina* sp. I32.4

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Author contributions

H.J.R. designed and performed the experiments. J.L. aided in genomic DNA isolation methods. H.J.R. and E.S. carried out the sequence assembly. A.Y.C provided the strain. H.J.R. wrote the manuscript. M.G.C., D.R.N. and D.C.R. supervised the study and managed the projects.

Aquimarina sp. I32.4 (formerly *Aquimarina* sp. ‘*homaria*’) is a putative pathogen involved in epizootic shell disease in the American lobster *Homarus americanus*. We report here the draft genome sequence for *Aquimarina* sp. I32.4 and describe virulence factors that may provide insight into its mechanism of pathogenicity.

Epizootic shell disease (ESD) is a prevalent and uncontrolled disease affecting the carapace of the American lobster *Homarus americanus*. ESD is characterized by formation of lesions on the carapace that exhibit bacterial counts 2-4 orders of magnitude higher than the surrounding area (1). The cause of ESD is still debated within the scientific literature, but recent studies have suggested the presence of *Aquimarina* sp. ‘*homaria*’ results in the exacerbation of lesions (2,3,4). *Aquimarina* sp. I32.4 is a Gram negative, chitin degrading bacterium (5). To better understand the mechanism of pathogenicity we announce the draft genome sequence of *Aquimarina* sp. I32.4 and describe some potential virulence factors.

Aquimarina sp. I32.4 was grown in artificial seawater (Instant Ocean) supplemented with yeast extract (1 g/L) and peptone (5 g/L) at 25 °C on an elliptical shaker (New Brunswick) for 48 hours. Genomic DNA was isolated using Promega Wizard DNA purification kit (Promega, USA) and DNA was resuspended in 2 mM Tris-HCL buffer (Bio Basic). DNA was quantified using a NanoDrop 1000 spectrophotometer (ND-1000) and checked for quality on a 1% agarose gel stained with ethidium bromide. DNA was sequenced on an Illumina MiSeq sequencer at the Genomics and Sequencing Center at the University of Rhode Island. Reads were

trimmed using the CLC Genomic Workbench (version 8.5.1) for quality, ambiguous base pairs, adaptors, duplicates and size resulting in 1,329,308 paired-end reads. The draft genome was assembled using the *de novo* assembly algorithm of SPAdes assembler (version 3.1.1). Contigs with coverage of >22 reads were processed using the CLC Microbial Genome Finishing module with *Aquimarina macrocephali* JAMB N27 (GCA_000520995.1) used as a reference genome. The completed draft genome is composed of 244 contigs, averaging 20,446 bp in size (total genome = 4,988,869 bp), with an average G+C content of 32.4%. The draft genome was annotated using the Rapid Annotation Subsystem Technology (RAST) server and resulted in 4,469 open reading frames (6). The closest neighbor identified by SEED viewer 2.0 (7) was *Gramella forsetii* KT0803 (score = 541).

The annotated draft genome of *Aquimarina* sp. I32.4 encodes for type III and IV secretion systems. It has previously been reported that *Aquimarina* sp. can degrade the carapace of *H. americanus* (1). Accordingly, sixteen putative chitinase genes were found, along with three hemolysins and three metalloproteases. Three iron acquisition systems were annotated: TonB, including the full complement of proteins responsible for the formation of the TonB-ExbB-ExbD complex, hemin and ferric siderophores were identified and could contribute to virulence associated with this strain. The RAST annotated draft genome was submitted to antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) for secondary metabolite biosynthesis gene cluster analysis (8). Identification of ten clusters for secondary metabolism including, T1PKS-NRPS biosynthesis, NRPS and Trans-ATPKS were categorized by

antiSMASH. Trans-ATPKS biosynthesis produces structurally diverse molecules in other bacteria, including the cytotoxic and antitumor compounds pederin and onnamide (9). Other species in this genus have been shown to have algicidal activity against toxic cyanobacterium strains (10).

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession number PGUB000000000. The version described in this paper is version PGUB000000000.

ACKNOWLEDGEMENTS

This work was supported by funding from the Rhode Island Sea Grant (RISG18-R/F-1618-31-1). This research is based upon the work conducted using the Rhode Island Genomics and Sequencing Center, which is supported in part by the National Science Foundation under EPSCoR grants no. 0554548 and EPS-1004057.

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synthesized by bacterium *Aquimarina* sp. Enzyme Microb Technol 49: 372 – 379.

Draft Genome Sequence of the Putative Marine Pathogen *Thalassobius* sp. I31.1

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Running Head: Draft Genome of *Thalassobius* sp. I31.1

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H.J.R. designed and performed the experiments. J.L. aided in genomic DNA isolation methods. H.J.R. and E.S. carried out the sequence assembly. A.Y.C. provided the strain. H.J.R. wrote the manuscript. M.G.C., D.R.N. and D.C.R. supervised the study and managed the projects.

ABSTRACT

***Thalassobius* sp. 31.1 is a putative pathogen involved in epizootic shell disease in the American lobster *Homarus americanus*. We report here the draft genome sequence for *Thalassobius* sp. 31.1 and provide insight into its metabolism and links to environmental pollutant degradation.**

Thalassobius sp. 31.1 is a Gram-stain-negative, aerobic, motile, rod-shaped bacterial strain. This genus is of the class *Alphaproteobacteria*, family *Rhodobacteraceae*, and order *Rhodobacterales*. *Thalassobius* sp. 31.1 was isolated from a lesion on the carapace of the American lobster *Homarus americanus* (1). Lesions are the characteristic symptom of epizootic shell disease (ESD) and *Thalassobius* has been indicated as a putative pathogen in lesion formation. Strains of the genus *Thalassobius* have previously been studied for the inability to utilize multiple carbon sources (2). However, *Thalassobius* has been shown to utilize the speculative environmental carcinogen phthalate, a chemical widely used in the manufacture of plastics, lubricants and textiles (3). These strains contain genes required for poly-beta-hydroxybutyrate (PHB) synthesis and genes relating the degradation of phenylacetates, an abundant class of environmental pollutants (4).

Thalassobius sp. I31.1 was grown in artificial seawater (instant ocean) supplemented with yeast (1 g/L) and peptone (5 g/L) at 25 °C on an elliptical shaker (New Brunswick) for 48 hours. Genomic DNA was isolated using Promega Wizard DNA purification kit (Promega, USA) and DNA was resuspended in 2 mM Tris-HCL

buffer (Bio Basic). DNA was quantified using a NanoDrop 1000 spectrophotometer (ND-1000) and checked for quality on a 1% agarose gel stained with ethidium bromide. The DNA was sequenced on an Illumina MiSeq sequencer at the Genomics and Sequencing Center at University of Rhode Island. Reads were trimmed using the CLC Genomic Workbench (version 8.5.1) for quality, ambiguous base pairs, adaptors, duplicates and size resulting in 2,277,834 paired-end reads. The draft genome was assembled using the *de novo* assembly algorithm of SPAdes assembler (version 3.1.1). Contigs with coverage of >44 reads were processed using the CLC Microbial Genome Finishing module. The completed draft genome is composed of 62 contigs, averaging 57,385 bp in size, with an average G+C content of 55.1%. The draft genome was annotated using the Rapid Annotation Subsystem Technology (RAST) server and resulted in 3,749 open reading frames (5). The closest neighbor identified by SEED viewer 2.0 (6) was *Silicibacter pomeroyi* DSS-3 (score = 508).

The annotated draft genome of *Thalassobius* sp. 31.1 encodes for type I and II/IV secretion systems. RAST annotation identified a suite of *paa* genes involved in phenylacetate degradation, two alkane monooxygenase genes, *phbF*, a transcriptional regulator involved in PHB synthesis (7) and one toluenesulfonate dehydrogenase. The annotated draft genome was submitted to antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) for secondary metabolite biosynthesis gene cluster analysis (8). Identification of six clusters for secondary metabolism including, homoserine lactone, bacteriocin and lantipeptide-bacteriocin were identified.

This Whole-Genome Shotgun project has been deposited in DDBJ/EMBL/GenBank under accession number PWAA000000000. The version described in this paper is the first version, PWAA01000000.

ACKNOWLEDGMENTS

This work was supported by funding from the Rhode Island Sea Grant. This research is based upon the work conducted using the Rhode Island Genomics and Sequencing Center, which is supported in part by the National Science Foundation under EPSCoR grants no. 0554548 and EPS-1004057.

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Draft Genome Sequence of *Loktanella maritima* YPC211, a commensal bacterium of the American lobster (*Homarus americanus*).

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Author Contributions

H.J.R. isolated the strain, designed, and performed the experiments. J.L. aided in genomic DNA isolation methods. H.J.R. and E.S. carried out the sequence assembly. H.J.R. wrote the manuscript. M.G.C., D.R.N. and D.C.R. supervised the study and managed the projects.

Loktanella maritima YPC211 was isolated from the American lobster *Homarus americanus*. We report here the draft genome sequence for *L. maritima* YPC211 and identify genes of potential importance to its role within the microbial community.

Loktanella maritima is a Gram negative aerobic bacterium that was originally isolated from shallow marine sediments in the Sea of Japan (1). *Loktanella* spp. appear to be ubiquitously distributed in the oceans and have been isolated from Antarctic to sub-tropical environments (6). The genus belongs to the class *Alphaproteobacteria*, family *Rhodobacterales*, and order *Rhodobacteraceae*. Trappen *et al.* (2) originally proposed the genus to accommodate three species but it has since been expanded and modified to contain 15 species (3, 4, 5). The strain Ha06YPC211 produces a beige/yellow pigment and was isolated from an egg mass of an American Lobster (*Homarus americanus*) from Jamestown, RI.

L. maritima YPC211 was grown in artificial seawater (Instant Ocean) supplemented with yeast extract (1 g/L) and peptone (5 g/L) at 25 °C on an elliptical shaker (New Brunswick) for 24 hours. Genomic DNA was isolated using Promega Wizard DNA purification kit (Promega, USA) and DNA was resuspended in 2 mM Tris-HCL buffer (Bio Basic). DNA was quantified using a NanoDrop 1000 spectrophotometer (ND-1000) and checked for quality on a 1% agarose gel stained with ethidium bromide. The DNA was sequenced on an Illumina MiSeq sequencer at the Genomics and Sequencing Center at University of Rhode Island. Reads were

trimmed using CLC Genomic Workbench (version 8.5.1) for quality, ambiguous base pairs, adaptors, duplicates and size resulting in 4,491,164 paired-end reads. The draft genome was assembled using the *de novo* assembly algorithm of CLC Genomic Workbench and SPAdes assembler (version 3.1.1). Contigs with coverage of >76 reads were processed using the CLC Microbial Genome Finishing module. The completed draft genome is composed of 13 contigs averaging 264,067 bp in size and 3 plasmids of 23,839, 6,277 and 215,371 bp in size (total genome = 3,678,360 bp), with an average G+C content of 53.5%. The draft genome was annotated using the Rapid Annotation Subsystem Technology (RAST) server and resulted in 3,681 open reading frames (7).

The annotated draft genome of *L. maritima* YPC211 encodes for type I, II, III, and IV secretion systems. It has previously been reported that *Loktanella* spp. can survive temperature extremes and have been isolated from Antarctic mats (2). Accordingly, five putative cold-shock proteins (including: CspA, CspB and CspC) were annotated. Additionally, the genome encodes for four hemolysins, two metalloproteases and one extracellular protease. Two iron acquisition systems, TonB and Hemin (HmuS), were annotated as well as iron-specific hemin and ABC transporters. Ferric siderophore related genes were also identified. The RAST annotated draft genome was submitted to antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) for secondary metabolite biosynthesis gene cluster analysis (8), leading to the identification of four clusters, including a non-ribosomal peptide synthetase, type I polyketide synthase, bacteriocin and homoserine lactone

biosynthetic gene clusters. *L. maritima* YPC211 inhibits the growth of the shrimp pathogen *Vibrio parahaemolyticus* when co-cultivated on an agar surface, as determined by a zone of inhibition assay (9). Further investigation of these biosynthetic gene clusters may aid in characterization of compounds responsible for this antibiosis.

This Whole Genome Shotgun project has been deposited in DDBJ/ENA/GenBank under accession number PKFN00000000. The version described in this paper is the first version, PKFN01000000.

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CONCLUDING REMARKS

Antibiotics have been our frontline defense against bacterial infections for decades. However, the increased emergence of antibiotic resistant infections has led to a world health crisis. Continued use of broad spectrum antibiotics has caused an increase in untreatable and fatal bacterial infections. In this dissertation I have explored the potential use of chemical signals to help mediate pathogenic bacterial infections. Understanding these chemical signaling strategies may help facilitate new treatment strategies.

Nature has had billions of years of natural selection to perfect chemical communication. Bacteria are particularly interesting as their rate of genetic change is so high. We are continually reminded that this has driven systems that have no redundancy; nothing is made by accident or without purpose. This dissertation shows that even very small molecules such as peptidoglycan fragments, previously thought to be innocuous, modulate extremely important communications in bacteria. Understanding these natural communication pathways could be extremely important in treating complex diseases. Bacteria depend on chemical cues for cell-cell communications to facilitate group decision making and thus are key to survival. All bacteria are capable of manipulating their chemical environment to survive.

This dissertation works across human health and marine bacterial biodiversity to harness the potential of genetically unique bacteria to produce novel chemical scaffolds. Most of the world's microorganisms have yet to be cultured; many of these are likely to be in a quiescent state and could harbor therapeutically useful natural

products. Genomic data is extremely powerful in predicating biosynthetic pathways from unculturable bacteria, allowing targeting of novel natural products.

Swarming motility and quiescence are phenomenon observed in disease causing bacteria. Targeting these strategies for disease persistence as an alternative or in combination with antibiotic treatment could significantly decrease the rate of antibiotic consumption.

We are currently in the “post antibiotic era” and the one size fits all approach to medicine is being overtaken by personalized medical treatment. Approaching bacterial infections through phenotype modulation and control of signaling pathways could allow prevention of disease progression without the threat of developing drug resistance. This body of work helps to increase our understanding of how specific chemical signaling pathways work and provides knowledge of potential avenues for alternative treatment of bacterial infections.

APPENDICIES

Appendix A: Manuscript 1

Peptidoglycan sensing prevents quiescence and promotes quorum-independent growth of uropathogenic *Escherichia coli*

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Additional Results

Additional figures include isolation schemes for peptidoglycan from live cell culture and support structure assignments for mucopeptides based on MS/MS fragmentation. Further figures show peptidoglycan recycling within the cell and indicate *E. coli* knockout mutants that were made to strengthen this manuscript.

Figure 1: Peptidoglycan isolation scheme indicating bioassay guided fractionation of crude peptidoglycan digestion product from *E. coli* CFT073.

E. coli CFT073 plated on LB and incubated for 24 h at 37 °C.

Single colony picked and inoculated into 10 mL of M9 broth and incubated at 37 °C shaking 150 rpm for 24 h.

5 mL of broth culture inoculated into 1 L of M9 broth media and incubated at 37 °C at 150 rpm for 24 h.

All cells collected into 4 x 50 mL falcon tubes by multiple rounds of centrifugation at 5,000 g for 10 min.

Each pellet was resuspended in 3 mL of M9 media.

6 mL of 6 % SDS was added to each tube while being boiled and stirred. This was allowed to continue for 3 h.

Centrifuged repeatedly at 262,000 x g until all of the SDS was removed by washing with LCMS water.

Pellet resuspended in 10 mM Tris-HCL with 0.06% w/v NaCL.

100 µg of Pronase E added and incubated for 2 h at 60 °C.

200 µL of 6 % SDS was added and pellets were boiled at 100 °C for 30 min.

Centrifuged at 262,00 x g for 30 min to remove SDS with LCMS water.

Pellet re-suspended in 200 µL of PBS.

Digested with 200 U of mutanolysin at 37 °C overnight.

Centrifuged at 16,000 x g for 10 min.

‘Crude’ peptidoglycan.

Figure 2: Isolation methods for peptidoglycan from *E. coli* CTF073 cells to produce ‘crude’ peptidoglycan preparations used for testing in the quiescence bioassay.

Figure 3: Synthesis, recycling and catabolism pathways for peptidoglycan in the cell.

Indicating transporters on the cellular membrane involved in uptake and regulation into the cell. All mutant knockout (MppA, Opp and AmpG) pathways target different sources of peptidoglycan transport.

Figure 4: High resolution tandem mass spectrometry for disaccharide tetrapeptide (NAG-NAM-Ala-Glu-DAP-Ala) as a potential constituent of crude peptidoglycan preparations. Total ion counts (TIC) for crude peptidoglycan (a), extracted ion counts (XIC) for disaccharide tetrapeptide (b), and MS/MS fragmentation of disaccharide tetrapeptide mass to confirm its structure (c).

Figure 5: High resolution tandem mass spectrometry for disaccharide tripeptide (NAG-NAM-Ala-Glu-DAP) as a potential constituent of crude peptidoglycan preparations. Total ion counts (TIC) for crude peptidoglycan (a), extracted ion counts (XIC) for disaccharide tripeptide (b), and MS/MS fragmentation of disaccharide tripeptide mass to confirm its structure (c).

Figure 6: High resolution tandem mass spectrometry for disaccharide dipeptide (NAG-NAM-Ala-Glu) as a potential constituent of crude peptidoglycan preparations. Total ion counts (TIC) for crude peptidoglycan (a), extracted ion counts (XIC) for disaccharide dipeptide (b), and MS/MS fragmentation of disaccharide dipeptide mass to confirm its structure (c).

Figure 7: High resolution tandem mass spectrometry for pentapeptide (Ala-Glu-DAP-Ala-Ala) as a potential constituent of crude peptidoglycan preparations. Total ion counts (TIC) for crude peptidoglycan (a), extracted ion counts (XIC) for pentapeptide (b). Ion abundance was too low to accurately assess the structure by MS/MS.

Figure 8: High resolution tandem mass spectrometry for tetrapeptide (Ala-Glu-DAP-Ala) as a potential constituent of crude peptidoglycan preparations. Total ion counts (TIC) for crude peptidoglycan (a), extracted ion counts (XIC) for tetrapeptide (b), and MS/MS fragmentation of tetrapeptide mass to confirm its structure (c).

Figure 9: High resolution tandem mass spectrometry for tripeptide (Ala-Glu-DAP) as a potential constituent of crude peptidoglycan preparations. Total ion counts (TIC) for crude peptidoglycan (a), extracted ion counts (XIC) for tripeptide (b). Ion abundance was too low to accurately assess the structure by MS/MS.

APPENDICES

Appendix B: Manuscript 2

Isolation, chemical screening and *in vivo* trials of probiotics isolated from the American lobster *Homarus americanus* in Narragansett Bay to treat epizootic shell disease.

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Author Contributions

Bacterial strains were isolated by H.J.R. Antimicrobial testing, chemical isolation, and genome sequencing were carried out by H.J.R. NO experiments were carried out by

Cam Pickard, 16S sequencing was done by Grace Underwood. All adult and PL lobster trials were carried out by M.H., B.S., M.H., and K.C.

Additional Results

1.1 Bacterial isolation and culture conditions

Bacterial isolations were carried out from field collection swabs of live wild lobsters in Narragansett Bay (12/10/15; Fort Wetherall, Jamestown, RI, USA). Swabs were transported from the field site to the lab in sterile Whirl-Pak® sampling bags and transferred into 1.5 mL of sterile artificial seawater (Instant Ocean™; Spectrum Brands) under aseptic conditions. These stocks were then vortexed and diluted 10- and 100-fold and stored at 4 °C. Each sample was plated onto artificial seawater (Instant Ocean) agar plates (ASW), ASW supplemented with yeast (1 g/L) and peptone (5 g/L) (YP30) and, YP30 with chitin (30 g/L). Single, morphologically unique colonies were picked, streaked to purity (twice on YP30) and cryogenically stored at -80 °C in 10 % glycerol. A total of 211 bacterial isolates were collected and cataloged for probiotic screening.

1.2 Screening against putative pathogens

All 211 bacterial isolates were assessed for antimicrobial activity against the putative pathogens involved in lobster shell disease (LSD) (*Aquimarina* sp. strain I32.4 and *Thalassobius* sp. strain I31.1) and a common fish pathogen *Vibrio*

anguillarum NB10Sm through a modified diffusion assay. A 24 h culture of the pathogen was spread over the surface of the agar plates to form a lawn and 5 μ L spots from 24 h broth cultures of each isolate were added in triplicate and incubated at 25 °C. Zones of inhibition (ZOIs) were measures after 24 and 48 h under optimal growth conditions for the indicator strains (Table 1). Inhibitory zones were measures using EVOS light microscope (Life Technologies). Tests were performed in triplicate with measurements taken from the edge of the test colony to the first viable colony of the indicator strain.

1.3 Characterization of pathogenic bacteria

Initial efforts concentrated on optimization of growth conditions for these pathogenic bacteria. *Thalassobius* could be easily and rapidly grown in liquid media, however *Aquimarina* could not. Table 2 summarizes some of the growth condition attempted to remove the clumping nature of the strains as it interfered with an accurate assessment of cellular growth by both OD₆₀₀ and CFU/mL counts. Both are important for developing bioassays for probiotic testing (work carried out by Jason LaPorte and Edward Spinnard).

A computational approach and whole genome sequence analysis was undertaken to gain a better understanding of the chemical diversity of these strains. Although the genomes for the wild type strains were not available, closely related species of *Aquimarina* showed the potential for extreme chemical diversity in their biosynthetic gene clusters (GCGs) (Table 3). Comparably fewer genomes were available for

Thalassobius sp. but many important BCGs were identified (Table 4). The methods and results obtained from this analysis are published in manuscripts 2, 3, 4, and 5.

1.4 Characterization of probiotic bacteria

Initial screening resulted in the identification of 26 putative probiotic strains. These strains were sequenced according to 16S rRNA PCR. Once potential phylogeny was substantiated whole genome sequencing was carried out for phylogenetically and chemically unique organisms *Bacillus pumilus* YP001, *Loktanella maritima* YPC210 and YPC211.

1.5 Chemical isolations from probiotic bacteria

Putative probiotic strains were grown in 1L YP30 broth cultures in the presence of XAD16 resin for 1 week, after which the resin was filtered through a double layer of cheese cloth and extracted with 50:50 dichloromethane (DCM) and methanol (MeOH). These ‘crude’ extracts were subsequently tested in a modified disc diffusion assay to assess their antibiotic effects against *V. anguillarum* (Table 5). Neither *Loktanella* strain was able to produce a ZOI from monoculture extracts. This topic is covered more thoroughly in appendix B.

1.6 NO to stimulate chemical diversity

To stimulate the expression of potentially silent BSGs, literature reports have suggested the use of oxidative stress to produce novel compounds. Preliminary testing indicated that sodium nitroprussate concentrations of 1 μ M and 10 μ M were sublethal where as 100 μ M and 1,000 μ M were both lethal for probiotic strain. Bacterial strains (*Thalassobius* sp. I31.1, *Bacillus pumilus* YP001, *Pseudoalteromonas* sp. YPC21, *Loktanella maritima* YPC210 and, *Loktanella maritima* YPC211) were streaked from freezer stocks onto YP30 agar and incubated for 48 h, after which a single colony was inoculated into 5 mL of YP30 broth and further incubated for 48 h at 25 °C 150 rpm. The cultures were assessed for CFU/mL using methods outlined previously. Cultures were then spiked with sodium nitroprussate (SNP) at final concentrations of 1 μ M and 10 μ M. Cultures were allowed to incubate for a further hour before the addition of sterile XAD16 resin and a further 24 h before chemical extraction. Resin was filtered through a double layer of cheesecloth and extracted with 100 mL of 50:50 dichloromethane and methanol. Extracts were assessed for their novel metabolite profiles via high performance liquid chromatography (HPLC) when compared to the same strain under optimal growth conditions. Initial small-scale extraction cultures showed no oxidative stress induced novel secondary metabolite production (Figure 1). Chromatographic methods utilized a gradient solvent scheme, maintaining 95 % water modified with 0.1 % formic acid and 5 % methanol for 4 minutes. Gradient then ran for 20 minutes up to 100 % methanol, where it was held for an additional 4 minutes. Experiments were carried out on a Shimadzu prominence-I LC-2030C equipped with a PDA detector model LC-2030/2040, pump LC-2030, and autosampler LC-2030. The column used was XBridge BEH 5 μ m C₁₈, 130 Å, column (4.6 mm X 250 mm).

*1.7 Preparation of combined *Loktanella maritima* (strains YPC210 and YPC211) probiotics for in vivo trials of adult *H. americanus**

Probiotic bacteria were grown in YP30 media for 48 h shaking at 25 °C until a dense culture was formed. 500 µL of culture stock was inoculated into 100 mL of fresh YP30 media and incubated at 25 °C and 125 rpm for 24 h. Cell density was measured by absorbance at 600 nm from 100 µL samples on a 96 well plate reader (Molecular Devices, Spectromax M2). Cultures were also plated to count CFU/mL and form a growth curve from which to measure the probiotic inoculum (Figure 2)

Each adult lobster was contained in individual 10 gallon aquarium tanks with an ambient water flow through system and a day/night light cycle at the Graduate School of Oceanography. Before treatment each lobster was scrubbed with fresh water to control for native biofilms. Ten lobsters were kept as a control and an addition 10 were treated with a probiotic cocktail consisting of 2×10^5 CFU/mL *L. maritima* strains YPC210 and YPC211 in a 50:50 ratio. Treatments occurred weekly both the day before and the day after feeding. Additionally, 10 lobsters received no treatment. All aquaria tanks were cleaned once a week to maintain the health of the adult lobsters. All lobsters showed little/moderate shell disease, while probiotic treatment appeared to induced both egg bearing and mortality. The trial was discontinued after 12 days due to 40% mortality with the probiotic treatment group (Table 6). Haemolymph was sent for pathology to see if the probiotics are responsive for the mortality incurred in this trial.

Table 1: Zones of inhibition (mm) from screened lobster isolates (5 μ L spot plates) against *Aquimarina* sp. strain I32.4, *Thalassobius* sp. I31.1 and, *Vibrio anguillarum* NB10Sm. ZOI assessed by EVOS light microscope (Life technologies). [–] Indicates that no ZOI was observed blanks indicate that the test has not been done.

Strain Identifier	Species (16S rRNA sequence data)	<i>Aquimarina</i> 'homaria' (mm)	<i>Thalassobius</i> sp. (mm)	<i>Vibrio anguillarum</i> (mm)
S4	<i>Phaeobacter inhibens</i>	ZOI	ZOI	
Ha021151216YP012	<i>Pseudoalteromonas</i> sp.	–	2.00	
Ha021151216YPC22	<i>Pseudoalteromonas</i> sp.	–	2.00	
Ha031151216YP014	<i>Pseudoalteromonas</i> sp.	–	2.00	
Ha031151216YPC23	<i>Alteromonas</i> sp.	–	ZOI	
Ha061151216YP001	<i>Bacillus</i> sp. PVR03 (98%)	1.25	–	ZOI
Ha061151216YPC21	<i>Pseudoalteromonas espejiana</i> (95%)	–	1.66	–
Ha061151216YPC22	<i>Pseudoalteromonas</i> sp. (89%)	–	2.00	ZOI
Ha061151216YPC210	<i>Loktanella maritima</i> strain KMM 9530 (97%)	–	2.00	ZOI
Ha061151216YPC211	<i>Loktanella maritima</i> strain KMM 9530 (98%)	–	1.66	ZOI
Ha061151216SWC23	<i>Pseudoalteromonas</i> sp.	–	ZOI	
Ha081151216YPC21	<i>Pseudoalteromonas</i> sp.	–	2.00	
Ha081151216SWC21	<i>Pseudoalteromonas</i> sp.	–	2.00	
Ha081151216SWC22	<i>Pseudoalteromonas</i> sp.	–	2.00	
Ha091151216YP001	<i>Pseudoalteromonas</i> sp.	–	4.32	–
Ha091151216YP018	<i>Pseudoalteromonas</i> sp.	1.60	–	ZOI
Ha091151216YP019	<i>Shewanella</i> sp. (77%)	0.63	–	ZOI
Ha091151216YPC23	<i>Pseudoalteromonas</i> sp.	–	–	ZOI
Ha091151216YPC25	<i>Pseudoalteromonas</i> sp.	–	–	ZOI
Ha091151216YPC29	<i>Pseudoalteromonas</i> sp.	–	–	ZOI
Ha10b1151216YP011	<i>Pseudoalteromonas</i> sp.	1.60	3.11	1.92
Ha10b1151216YPC21	<i>Cobetia</i> sp.	–	2.24	
Ha111151216YP013	<i>Pseudoalteromonas</i> sp.	–	ZOI	1.70
Ha111151216YP016	<i>Pseudoalteromonas</i> sp.	–	2.01	0.30
Ha111151216YPC25	<i>Pseudoalteromonas</i> sp.	–	ZOI	
Ha121151216YP001				1.50
Ha121151216YP011				0.50

Table 2: Optimizing growth conditions for *Aquimarina 'homaria'*. Symbols correspond with + (Low Clumps), ++ (Clumps), +++ (High Clumps) and + (Low Growth), ++ (Growth), +++ (High Growth). Data from Edward Spinnard.

Media/Conditions	Growth	Clumps
YP-30 (1μM EDTA)	+++	+++
YP-30 (5μM EDTA)	+++	+++
YP-30 (10μM EDTA)	+++	+++
YP-30 Small Stir Rod	+	-
YP-30 Large Stir Rod	+	-
Marine Minimal Media (3M)	+	-
YP-10	+	-
YP-20	++	+
YP-30 (175 RPM)	+++	+++
YP-30 (300 RPM)	+++	+
YP-30 Static	+++	-
Marine Broth	+++	+++
LB-10	+	-
LB-20	+	-
LB-30	+	-

Table 3: Data obtained from NCBI for all known *Aquimarina* genomes and run through antiSMASH cluster analysis database.* *Aquimarina pacifica* and *Aquimarina agarilytica* possess no know biosynthetic gene clusters (BGCs).

	TRKS	Sitrophore	Lanipeptide	Terpene	Sitrophore-Terpene	Anglycone	Alipolyene-Resorcinol	Alipolyene-Resorcinol-TRKS	NRPS-Lanipeptide	Resorcinol	NRPS-TRKS	NRPS	Bactacin	Bactacin-Lanipeptide	Orbital-Terpenoids	Terpenoids	Other
<i>Aquimarina longa</i>	✓ (cluster 3)		✓ (cluster 1)	✓ (cluster 2)													
<i>Aquimarina pacifica</i> *																	
<i>Aquimarina norecepahli</i>			✓ (cluster 1)														
<i>Aquimarina megastriatum</i>		✓ (cluster 1)															
<i>Aquimarina agarilytica</i> *																	
<i>Aquimarina medleri</i>	✓ (cluster 2)	✓ (cluster 9)	✓ (cluster 10 & 12)	✓ (cluster 4 & 5)				✓ (cluster 6)	✓ (cluster 7)	✓ (cluster 1)	✓ (cluster 3)	✓ (cluster 6, 13, 15, 16, 18, 20, 22, 23 & 24)			✓ (cluster 14)	✓ (cluster 11, 17 & 21)	✓ (cluster 25 & 26)
<i>Aquimarina lateralis</i>	✓ (cluster 2)	✓ (cluster 2)	✓ (cluster 1)	✓ (cluster 4, 6 & 9)			✓ (cluster 7)		✓ (cluster 5)								
<i>Aquimarina rubicunda</i>	✓ (cluster 2)	✓ (cluster 6)	✓ (cluster 4)	✓ (cluster 8 & 10)				✓ (cluster 7)				✓ (cluster 5)	✓ (cluster 1)	✓ (cluster 3)	✓ (cluster 2)		
<i>Aquimarina agarilytica</i>					✓ (cluster 2)	✓ (cluster 1)						✓ (cluster 3)					

Table 4: Data obtained from NCBI for all known *Thalassobius* sp. genomes and run through antiSMASH cluster analysis database.

Species	T1PKS (cluster 1)	T1PKS (cluster 2)	T1PKS (cluster 4)	T3PKS (cluster 3)	Terpene (cluster 3)	Bacteriocin	Lasso peptide	Hserlactone
<i>Thalassobius gelatinovor</i>	✓							
<i>Thalassobius mediterraneus</i>		✓			✓	✓ (cluster 4)		✓ (cluster 1)
<i>Thalassobius</i> sp. CECT 5114	✓		✓			✓ (cluster 2)	✓ (cluster 5)	✓ (cluster 3)

Table 5: Monoculture XAD16 extracts activity against *V. anguillarum* NB10Sm.

Bacteria were grown in 1L YP30 shaking cultures for 1 week. Cultures contained XAD16 throughout the growth period. Cultures inoculated from 5 mL broth cultures with 1 mL of dense cell media.

Strain Identifier	Species	Disc Diffusion (200 µg)	Disc Diffusion (400 µg)
Ha031151216YPC23	<i>Alteromonas</i> sp.	✓	✓
Ha061151216YP001	<i>Bacillus pumilus</i> YP001	✓	✓
Ha061151216YPC21	<i>Pseudoalteromonas</i> sp.	✓	✓
Ha061151216YPC22	<i>Pseudoalteromonas</i> sp.	✓	✓
Ha061151216YPC210	<i>Loktanella maritima</i> strain YPC210	✗	✗
Ha061151216YPC211	<i>Loktanella maritima</i> strain YPC211	✗	✗
Ha091151216YP019	<i>Shewanella</i> sp.	✓	✓
Ha10b1151216YPC21	<i>Cobetia</i> sp.	✓	✓

Figure 1. Overlaid chromatograms for SNP spiking experiment at doses 0, 1 and 10 μ M. (A) *B. pumilus* YP001, (B) *Pseudoalteromonas* sp. YPC21, (C) *Pseudoalteromonas* sp. YPC22, (D) *L. maritima* YPC210 (E) *L. maritima* YPC210.

Figure 2. Growth of putative probiotics by measurement of wavelength (600 nm) as a proxy for cell density over time. Standard deviation from triplicates calculated for each time point.

Table 6: Adult *H. americanus* trial carried out over 12 days. Data from Kathy Castro, Barbara Somers, and Mitch Hatzipetro.

Strains Tested	N of Lobsters	Mortality (N) of Lobsters	Number of Egg Bearing Lobsters
<i>Loktanella maritima</i> strains YPC210 and YPC211 combined	10	4	3
Freshwater Scrub	10	0	2
Control	10	0	0

Table 7: Single strain testing of potential probiotics against PL lobsters assessing mortality over 3 days. All putative probiotics were tested at 1×10^6 cell/mL. Work carried out by Melissa Hoffman, Kathy Castro, Barbara Somers, and Mitch Hatzipetro.

Strains Tested	16S Sequence	PL Lobsters (N)	Mortality (5 days) Trial
Ha031151216YP014	<i>Pseudoalteromonas sp.</i>	12	0
Ha061151216YPC22	<i>Pseudoalteromonas sp.</i>	12	3
Ha061151216YP001	<i>Bacillus sp.</i>	12	1
Ha061151216YPC210	<i>Loktanella sp.</i>	12	0
Ha061151216YPC211	<i>Loktanella sp.</i>	12	1
Ha081151216YPC21	<i>Pseudoalteromonas sp.</i>	12	1
S4	<i>Phaeobacter inhibens</i>	12	2

Table 8. Probiotic treatments of PL lobsters with concomitant challenge with the putative pathogen *Thalassobius* I31.1. Mortality was assessed after 3 days. All probiotics were tested at 1×10^6 cell/mL. Melissa Hoffman, Kathy Castro, Barbara Somers, and Mitch Hatzipetro.

Strains Tested	16S Sequence	PL Lobsters (N)	Mortality (3 days)
Ha031151216YP014	<i>Pseudoalteromonas sp.</i>	12	0
Ha061151216YPC22	<i>Pseudoalteromonas sp.</i>	12	0
Ha061151216YP001	<i>Bacillus sp.</i>	12	0
Ha061151216YPC210	<i>Loktanella sp.</i>	12	0
Ha061151216YPC211	<i>Loktanella sp.</i>	12	0
Ha081151216YPC21	<i>Pseudoalteromonas sp.</i>	12	0
S4	<i>Phaeobacter inhibens</i>	12	0

APPENDICES

Appendix C: Manuscript 5

Production of known siderophore agrobactin by the marine bacterium *Loktanella maritima* YPC211 when grown in co-culture with *Thalassobius* sp. I31.1 a putative pathogen involved in lobster shell disease.

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Author Contributions

Bacterial strains were isolated by H.J.R. Antimicrobial testing, chemical isolation, and genome sequence analysis were carried out by H.J.R. C.P. helped with large scale chemical extraction and purification. J.L. and E.S. aided in genome sequencing.

Additional Results

Loktanella maritima is a Gram-negative, aerobic bacterium originally isolated from shallow marine sediments in the Sea of Japan (1). The genus is of the class *Alphaproteobacteria*, family *Rhodobacterales*, order *Rhodobacteraceae*. Von Trappen *et al.* (2) originally proposed the genus to accommodate three species but it has since been expanded and modified to contain 15 species (3-5). *Loktanella* appear to be ubiquitously distributed in the oceans and have been isolated from Antarctic to sub-tropical environments (1,2). The strain YPC211 produces a beige/yellow pigment and was isolated from the egg mass of an American Lobster *Homarus americanus* collected in Narragansett Bay near Jamestown Island, Rhode Island, USA.

No natural products have been isolated from this genus despite widespread genomic sequencing. We present the first documented natural product, biosynthesis and bioactivity for this strain. This strain was isolated from eggs, which often retain a specific communities of microorganisms that contribute to the protection against infection. Bacteria isolated from crustacean egg masses have previously been found to produce secondary metabolites that aid in the protection of the eggs. Initial investigation of extracts from YPC211 monocultures did not yield antimicrobial bioactivity. Therefore a co-culturing approach was undertaken since such approaches have previously been successful in activating silent biosynthetic gene clusters (6).

1.1 Sampling and Isolation

Bacterial isolations were carried out from field collection swabs of live wild Lobsters in Narragansett Bay (12/16/15; Fort Weatherall, Jamestown, RI, USA) as outlined in appendix I. This particular isolated was from an egg mass which harbored a diverse range of antimicrobial producing bacteria and was isolated on YP30 agar supplemented with chitin (30 g/L) from a 100-fold dilution.

1.2 Genomic Sequencing and Biosynthetic Gene Cluster Analysis

Loktanella sp. constitutes a biosynthetically diverse and relatively unstudied genus, therefore whole genome sequencing was carried out for strain YPC211 and published as manuscript 5. A comparison of secondary metabolite gene clusters was made across the 12 genomes sequenced for *Loktanella* and available through NCBI (Table 1). Further computational approaches were used to determine potential BGCs responsible for the antibiotic effects observed lead to the investigation of a non-ribosomal peptide synthase (NRPS) BGC (Figure 1). Sequence antibiotic biosynthetic cassettes were analyzed for identity through Geneious and NCBI-delta-blastp.

1.3 CAS Iron Binding Affinity Assay

To qualitatively detect siderophore production in *Loktanella maritima* YPC211 the strain supernatant was tested in liquid chrome azurol S (CAS) indicator. A CAS solution was prepared according to the reported method (Schwyn & Neilands;

1987). Overnight liquid cultures grown in iron-limited media were centrifuged 10,000 rpm for 1 minute and supernatant was combined in a 1:1 ratio with CAS reagent.

1.4 Chemical Characterization of *Loktanella maritima* strain YPC211

Initial screenings outlined in appendix I indicated that the strain YPC211 was able to produce a ZOI when spot plated against putative pathogens. However, no monoculture secondary metabolite extract was able to induce a ZOI. To tackle this problem *Thalassobius sp.* I31.1 and bacterial isolate *Loktanella maritima* YPC211 were grown from stock on YP30 agar plates at 25 °C for 48 h. Single colonies were picked and inoculated into 5 mL of sterile artificial seawater supplemented with yeast (1 g/L) and peptone (5 g/L) and incubated at 25 °C with shaking at 150 rpm for 48 h. Two 2 L Erlenmeyer flasks containing YP30 media were inoculated with 1 mL of dense culture and allowed to grow under the same conditions for 48 h. Two 50 mL samples of equally dense cultures of *Thalassobius sp.* and *Loktanella sp.* were taken according to OD₆₀₀ readings. The *Thalassobius sp.* was autoclaved and the samples were centrifuged at 5,000 rpm for 30 minutes. The cells were washed 3 times with ASW and combined in 1 L of fresh YP30 incubated for 5 days at 25 °C and 150 rpm (New Brunswick) in the presence of Amberlite™ XAD-16 resin (Sigma Aldrich). Resin was filtered through a double layer of cheesecloth, washed with 700 mL of DI water and dried.

Resin was eluted with a first wash of 250 mL DCM and 250 mL of methanol followed by 250 mL of methanol. The crude extract was concentrated via rotary

evaporation. The resulting extract was partitioned between ethyl acetate (EtOAc) and water 3 times in a 2 L glass separatory funnel. The organic fraction was concentrated by rotary evaporation before further purification. Preliminary screening for bioactivity utilized bioassay guided 96 well plate fractionation from the crude extract and tested all peaks against *V. anguillarum* NB10Sm. Results from this indicated a single active peak (Figure 2). The active peak showed a strong 254 nm absorbance used to target it in further purifications.

Large-scale chemical separation methods are summarized in figure 3. Once the active peak was established a high throughput targeted fractionation strategy could be employed using solid-phase extraction (SPE) and carried out on a CombiFlash Rf200 system equipped with a 13 g C18 RediSepRf High Performance Gold column (Teledyne ISCO). Solvent gradient of 5-100% B (methanol with 0.1% water formic acid) was run over 28 minutes. The mobile phase was initially held at 95 % solvent A (water with 0.1% water formic acid) for 4 minutes before starting the gradient and was held at 100% solvent B for 4 minutes after the gradient was completed. Individual peaks were collected according to absorbances at 254 nm and then concentrated *in vacuo*. This yielded 6 fractions which were further separated using high performance liquid chromatography (HPLC). HPLC experiments were performed on a Shimadzu prominence-I LC-2030C equipped with a PDA detector model LC-2030/2040, pump LC-2030, and autosampler LC-2030. The HPLC method included a flow rate of 1 mL/min, a linear gradient of MeOH against H₂O (0.1% formic acid) and an XBridge BEH 5 μ m C₁₈, 130 Å, column (4.6 mm X 250 mm) as the stationary phase. Chromatography method: 5% MeOH for 4 minutes, linear gradient to 100% MeOH

over 16 minutes, maintain 100% MeOH for 4 minutes, return to 5% MeOH for 4 minutes.

Chemical characterization of pure compounds was undertaken using mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy and optical rotations. An accurate mass spectrum for agrobactin was acquired by electrospray ionization mass spectrometry (ESIMS) using an AB Sciex TripleTOF 4600 spectrometer in the positive ion mode (Figure 4). Validation of agrobactin structure was carried out by MS/MS fragmentation (Figure 5). ¹H NMR experiments were conducted using a 500 MHz Varian NMR (Varian Inc., Palo Alto, CA, USA) with DMSO-*d*₆ as the solvent at 25 °C (Figure 6).

1.5 Bioassay guided fractionation, disc diffusion and minimum inhibitory concentration (MIC) antibiotic determination and quantification

Once purity of the active peak was established (Figure 7) disc diffusion assays were carried out as a bioactivity guided fractionation strategy. *V. parahaemolyticus* was grown from stock on YP30 agar at 25 °C for 24 h, then in 5 mL of YP30 at 25 °C shaking at 150 rpm for 24 h. The dense culture was spread onto fresh YP30 agar. Organic fractions were dissolved in MeOH at a concentration of 20 mg/mL and loaded onto discs at final concentrations of 100 µg and 200 µg, dried, and placed onto the plate, and the plate was grown for 24 h at 25 °C. Plates were observed for zones of inhibition (Figure 8).

Minimum inhibitory concentration (MIC) was determined using a broth dilution assay in a 96-well plate, methanol was the vector for compound testing, and ciprofloxacin at 0.4 µg/mL was a positive control. Plate was incubated shaking at room temperature. Absorbance readings were taken hourly at 600 nm for 24 h (Spectromax). The MIC of agrobactin (HR1-149-2) was determined to be 50 µg/mL against *V. parahaemolyticus* (Figure 9).

References

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Table 1: Data obtained from NCBI for all known genomes of *Loktanella* and run through antiSMASH cluster analysis. *indicates no biosynthetic gene clusters identified by antiSMASH.

Strain	T1PKS	T3PKS	Terpene	Bacteriocin	Lantipeptide	Hserlactone	Siderophore	NRPS	Other
<i>Loktanella vestfoldensis</i>			✓ (cluster 1 & 4)	✓ (cluster 3)		✓ (cluster 2)			
<i>Loktanella cinnabarina</i>	✓ (cluster 2)	✓ (cluster 3)	✓ (cluster 1)						
<i>Loktanella hongkongensis</i>	✓ (cluster 4)		✓ (cluster 1 & 3)		✓ (cluster 2)				
<i>Loktanella litorea</i>				✓ (cluster 2)		✓ (cluster 1)			
<i>Loktanella tamensis</i>				✓ (cluster 2)		✓ (cluster 3 & 4)			✓ (cluster 1)
<i>Loktanella salsilacus*</i>									
<i>Loktanella koreensis</i>			✓ (cluster 3)			✓ (cluster 2 & 4)			✓ (cluster 1)
<i>Loktanella fryxellensis*</i>									
<i>Loktanella rosea</i>				✓ (cluster 3)	✓ (cluster 4)	✓ (cluster 2)	✓ (cluster 1)		
<i>Loktanella atrilutea</i>		✓ (cluster 5)	✓ (cluster 1 & 3)			✓ (cluster 2 & 4)			
<i>Loktanella sediminum</i>			✓ (cluster 2 & 5)	✓ (cluster 4)		✓ (cluster 3,6 & 7)			✓ (cluster 1)
<i>Loktanella pyoseonensis</i>			✓						
<i>Loktanella maritima</i> YPC210	✓ (cluster 4)			✓ (cluster 1)		✓ (cluster 2 & 3)		✓ (cluster 5)	
<i>Loktanella maritima</i> YPC211	✓ (cluster 4)			✓ (cluster 1)		✓ (cluster 2 & 3)		✓ (cluster 5)

Figure 1: NRPS biosynthetic gene cluster analysis and alignment to known gene clusters. Binding pocket sequence analysis was used to predict the substrate specificity.

Figure 2: Initial chemical screening, 96 well plate fractionation from crude extract to identify the peak responsible for the observed antibiotic activity.

Figure 3: Large scale chemical isolation of agrobactin from a co-culture of *Loktanella maritima* YPC211 and *Thalassobius* sp. strain I31.1.

Figure 4: High resolution tandem mass spectrometry intensity from direct injection ESI in positive ion mode (declustering potential 150 collision energy 30) for agrobactin m/z 637.2566 $[M+H]^+$, 659.2373 $[M+Na]^+$, and 681.2185 $[M+Fe]^+$.

Figure 5: Direct injection TOF MS/MS fragmentation positive ion mode of agrobactin m/z 637.2 $[H+H]^+$ to produce identifiable building blocks and structure confirmation.

Figure 6: Proton NMR spectrum of agrobactin Varian NMR 500 MHz with DMSO- d_6 as the solvent at 25 °C.

Figure 7: Analytical HPLC chromatogram of agrobactin as purified product.

Figure 8. Disc diffusion assay indicating that only crude extracts from co-cultures of *Thalassobius* I31.1 and *L. maritima* YPC211 are able to elicit an antibiotic effect against *V. anguillarum* NB10Sm. Positive control is ciprofloxacin, negative controls are crude media extract and methanol blank. Testing monoculture extracts of *Thalassobius* I31.1 and *L. maritima* YPC211.

Figure 9. MIC assay for agrobactin (HR1-149-2) against MRSA plotted as averages and standard deviation * $P < 0.05$ ** $P < 0.01$ from agrobactin (methanol) and ciprofloxacin (DMSO). Cell density measured by optical density (600 nm absorbance).