Final Program and Abstracts

ASM CONFERENCES

5th ASM Conference on

Cell-Cell Communication in Bacteria

October 18 – 21, 2014

San Antonio, Texas

1752 N Street, N.W.
Washington, DC 20036-2904
Phone: 202-737-3600
World Wide Web: www.asm.org

ASM CONFERENCES

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To identify emerging or underrepresented topics of broad scientific significance.

To facilitate interactive exchange in meetings of 100 to 500 people.

To encourage student and postdoctoral participation.

To recruit individuals in disciplines not already involved in ASM to ASM membership.

To foster interdisciplinary and international exchange and collaboration with other scientific organizations.

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Acknowledgments

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General Information

REGISTRATION AND NAME BADGES

ASM Staff will be available at the registration desk in the Grand Ballroom Foyer on the 3rd Floor at the San Antonio Marriott Rivercenter during posted registration hours. Participants may collect name badges and program materials at the registration desk. A name badge is required for entry into all sessions and meals. Each participant may register one guest to attend the welcome reception. Guest tickets are $50. Guests may not attend sessions, poster sessions, lunches or coffee breaks.

GENERAL SESSIONS

All general sessions will be held in the Salon E of the Grand Ballroom at the San Antonio Marriott Rivercenter.

POSTER SESSIONS

Poster boards are located in the Grand Ballroom at the San Antonio Marriott Rivercenter. Posters will be available for viewing informally throughout the conference, with official poster sessions scheduled on Sunday and Monday.

All posters should be mounted on Sunday morning, October 19, between 7:30 and 8:30 am, and should be removed by 1:00 pm on Tuesday, October 21. Posters which are not removed in time will be discarded.

Odd-numbered posters (1,3,5…) will be officially presented in Session A on Sunday, October 19, and even-numbered posters (2,4,6…) will be officially presented in Session B on Monday, October 20.

Please check your assigned number in the abstract index. The same number is used for the presentation and board number.

NETWORKING MEALS AND SOCIAL EVENTS

Registration includes attendance at the Welcome Reception on Saturday, Networking Lunches on Sunday and Monday, and Poster Session Mixers on Sunday and Monday. Ample time has also been scheduled for participants to network during coffee breaks.

CERTIFICATE OF ATTENDANCE

Certificates of Attendance can be found in the registration packet received at the registration desk.
Note: Certificates of Attendance do not list session information.

CAMERAS AND RECORDINGS POLICY

Audio/video recorders and cameras are not allowed in session rooms or in the exhibit and poster areas. Taking photographs with any device is prohibited.

CHILD POLICY

Children are not permitted in session rooms, poster sessions, conference meals or social events. Please contact the hotel concierge to arrange for babysitting services in your hotel room.

Travel Grants

ASM TRAVEL GRANTS

ASM encourages the participation of graduate students and new postdocs at ASM Conferences. To support the cost of attending the conference, ASM has awarded travel grants of $500 to each of the following individuals:

Kyle Asfahl
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Rebecca Scholz
Joe Sexton
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Jiayue Yang
Liqin Zhou

NIH TRAVEL GRANTS

The NIAID – NIH has provided a conference grant to ASM to support travel of junior investigators, postdoctoral fellows and students. The following individuals have received travel grants administered by ASM which are supported by NIH funds:

Gilles Brackman
Daniel Cornforth
Michael Federle
Maria Hadjifrangiskou
Beth Lazazzera
Charlotte Michelsen
Philip Rather
Lynne Turnbull
Julie Valastyan
Karina Xavier

NSF TRAVEL GRANTS

The National Science Foundation has provided a conference grant to ASM to support travel of junior investigators, postdoctoral fellows and students. The following individuals have received travel grants administered by ASM which are supported by NSF funds:

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SGM TRAVEL GRANTS

The Society for General Microbiology (SGM) has sponsored travel grants for the following individuals to support their participation in the conference:

Luke McNally
Rita Monson
Roman Popat
Aled Roberts

Scientific Program

Saturday, October 18, 2014

6:00 – 7:30 pm Grand Ballroom, Salon E	**Opening Session**
6:00 – 6:15 pm	Welcome Remarks ***Marvin Whiteley***, ***Eric Stabb***, ***Joanna Goldberg***
6:15 – 6:30 pm	Tribute to Woody Hastings ***Pete Greenberg***, *University of Washington, Seattle, WA*
6:30 – 7:30 pm	Keynote Lecture ***Bonnie Bassler***, *Princeton University, Princeton, NJ*
7:30 – 9:00 pm Grand Ballroom, Salon E	**Session 1: Signal Generation and Perception**
7:30 – 8:00 pm	Title to be announced ***Steve Winans***, *Cornell University, Ithaca, NY*
8:00 – 8:20 pm	Screening and Mutual Information Theory Reveal Single Nucleotides in Qrr4 Important for LuxR Repression ***Julie Valastyan***, *Princeton University, Princeton, NJ*
8:20 – 8:40 pm	Phase Variation Controls Quorum Sensing in *Acinetobacter baumanii* ***Philip Rather***, *Emory University, Atlanta, GA*
8:40 – 9:00 pm	Quorum Sensing Control of a Lantibiotic Gene Cluster in *Streptococcus pneumonia* ***Beth Lazazzera***, *University of California, Los Angeles, CA*
9:00 – 10:00 pm Sazo's Latin Grill	**Welcome Reception**

Sunday, October 19, 2014

8:30 – 11:40 am Grand Ballroom, Salon E	**Session 2: Interference of Signaling**
8:30 – 9:00 am	Title to be announced ***Michael Givskov**, University of Copenhagen, Copenhagen, DENMARK*
9:00 – 9:20 am	How Does the Quorum Sensing Inhibitor Hamamelitannin Increase *Staphylococcus aureus* Biofilm Susceptibility Towards Glycopeptides ***Gilles Brackman**, Ghent University, Gent, BELGIUM*
9:20 – 9:40 am	Growth Substrate Topography Switches an Interspecies Signaling Pathway in Co-Culture Biofilms ***Arunima Bhattacharjee**, University of California, Irvine, Irvine, CA*
9:40 – 10:00 am	Combinatorial Quorum Sensing Can Allow Bacteria to Resolve Their Social and Physical Environment ***Daniel Cornforth**, The University of Texas at Austin, Austin, TX*
10:00 – 10:30 am Grand Ballroom, Salons A & B	Coffee break
10:30 – 11:00 am	Synthetic Ligands for the Interception of Bacterial Communication ***Helen Blackwell**, University of Wisconsin, Madison, WI*
11:00 – 11:20 am	Cell-Cell Communication Among the Streptococci: Rgg Pheromones, Inhibitors and Regulated Behaviors ***Michael Federle**, University of Illinois at Chicago, Chicago, IL*
11:20 – 11:40 am	Investigating the Quorum Quenching Ability of Bacteria from Gastrointestinal Tracts of Cultured Ornamental Freshwater-Fish and Marine Farmed Fish ***Zohra Kajee**, University of KwaZulu-Natal, Durban, SOUTH AFRICA*

12:00 – 1:30 pm Grand Ballroom, Salon M	Lunch
1:30 – 4:20 pm Grand Ballroom, Salon E	**Session 3: Bacterial Development and Signaling**
1:30 – 2:00 pm	Cell-cell Recognition by the Polymorphic Myxobacterial TraA Receptor Leads to Beneficial and Adversarial Interactions ***Dan Wall,*** *University of Wyoming, Laramie, WY*
2:00 – 2:20 pm	Intercellular Signalling Using Extracellular DNA Facilitates Active Biofilm Expansion ***Lynne Turnbull****, University of Technology Sydney, Sydney, AUSTRALIA*
2:20 – 2:40 pm	Characterization of Quorum Sensing-Regulated Gas Vesicle Morphogenesis in Enterobacteria ***Rita Monson****, University of Cambridge, Cambridge, UNITED KINGDOM*
2:40 – 3:10 pm Grand Ballroom, Salons A & B	Coffee break
3:10 – 3:40 pm	Jekyll and Hyde: Microbial Metabolites that Both Kill and Stimulate Biofilm Formation ***Beth Shank****, University of North Carolina, Chapel Hill, NC*
3:40 – 4:00 pm	Kin-Specific Binding Between Two Proteins Correlates with in vivo Self Identity and Wild-Type Swarm Expansion in *Proteus mirabilis* ***Karine Gibbs****, Harvard University, Cambridge, MA*
4:00 – 4:20 pm	*Staphylococcus aureus* Enhance Growth and Antibiotic Tolerance in a Human Host Adapted *Pseudomonas aeruginosa* Lineage ***Charlotte Michelsen****, Technical University of Denmark, Kgs. Lyngby, DENMARK*
4:30 – 6:30 pm Grand Ballroom, Salons A & B	**Poster Session A and Networking Mixer** *Odd-numbered posters will be formally presented*

Monday, October 20, 2014

8:30 – 11:40 am Grand Ballroom, Salon E	**Session 4: Host-Pathogen Interactions and Signaling**
8:30 – 9:00 am	The Curious Case of Death by Seduction: How the Siren Song of Commensal *E. faecalis* Drove VRE to Suicide ***Michael Gilmore**, Harvard Medical School, Boston, MA*
9:00 – 9:20 am	Bioluminescence Based Biosensors for the Detection of Peptide Pheromone Production and Cell-Cell Communication in *Enterococcus faecalis* ***Sabina Leanti La Rosa**, Norwegian University of Life Sciences, Aas, NORWAY*
9:20 – 9:40 am	Understanding the Role of Diffusible Signalling Factor (DSF) in Virulence of Xanthomonas Plant Pathogens ***Rikky Rai**, CDFD, Hyderabad, INDIA*
9:40 – 10:00 am	Integration of Multiple Sensory Inputs in the *Vibrio cholerae* Quorum-Sensing Circuit and the Impact on Virulence and System Robustness ***Sarah Jung**, Tufts University, Boston, MA*
10:00 – 10:30 am Grand Ballroom, Salons A & B	Coffee break
10:30 – 11:00 am	Unravelling the Interplay Between Yersinia and *Caenorhabditis elegans* during Quorum Sensing-dependent in vivo Biofilm Formation ***Paul Williams**, University of Nottingham, Nottingham, UNITED KINGDOM*
11:00 – 11:20 am	Resolving the Contribution of Quorum Sensing and Twitching Motility in the Systemic Spread of *Pseudomonas aeruginosa* in Burn Wound Infections ***Jake Everett**, Texas Tech University Health Sciences Center, Lubbock, TX*

11:20 – 11:40 am	The PmrB histidine Kinase Controls Expression of the qseBC Two-Component System via a Cognate and a Non-Cognate Response Regulator: Cross-Regulation or a Four-Component System? ***Maria Hadjifrangiskou**, Vanderbilt University School of Medicine, Nashville, TN*
12:00 – 1:30 pm Grand Ballroom, Salon M	Lunch
1:30 – 4:20 pm Grand Ballroom, Salon E	**Session 5: Systems Biology and New Technologies**
1:30 – 2:00 pm	Opening Lines of Communication: Quorum Sensing at the Intersection of Synthetic Biology and Microelectronics ***William Bentley**, University of Maryland, College Park, MD*
2:00 – 2:20 pm	A Systems Analysis of Dual Signaling Control of Conjugative Drug Resistance Transfer in *Enterococcus faecalis* ***Arpan Bandyopadhyay**, University of Minnesota, Minneapolis, MN*
2:20 – 2:40 pm	A Modular View on Cell Density Encoding Schemes in Bacterial Quorum Sensing ***Ilka Bischofs**, University of Heidelberg, Heidelberg, GERMANY*
2:40 – 3:10 pm Grand Ballroom, Salons A & B	Coffee break
3:10 – 3:40 pm	Title to be announced ***Peter Dorrestein**, University of California, San Diego, CA*
3:40 – 4:00 pm	Electrochemical Quantification of Secondary Metabolites Produced by 3D-Printed Bacterial Aggregates ***Jodi Connell**, The University of Texas at Austin, Austin, TX*

4:00 – 4:20 pm	Quorum Sensing of *Pseudomonas putida* IsoF ***Burkhard Hense**, Helmholtz Zentrum Muenchen, Neuherberg/ Munich, GERMANY*
4:30 – 6:30 pm Grand Ballroom, Salons A & B	**Poster Session B and Networking Mixer** *Even-numbered posters will be formally presented*

Tuesday, October 21, 2014

8:30 – 10:30 am Grand Ballroom, Salon E	**Session 6: Symbiosis and Mutualism**
8:30 – 9:00 am	SypF, An Unusual Two-Component Regulator of Biofilm Formation and Colonization ***Karen Visick**, Loyola University Chicago, Maywood, IL*
9:00 – 9:20 am	Interaction between *Pseudomonas fluorescens* Pf0-1 and *Pedobacter sp. V48* Induces Social Motility ***Lucy McCully**, University of Massachusetts Dartmouth, North Dartmouth, MA*
9:20 – 9:40 am	Manipulating the Interspecies Quorum Sensing Signal AI-2 in the Mouse Gut ***Karina Xavier**, Instituto Gulbenkian de Ciencia, Oeiras, PORTUGAL*
9:40 – 10:00 am	A Plant-Responsive LuxR Homolog in a Cottonwood Tree Root Endophyte ***Amy Schaefer**, University of Washington, Seattle, WA*
10:00 – 10:30 am Grand Ballroom, Salons A & B	Coffee break

10:30 am – 12:30 pm Grand Ballroom, Salon E	**Session 7: Evolution and Signaling**
10:30 – 11:00 am	Title to be announced ***Pete Greenberg**, University of Washington, Seattle, WA*
11:00 – 11:20 am	The Evolutionary Origins of Quorum Sensing ***Luke McNally**, University of Edinburgh, Edinburgh, UNITED KINGDOM*
11:20 – 11:40 am	The Evolution of Quorum Sensing in a Naturally Co-Evolved Host-Pathogen Model ***Liqin Zhou**, Imperial College London - Silwood Park Campus, Ascot, UNITED KINGDOM*
11:40 – 12:00 pm	Exploiting Combinatorial Signalling - A New Type of Cheating Strategy in *Pseudomonas aeruginosa* ***James Gurney**, University of Nottingham, Nottingham, UNITED KINGDOM*
12:00 – 12:30 pm	Facultative Cheating Drives the Maintenance of Quorum-Sensing Pherotype Diversity in Structured Populations ***Avigdor Eldar**, Tel Aviv University, Tel Aviv, ISRAEL*
12:30 – 1:00 pm	**Closing Remarks**

Speaker Abstracts

OS:1

B. Bassler;
Department of Molecular Biology, Howard Hughes Medical Institute-Princeton University, Princeton, NJ.

S1:1

S. Winans;
Cornell University, Ithaca, NY.

S1:2

SCREENING AND MUTUAL INFORMATION THEORY REVEAL SINGLE NUCLEOTIDES IN QRR4 IMPORTANT FOR LUXR REPRESSION

J. S. Valastyan*, S. T. Rutherford, T. O. Taillefumier, C. G. Callan, N. S. Wingreen, B. L. Bassler;*
Princeton University, Princeton, NJ.

Five small regulatory RNAs, the Qrr sRNAs, are expressed at low cell density in Vibrio harveyi and regulate multiple RNA targets whose encoded proteins function in the quorum-sensing pathway. While the Qrr sRNAs are highly conserved and additively suppress luciferase production at low cell density, their expression levels and roles in the regulation of specific targets are divergent. We sought to more fully understand the contribution of individual nucleotides within one Qrr family member - Qrr4 - to the repression of LuxR, the master regulator of the lux operon. To this end, we used saturating mutagenesis, fluorescence-activating cell sorting, and mutual information analysis to identify individual mutations that strongly inactivate Qrr4. Further analysis of the mutants determined to have high mutual information revealed differential roles for various bases in Qrr4 function; we identified bases important for base-pairing with the target RNA, forming stem-loops, and interacting with the chaperone Hfq. Complementary studies elucidated nucleotides important for Qrr4's repression of LuxO. Comparison of these two data sets aided in defining sequences important for target binding, but revealed significant functional homology throughout the rest of the sequence. While our studies were able to ascribe function to most of the bases determined to be important, some remain elusive. However, we were able to show that double mutant analysis may allow even deeper understanding of the mechanism by which a single mutation disrupts Qrr4 function. Additionally, expanding this analysis to other targets and the other Qrr sRNAs will elucidate more fully the individual roles of these highly conserved small RNAs, aiding in our understanding of their role in regulation of quorum sensing.

S1:3

PHASE VARIATION CONTROLS QUORUM SENSING IN ACINETOBACTER BAUMANII

K. M. Clemmer[1], ***P. N. Rather[2];***
[1]Atlanta VA Medical Center, Atlanta, GA,
[2]Emory University, Atlanta, GA.

The Gram-negative bacterium *Acinetobacter baumannii* has become a major cause of nosocomial infections, which are exceedingly difficult to treat due to the extensive array of antibiotic resistance genes present in this bacterium. In *A. baumannii,* a LuxI/R pair of proteins designated AbaI/AbaR mediate a quorum sensing response via the signaling molecule 3-OH C_{12}-HSL. Our lab has found that the highly virulent *A. baumannii* strain AB5075 exhibits two colony opacity phenotypes, opaque and translucent. Opaque and translucent variants interconvert at frequencies ranging from $1/10^3$-10^4 indicating a phase variable mechanism was responsible. Using an *Agrobacterium tumefaciens traG-lacZ* biosensor, the translucent colony variants were shown to produce greatly reduced levels of 3-OH C_{12}-HSL, which are restored in the opaque form. However, the translucent colony

phenotype was not converted to opaque by the addition of exogenous 3-OH C_{12}-HSL. Therefore, the translucent colony phenotype does not result from decreased signal production and it is hypothesized that 3-OH C_{12}-HSL signal production is coordinately regulated with the opaque/translucent switch by a global regulatory mechanism. Consistent with this, semi-quantative RT-PCR demonstrated translucent colonies exhibited reduced expression of the *abaI* gene. An *abaR* mutant was capable of colony switching, although at significantly reduced frequencies indicating that quorum sensing reciprocally regulates phase variation. Whole genome sequencing and RNA-Seq analysis are currently being used to determine the genetic and expression differences between each colony type.

S1:4

QUORUM SENSING CONTROL OF A LANTIBIOTIC GENE CLUSTER IN STREPTOCOCCUS PNEUMONIAE

S. Hoover[1], T. Ho-Ching[2], M. Winkler[2], ***B. Lazazzera[1];***
[1]Univ. of California, Los Angeles, CA, [2]Indiana University, Bloomington, IN.

Quorum sensing in Gram-positive bacteria is mediated by small-secreted peptides that diffuse through the environment. One such group of quorum sensing peptides is the Phr family of peptides, originally characterized in Bacillus subtilis. We have identified in Streptococcus pneumoniae gene cassettes that appear to encode Phr peptides. Here, we report the characterization of one of these Phr peptide cassettes, which like the others, encodes a gene for a secreted Phr peptide (phrA) and a putative transcription factor (tprA). The TprA protein shares the greatest similarity with the PlcR transcription factor of B. cereus, which is activated by a Phr peptie. In contrast, TprA was found to acts as a transcriptional repressor and the PhrA peptide antagonized its activity. The tprA/phrA regulon was found to include the phrA gene itself and a putative lantibiotic biosynthesis operon. We further found that this tprA/phrA system is under strong catabolite repression, such that PhrA peptide signaling only occurred in the presence of a poor carbon source, galactose. As galactose is primarily encountered by S. pneumoniae in the nasopharynx, and glucose is a sugar encountered in the lungs and the bloodstream, our data suggest that this quorum-sensing cassette may be important during colonization of the nasopharynx. Our currently model is that the metabolic signals of the nasopharynx combined with a high cell density signal, mediated by phrA, induce production of a lantipeptide that allows S. pneumoniae to compete with other organisms of the nasopharynx microbiota.

S2:1

M. C. Givskov;
Intl. Hlth. Immunol. and Microbiol., Univ. of Copenhagen, Copenhagen, DENMARK.

S2:2

HOW DOES THE QUORUM SENSING INHIBITOR HAMAMELITANNIN INCREASE *STAPHYLOCOCCUS AUREUS* BIOFILM SUSCEPTIBILITY TOWARDS GLYCOPEPTIDES

G. Brackman*, F. Van den Driessche, T. Coenye;*
Lab Pharm. Microbiology, Ghent University, Gent, BELGIUM.

Background: Biofilm-associated infections caused by *Staphylococcus aureus* are often very difficult to treat and novel targets are needed to combat these infections. We have previously shown that the quorum sensing (QS) modulator 2',5-di-O-galloyl-D-hamamelose (hamamelitannin, HAM) increases the susceptibility of *S. aureus* biofilms towards vancomycin (VAN) *in vitro* as well as *in vivo*. However, the mechanism of action of HAM at the molecular level has not yet been elucidated. **Methods:** Two parallel strategies were followed in order to gain insights in the

way HAM affects QS. First, we evaluated the effect of HAM on the susceptibility of biofilms of *S. aureus* strains with mutations in the QS systems (e.g. *agrBCDA*, *trap*, *luxS*) or in genes involved in biofilm formation and virulence (e.g. *icaA*, *sarA*, *codY*). Secondly, using illumina sequencing we identified genes that were differentially expressed in untreated biofilms and biofilms treated with VAN alone or in combination with HAM. Results obtained with both strategies were further investigated using the appropriate tools. **Results:** No loss in HAM activity was observed for most of the mutants. In contrast, HAM did not affect biofilm susceptibility of *S. aureus* strains with mutations in *agrA* or *trap* gene. This suggests that these genes are involved in mediating the activity of HAM. Using sequencing, we identified a large number of genes that were differentially regulated after treatment. Treatment with HAM (alone or in combination with VAN) resulted in a downregulation of genes involved in biosynthesis of lysine, diaminopimelate and D-alanine and an upregulation of genes involved in glutamine consuming pathways. In addition, the upregulation of genes encoding virulence factors (e.g. *tst*, *hla*, *yent1*, *hlgB*) during VAN treatment was not observed when VAN was combined with HAM. **Conclusion:** HAM reduces the upregulation of peptidoglycan biosynthesis normally observed after treatment with VAN. This possibly leads to the increased susceptibility of *S. aureus* biofilm cells towards VAN. Our results further indicate that combination therapy could positively affect morbidity since the upregulation of virulence factors observed for VAN treatment are not observed when VAN is combined with HAM.

S2:3

GROWTH SUBSTRATE TOPOGRAPHY SWITCHES AN INTERSPECIES SIGNALING PATHWAY IN CO-CULTURE BIOFILMS

***A. Bhattacharjee**, A. Hochbaum;*
University of California, Irvine, Irvine, CA.

Bacterial biofilms are sources of persistent infection and resist antibiotic treatment in medical environments. P. aeruginosa is an opportunistic pathogen associated with lung infections in cystic fibrosis and infections of indwelling device. Medical and environmental biofilms are often multi-species communities of bacteria which are maintained through chemical signaling and metabolite exchange. Most studies of multispecies interactions are in isotropic or homogeneous environments. Here we demonstrate that growth substrate topography is capable of switching an interspecies chemical signaling pathway in co-culture biofilms composed of Escherichia coli and Pseudomonas aeruginosa. Typically, an E. coli receptor, SdiA, recognizes quorum sensing (QS) compounds from P. aeruginosa and triggers a biofilm dispersal response, resulting in P. aeruginosa colonization of the growth substrate. Periodically structured substrates, on the other hand, are shown to modulate this pathway by metabolite accumulation in engineered microenvironments. Substrate structures induce changes in E. coli biofilm morphology, which in turn increase the concentration of indole, a constitutively produced metabolite and active signaling molecule, within the biofilm. The extent of indole accumulation is responsible for modulating the dispersal response of E. coli to P. aeruginosa QS signals. In contrast to transient surface chemistry or antibiotic leaching strategies, substrate topography generates persistent, physical cues that are promising for long-term control of biofilm structure and properties. We show that these materials are able to produce E. coli biofilms that are resistant to P. aeruginosa colonization for at least three weeks. Moreover, these surfaces render the resulting biofilm, almost exclusively composed of E. coli, more susceptible to antibiotic treatment than co-cultures grown on flat surfaces. These results suggest the potential for physical structures to modify signaling dynamics in multi-species communities and to engineer structure-property relationships in biofilms to exhibit useful behavior.

S2:4

COMBINATORIAL QUORUM SENSING CAN ALLOW BACTERIA TO RESOLVE THEIR SOCIAL AND PHYSICAL ENVIRONMENT

D. Cornforth[1], *R. Popat*[2], *L. McNally*[2], *J. Gurney*[3], *T. C. Scott-Phillips*[4], *A. Ivens*[2], *S. P. Diggle*[3], *S. P. Brown*[2];
[1]*The University of Texas at Austin, Austin, TX,* [2]*The University of Edinburgh, Edinburgh, UNITED KINGDOM,* [3]*University of Nottingham, Nottingham, UNITED KINGDOM,* [4]*Durham University, Durham, UNITED KINGDOM.*

Many bacterial species engage in a form of cell-cell communication known as quorum sensing (QS). Despite great progress in unraveling the molecular mechanisms of QS, controversy remains over its functional role. There is disagreement over whether QS surveys bacterial cell density or rather environmental properties like diffusion or flow, and moreover there is no consensus on why many bacteria use multiple signal molecules. We develop and test a new conceptual framework for bacterial cell-cell communication, demonstrating that bacteria can simultaneously infer both their social (density) and physical (mass-transfer) environment, given combinatorial (nonadditive) responses to multiple signals as well as distinct half-lives of the signals. We test these predictions using the opportunistic pathogen Pseudomonas aeruginosa and demonstrate significant differences in signal decay between its two primary signal molecules, as well as diverse combinatorial responses to dual-signal inputs. We also demonstrate that secretome genes are preferentially controlled by synergistic "AND-gate" responses to multiple signal inputs which may help adaptively limit secretions to high cell density/ low mass-transfer environments.

S2:5

SYNTHETIC LIGANDS FOR THE INTERCEPTION OF BACTERIAL COMMUNICATION

H. E. Blackwell;
University of Wisconsin, Madison, WI.

We are developing chemical tools that attenuate cell-cell communication pathways in bacteria. Many bacteria communicate using small organic molecules and peptides to monitor their population densities in a process called "quorum sensing." At high cell densities, bacteria use this signaling network to switch from an isolated, nomadic existence to that of a multicellular community. This lifestyle switch is significant; only in groups will pathogenic bacteria turn on virulence pathways and grow into drug-impervious communities called biofilms that are the basis of myriad chronic infections. In turn, certain symbiotic bacteria will only colonize their hosts and initiate beneficial behaviors at high population densities. Our research is broadly focused on the design, synthesis, and characterization of non-native ligands that can intercept quorum sensing and provide new insights into its role in host/microbe interactions. These molecules provide a novel approach to study quorum sensing with both spatial and temporal control in a range of settings. We have developed a series of efficient synthetic methods that provide us with straightforward access to these ligands. In addition, we have applied our quorum sensing antagonists and agonists in vitro and in vivo to investigate quorum sensing as an anti-infective target. This talk will introduce our research approach and highlight recent results.

S2:6

INVESTIGATING THE QUORUM QUENCHING ABILITY OF BACTERIA FROM GASTROINTESTINAL TRACTS OF CULTURED ORNAMENTAL FRESHWATER-FISH AND MARINE FARMED FISH

***Z. Kajee**, H. Y. Chenia;*
University of KwaZulu-Natal, Durban, SOUTH AFRICA.

While many bacterial pathogens in aquaculture environments use quorum sensing (QS) for the regulation of processes including pathogenesis, competitor bacteria can inhibit QS *via* enzymatic quorum quenching (QQ). Gram-negative bacteria generally communicate using N-acyl homoserine lactones (AHLs) that vary in structure but which are susceptible to degradation by enzymes resulting in QS inhibition. QQ holds high value as an alternative mechanism in combatting bacterial infections without selecting for drug-resistant cells and thus, the QQ ability of gastrointestinal tract (GIT) bacteria from cultured fish environments were investigated as potential anti-virulence resources. Bacteria were isolated, enriched and purified from GIT of Malawian cichlids, koi carp, tank biofilms and GIT of marine cultured dusky kob using standard microbiological assays. Isolated bacteria were subjected to qualitative T-streak assays to identify potential QS activity, using three AHL biosensor systems to detect a wide spectrum of AHL molecules, viz., *Chromobacterium violaceum* CV026, VIR07, and *Agrobacterium tumefaciens* A136. Biosensor sandwich assays were used as a preliminary, qualitative screen to investigate the quenching activity. AHL degradation assays were used to investigate QQ substrate specificity of selected isolates using six different synthetic AHLs (C6 - C12). The ability to quorum sense, using AHL molecules of varying chain lengths, was observed for 51.72% of Gram-negative isolates. The qualitative QQ sandwich assays identified 87.96%, 78.24%, and 19.91% of isolates which were capable of quenching short chain, medium to long chain and long chain AHLs, respectively. Only 101 QQ isolates displaying no AHL-based QS were further investigated for substrate specificity. Complete degradation of medium to long chain AHLs were most commonly observed with majority of isolates degrading *N*-octanoyl-DL-homoserine lactone (96%) and *N*-(3-oxodecanoyl)-L-homserine lactone (68.31%). The use of AHL-degrading enzymes to inhibit QS displays great potential as these enzymes are not bactericidal and may thus reduce or inhibit the QS-regulated pathogenesis factors and need to be examined further. The QQ enzymes from these isolates could replace traditional antibiotics since the QQ strategy is not aimed at killing the pathogen or limiting cell growth but rather is focused on shutting down the expression of pathogenicity genes.

S3:1

CELL-CELL RECOGNITION BY THE POLYMORPHIC MYXOBACTERIAL TRAA RECEPTOR LEADS TO BENEFICIAL AND ADVERSARIAL INTERACTIONS

***D. Wall**;*
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Cooperative behavior among individuals often involves resource sharing which in turn provides fitness advantages to the community. Myxobacteria are a microbial example where individuals share their resources and build cooperative multicellular communities. In the case of Myxococcus xanthus, cells will transiently fuse their outer membranes (OMs) and exchange their OM proteins and lipids, a process that is mediated by the TraA polymorphic cell surface receptor and the TraB cohort protein. Resource sharing within diverse microbial communities found in native soil environments raises interesting questions about how partner cells are identified and whether such interactions are regulated. Through a series of chimeric and site directed mutagenesis studies we identify particular regions and amino acid residues within TraA that govern the specificity for cell-cell fusion. We also provide evidence

that among environmental populations there are hundreds of different TraA recognition/social groups. The ability of Tra-dependent interactions to lead to beneficial outcomes was also tested. Here, a healthy population was tested for their ability to repair a damage population. This was done with a conditional lpxC allele, an essential gene for lipopolysaccharide biosynthesis. The cell viability and antibiotic sensitivity defects of lpxC mutants were rescued when mixed with healthy tra+ donor cells, but not when mixed with healthy tra- donors. This result suggests OM exchange can rejuvenate a sub-population that contains a defective cell envelope by exchanging corresponding wild-type components from healthy cells. Although Tra-dependent interactions can lead to beneficial outcomes, in other cases they led to antagonism. The antagonistic interactions were triggered by disparate gliding motility phenotypes among sibling strains. Thus mixtures of nonmotile mutants with motile strains resulted in the latter strain being inhibited for motility and subsequently killed. In summary, OM exchange represents a new paradigm for how bacterial cells communicate and interact. These interactions are complex; in some cases they are constructive and lead functional communities or tissues. In other cases Tra-dependent interactions are antagonistic, suggesting populations regulate or 'police' their behaviors.

S3:2

INTERCELLULAR SIGNALLING USING EXTRACELLULAR DNA FACILITATES ACTIVE BIOFILM EXPANSION

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Introduction: Twitching motility-mediated biofilm expansion is a complex, multicellular behavior that enables the active colonisation of surfaces by many species of bacteria. Communication between individual bacteria must occur for successful active biofilm development. Both active biofilms and hydrated sessile biofilms of many bacterial species have been shown to contain complex polysaccharide matrix components including DNA. The role of extracellular DNA (eDNA) in early hydrated biofilms and active biofilm expansion is currently unclear. **Aim:** In this study we aim to explore the role of eDNA in actively expanding biofilms of Pseudomonas aeruginosa. **Methods:** We have used high resolution phase contrast time-lapse microscopy and developed sophisticated computer vision algorithms to track and analyse individual cell movements during expansion of P. aeruginosa biofilms in the presences and absence of eDNA. **Results:** Our analyses reveal that at the leading edge of the actively expanding biofilm, highly coherent groups of bacteria migrate across the surface of the semi-solid media. This leads to the emergence of a network of trails that guide the mass transit of cells toward the leading edges of the biofilm. We have determined that eDNA facilitates efficient traffic flow throughout the trail network by maintaining coherent cell alignments between neighbouring cells, thereby avoiding traffic jams and ensuring an efficient supply of cells to the migrating front. Our analyses reveal that eDNA also coordinates the movements of cells in the leading edge vanguard rafts and is required for the assembly of cells into the "bulldozer" aggregates that forge the interconnecting trails. **Conclusions:** Our observations have revealed that large-scale self- organization of multicellular communities occurs through construction of an intricate network of trails that is facilitated by eDNA. This reveals a new form of intercellular communication by bacteria using eDNA as a passive physical signal.

S3:3

CHARACTERIZATION OF QUORUM SENSING-REGULATED GAS VESICLE MORPHOGENESIS IN ENTEROBACTERIA

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Gas vesicles (GVs) are hollow, proteinaceous intracellular organelles produced by some bacteria and archaea that facilitate flotation because GVs are permeable to gas but not to liquid. Strains capable of producing GVs can use controlled production to manage vertical movement in an aquatic environment, presumably providing an adaptive competitive advantage for colonization of zones in a water column. Recently, it was discovered that GVs are produced in *Serratia* sp. ATCC39006 (S39006) - the first time such organelles were observed, naturally, in any enterobacterium. The production of GVs in S39006 is positively regulated in a density-dependent manner by a LuxIR-type quorum sensing system: SmaIR. SmaI produces *N*-butanoyl-L-homoserine lactone (BHL) and, at high cell densities, BHL derepresses the transcriptional regulator SmaR, leading to GV biogenesis. GV production in S39006 is also regulated by the small RNA-binding protein RsmA, and production increases in oxygen-depleted environments, suggesting that GVs promote and maintain the presence of S39006 at air-liquid surfaces. The 39006 GV cluster can be heterologously expressed in *Escherichia coli* to engineer floating recombinants. We have attempted to physically characterize GVs in S39006 to understand more about their development and their role in the cell. GVs are sensitive to external pressure and can have different structural robustness. Using pressure nephelometry, the collapse pressure of GVs within S39006 has been determined and the protein GvpC has been shown to enhance structural rigidity. GVs produced heterologously in *E. coli* display the same collapse pressure as those formed in S39006, suggesting that they are physically similar. We have used pressure nephelometry to calculate the instantaneous turgor pressure within both S39006 and *E. coli*. Finally, we have demonstrated that GVs can make up ~20% of the cell volume in S39006 and ~50% of the volume of an *E. coli* cell expressing the S39006 GV cluster. This suggests that, while GVs within S39006 and *E. coli* appear to be structurally similar, production of GVs may be "deregulated" in the synthetically engineered host where organelle morphogenesis may be liberated from the natural physiological control signals (such as quorum sensing or oxygen status sensing) operating in the cognate bacterium.

S3:4

JEKYLL AND HYDE: MICROBIAL METABOLITES THAT BOTH KILL AND STIMULATE BIOFILM FORMATION

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In nature, bacteria are rarely found in isolation; they are most often surrounded by other microorganisms. We are interested in how microbes in these complex communities alter their physiology and development in response to metabolites secreted by their microbial neighbors. To explore these interspecies interactions, we focus on the ability of *Bacillus subtilis* to respond to bacteria from its natural environment, the soil. *B. subtilis* is ideal for these studies because of its ability to differentiate into a variety of distinct cell types that can be monitored using fluorescent reporters. Using a co-culture screen, we previously identified numerous soil microbes secreting compounds that affect the differentiation of *B. subtilis* into biofilm-forming cells. One of the microbes that activated *B. subtilis* biofilm gene expression was *Bacillus cereus*. Using imaging mass spectrometry, we identified the thiocillins, a group of thiazolyl peptide antibiotics, as potential

biofilm-inducing compounds produced by *B. cereus*. Using bioassays and flow cytometry, we confirmed that purified thiocillin increased the biofilm-expressing population of *B. subtilis* cells. We also showed that biofilm-induction appears to be a general phenomenon common to a variety of structurally diverse thiazolyl peptides, and that biosynthesis genes encoding putative thiazolyl peptides were present in many bacterial species' genomes. Notably, although multiple alterations to the backbone of thiocillin abolished its ability to induce biofilm gene expression in *B. subtilis*, a mutation that eliminated its antibiotic activity did not. The antibiotic activity and biofilm-induction activity of thiocillin are therefore independent of one another. Our results thus indicate that thiocillin possesses dual bioactivities: acting not only as a killing agent, but also as a specific modulator of microbial cellular phenotypes. Future work is needed to determine whether other microbial metabolites initially identified as antibiotics might similarly possess dual bioactivities.

S3:5

KIN-SPECIFIC BINDING BETWEEN TWO PROTEINS CORRELATES WITH IN VIVO SELF IDENTITY AND WILD-TYPE SWARM EXPANSION IN PROTEUS MIRABILIS

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The ability to distinguish kin from others underlies many group behaviors, including territoriality. For the bacterium *Proteus mirabilis*, where self versus non-self recognition is exhibited through the physical exclusion of one strain from surfaces occupied by another, the Ids system contributes to this behavior and is associated with a type VI secretion (T6S) system. We have previously shown that of the *ids* genes, *idsD* and *idsE* encode strain-specific identifiers; however, it was unknown if and how the encoded proteins interact to define strain identity. Here we present evidence that *idsD* and *idsE* encode a T6S-associated protein pair that together determine identity and influence motility, and unlike many T6S-associated proteins, do not impact cell viability. IdsD and IdsE bind each other, and this interaction appears to be allele-specific, chiefly limited to the variants originating from the same strain. The specificity for binding between these proteins is primarily encoded in a stretch of distinctive amino acids, i.e. a variable region, within each protein. Exchange of these sequences to those of another strain changes the binding specificity *in vitro*. Moreover, the *in vitro* strain-specific binding interactions between these two proteins strongly correlate with *in vivo* self identity and wild-type swarm expansion. Lack of binding *in vitro* corresponded to atrophied swarm expansion. These two self-identity proteins, IdsD and IdsE, are examples of T6S-associated proteins that cause a behavioral change (i.e., altered swarm expansion) within a clonal population that is not associated with cell death. Moreover, the communication of identity among cells likely via the Ids system indicates that self-recognition events between kin can influence not only boundary formation, but also group motility.

S3:6

STAPHYLOCOCCUS AUREUS ENHANCE GROWTH AND ANTIBIOTIC TOLERANCE IN A HUMAN HOST ADAPTED PSEUDOMONAS AERUGINOSA LINEAGE

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Interactions among members of polymicrobial infections can result in altered pathogen behaviours such as enhanced virulence, biofilm formation or antibiotic tolerance, which may influence the disease phenotype and clinical outcome of the infection. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are important opportunistic human pathogens and are both part of the polymicrobial infection communities in human hosts. Previous studies

have revealed that cell-cell communication can play a major role in the interaction behaviors between these bacteria and subsequently can affect the behavior of the individual strains. However, the extent to which evolutionary processes may remodel interspecies interactions and cell-cell communication during the course of infection and therapy is currently not understood. Therefore, the aim of this study was to analyze the *in vitro* interaction between a community-associated methicillin-resistant *S. aureus* (CA-MRSA) and a collection of *P. aeruginosa* isolates representing different evolutionary steps of a dominant lineage, DK2, that have evolved through decades of growth in chronically infected patients. Although, the early-adapted *P. aeruginosa* DK2 strains outcompeted *S. aureus*, we found that the majority of the *P. aeruginosa* DK2 strain collection showed a mutualistic interaction with *S. aureus* during co-culturing on agar plates, where the cell density of *P. aeruginosa* was increased in the presence of *S. aureus*. Furthermore, *S. aureus* derived extracellular compounds were found to suppress *P. aeruginos*a autolysis, and to protect *P. aeruginosa* from being killed by clinically relevant antibiotics most likely through a selection for small-colony variants (SCVs). The *S. aureus* derived extracellular compounds were proteins controlled by the virulence regulators, SarA and *agr*, as well as by the proteolytic subunit, Clp, of the Clp protease. Our results suggest that *P. aeruginosa* host adaptation may be accompanied with remodeling of interspecies interactions, which further can affect bacterial signaling and development.

S4:1

THE CURIOUS CASE OF DEATH BY SEDUCTION: HOW THE SIREN SONG OF COMMENSAL E. FAECALIS DROVE VRE TO SUICIDE

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Enterococci are highly adapted members of the gastrointestinal tract consortia of many animals, from man to insects. It is very likely that they were members of the commensal consortia of the last common ancestor of terrestrial life, which existed about 450 MYA, and may go back further. However, in the antibiotic era, strains of enterococci have evolved into leading causes of multidrug resistant hospital acquired infection. One of our main research goals is to understand how human use of antibiotics has led to the changes in enterococci that have allowed them to become leading agents of hospital acquired infection. Comparative genomics is showing us that enterococcal adaptation to the hospital environment has involved the loss of mechanisms for protecting the fidelity of its genome, such as the CRISPR-cas system, and the concomitant acquisition of new traits, including antibiotic resistances and pathogenic traits, as well as phages, on mobile genetic elements. This has occurred to the extent that the genomes of many multidrug resistant hospital isolates are now more than 25% larger than commensal counterparts. Because of the large accumulation of mobile elements in these strains, it was of interest to know to what extent these hospital strains were capable of competing with commensal strains in the absence of antibiotic pressure. These studies showed that a prototype VRE *E. faecalis* strain could no longer compete in a healthy GI tract consortium, and in fact they were unexpectedly observed to be killed by it. The mechanism of killing was found to require the presence of commensal *E. faecalis*; and commensal *E. faecalis* were found to be capable of effecting this killing in pure culture. The mechanism of killing was further found to depend on the production of a pheromone signal by the commensal *E. faecalis*, which induced the suicidal death of the VRE. That suicide required the presence in the VRE of an integrated genetic element in its chromosome. This result shows that this VRE strain cannot co-exist with commensal enterococci, meaning that in the patient, either the VRE colonize a distinct microniche, or that they can only colonize patients in whom the

commensal enterococci have been eliminated altogether. This work highlights the importance of maintaining healthy commensal flora during antibiotic therapy, as well as new strategies for the elimination of this multidrug resistant hospital pathogen.
Portions of this work were conducted by: Marcus Rauch, Lynn Hancock, Matthew Ramsey, Paul Himes, Sriram Varahan, and Francois Lebreton.

S4:2

BIOLUMINESCENCE BASED BIOSENSORS FOR THE DETECTION OF PEPTIDE PHEROMONE PRODUCTION AND CELL-CELL COMMUNICATION IN ENTEROCOCCUS FAECALIS

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Background: Once considered a harmless commensal of the gastrointestinal tract, Enterococcus faecalis has become over the past three decades a challenging nosocomial problem due to the emergence of multi-antibiotic resistant isolates refractory to therapies and equipped with a plethora of pathogenicity determinants. The bacterium uses quorum-sensing systems to regulate physiological processes among which the expression of virulence traits to adapt and proliferate in the host. Aim. This study was carried out to develop two bioluminescence based reporter systems for the direct detection of the quorum-sensing regulated gelatinase biosynthesis activating pheromone (GBAP) and cytolysin small subunit (CylLS) in natural isolates. **Methods:** The two biosensors were constructed by cloning the cytolysin promoter and the CylLS responsive regulatory genes (Pcyl-cylR1R2) or the gelatinase promoter (PgelE) into a luxABCDE-containing vector. The resulting plasmids, pSL101cylR2R1Pcyl and pSL101PgelE were introduced in the Cyl- strain E. faecalis JH2-2 and the Gel- strain V583fsrB*, respectively. **Results:** E. faecalis cells containing the constructs conditionally expressed bioluminescence in the presence of GBAP and CylLS both in the supernatant of liquid cultures and in agar overlay assay in as little as three hours with high sensitivity. The biosensors were employed to investigate interaction between the fsr and cyl systems, revealing that fsr antagonizes CylLS activity by 75%. This effect towards cytolysin production was mediated by the metalloprotease GelE, presumably by degradation of CylLS. Furthermore, we show that the clinical isolate E. faecalis T2 acts as a biological cheater producing cytolysin only upon sensing CylLS producers in its environment. This isolate was thus able to enhance its virulence during polymicrobial systemic infection in the Galleria mellonella model. **Conclusions:** The reporter systems presented here are the first example of a bioluminescence-based screening method applied to the detection of cell-cell communication and virulence determinants in E. faecalis. These biosensors will be a very useful means to study the complex mechanisms underlying virulence in this prominent nosocomial pathogen.

S4:3

UNDERSTANDING THE ROLE OF DIFFUSIBLE SIGNALLING FACTOR (DSF) IN VIRULENCE OF XANTHOMONAS PLANT PATHOGENS

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Xanthomonas oryzae pv. *oryzae* (*Xoo*) and *Xanthomonas oryzae* pv. *oyzicola* (*Xoc*) are important members of the genus *Xanthomonas* which causes serious disease of rice. In *Xanthomonas* group of plant pathogens, a secreted fatty acid signalling molecule known as Diffusible Signalling Factor (DSF) regulates diverse virulence associated functions. Our studies have shown that DSF regulates virulence associated traits in an atypical fashion in rice pathogen *Xoo*. In *Xoo*, DSF promotes biofilm formation and regulates transition from planktonic to biofilm lifestyle. In *Xoc*, DSF

deficient mutants exhibit reduced virulence and *in planta* growth deficiency. To understand how DSF promotes *in planta* growth, we characterized the DSF deficient mutant of *Xoc*. Evidence will be provided which indicates that DSF in *Xoc* promotes virulence by regulating ferric iron uptake. We further show that ferric uptake is critical for virulence in *Xoc*. This is in contrast to *Xoo*, which is a vascular pathogen wherein, ferrous uptake system contributes to virulence. Our study revealed that closely related rice pathogens (*Xoo* and *Xoc*) fine tunes DSF mediated signalling to utilize alternate source of iron to suite their different lifestyle.

S4:4

INTEGRATION OF MULTIPLE SENSORY INPUTS IN THE VIBRIO CHOLERAE QUORUM-SENSING CIRCUIT AND THE IMPACT ON VIRULENCE AND SYSTEM ROBUSTNESS

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Bacteria use quorum sensing (QS) for cell-cell communication to carry out robust group behaviors. This inter-cellular signaling process relies on cell-density dependent production and detection of chemical signals, called autoinducers (AIs). *Vibrio cholerae*, the causative agent of cholera, controls biofilm formation in the environment and virulence factor production in the host via QS. Two QS sensor histidine kinases (HKs), CqsS and LuxQ, have been previously characterized and they detect two distinct AIs, CAI-1 and AI-2, respectively. These two receptors function in parallel to activate the key regulator of the *V. cholerae* QS response, LuxO, which is essential for virulence. Surprisingly, mutants lacking CqsS and LuxQ display a normal cell-density dependent QS response and remain virulent *in vivo*, suggesting that LuxO is activated by additional unidentified signaling pathways. We show that similarly to CqsS and LuxQ, two other HKs, VC1831 and VpsS, act upstream in the central QS circuit of *V. cholerae* to activate LuxO. *V. cholerae* strains expressing only one of these four receptors are QS proficient, displaying LuxO-dependent gene regulation with distinct temporal dynamics. Moreover, these single-receptor-expressing mutants are capable of colonizing animal hosts as well as the wild type. In contrast, mutants lacking all four receptors are phenotypically identical to LuxO-defective mutants both *in vitro* and *in vivo*. We suggest that these four parallel signaling systems integrate into the *V. cholerae* QS circuit to facilitate temporal coordination of multiple sensory inputs and modulate QS-dependent gene expression. We hypothesize that the presence of multiple functionally redundant receptors controlling a single common regulator increases the overall robustness of the system. To test this, we studied how LuxO-dependent gene expression is affected by exogenously adding a surplus amount of CAI-1, which inhibits LuxO activation by CqsS. We found that the overall QS response is unaffected by excess CAI-1 in wild type *V. cholerae* or in mutants missing single receptors LuxQ, VC1831, or VpsS. In contrast, the QS response is significantly induced by CAI-1 in a triple receptor mutant lacking LuxQ, VC1831, and VpsS. Therefore, by integrating multiple sensory inputs, the *V. cholerae* QS circuit has evolved to be refractory to sporadic fluctuation in the external environment, thus allowing robust and synchronous expression of QS-dependent genes.

S4:5

UNRAVELLING THE INTERPLAY BETWEEN YERSINIA AND CAENORHABDITIS ELEGANS DURING QUORUM SENSING-DEPENDENT IN VIVO BIOFILM FORMATION

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The formation of an incapacitating biofilm on Caenorhabditis elegans by Yersinia pseudotuberculosis that blocks nematode feeding, represents

a tractable model for investigating the genetic basis for host-pathogen interplay during the biofilm-mediated infection of a living surface. N-Acylhomoserine lactone-dependent quorum sensing (QS) via two pairs of LuxRI orthologues (YpsR/I and YtbR/I) and multiple AHLs plays a critical role in this process. Y. pseudotuberculosis strains expressing an AHL-degrading enzyme or in which the AHL synthase (ypsI and ytbI) or response regulator (ypsR and ytbR) genes have been mutated, are attenuated for biofilm formation. To gain more detailed information on the response of C.elegans to the presence of Yersinia biofilms and on the Yersinia QS regulatory hierarchy involved transcriptomic, reporter gene fusion and promoter pull down assays were employed in conjunction with electron and fluorescence microscopy. For C. elegans, infection with Y.pseudotuberculosis resulted in the differential regulation of numerous genes, including a distinct subset of nematode C-lectin and fatty acid desaturase genes, the mutation of which resulted in the abrogation of biofilm formation. For Yersinia, we have discovered that the QS-regulatory hierarchy is itself controlled via the sensing of N-acetyl glucosamine while in turn regulating the flagellar regulatory cascade in a pathway involving the histidine utilization (hut) genes such that QS, flagellar pathway, hut and nag mutants all fail to form biofilms on C.elegans. Collectively this work indicates that biofilm formation by Y. pseudotuberculosis on C. elegans is a sophisticated interactive process during which the initial attachment/recognition of Yersinia to/by C. elegans is followed by biofilm development during which QS plays a central role.

S4:6

RESOLVING THE CONTRIBUTION OF QUORUM SENSING AND TWITCHING MOTILITY IN THE SYSTEMIC SPREAD OF PSEUDOMONAS AERUGINOSA IN BURN WOUND INFECTIONS

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Thermal injuries represent a very unique type of physical trauma that are highly associated with the development of bacterial sepsis. *Pseudomonas aeruginosa* is a gram-negative, opportunistic pathogen that is exceptionally well adapted at establishing systemic infections in thermally-injured patients. Previous studies have described the importance of the bacterial communication conduit, quorum sensing (QS), in *P. aeruginosa* burn wound infections. These studies largely evaluated the QS mutant JP2, which is deficient in the LasI and RhlI QS systems. This QS-deficient mutant was much less virulent in thermally-injured mice compared to burn mice infected with the wild-type strain, PAO1. Additionally, numbers of bacteria recovered at distal locations within the burn wound and from the spleen were significantly lower in JP2-infected burn mice compared to those infected with PAO1. However, later characterization of JP2 revealed that, in addition to defective QS, JP2 also exhibits defective twitching motility, which is unrelated to the *lasI* and *rhlI* mutations. Given the importance of twitching motility in bacterial translocation, biofilm formation, and chemotaxis, it is necessary to reevaluate the independent contributions of QS and twitching motility to the virulence of *P. aeruginosa* in burn wound infections. Here, using a QS mutant with fully intact twitching motility, JM2, we report and compare the importance of QS and twitching motility in the local and systemic spread of *P. aeruginosa* in burn wound infections. *In vivo* examination revealed that thermally-injured mice infected with either JM2 or JP2 exhibited delayed mortality compared to burn mice infected with PAO1, supporting the previous findings stressing the importance of QS in thermal infections and that twitching motility is not necessary for this organism to cause systemic infections. These results, in combination with the ongoing evaluation of bacterial chemotaxis and dissemination of JP2 and JM2 in thermally-injured mice, should help us understand the individual and combined effects of QS and twitching motility on the systemic spread of *P. aeruginosa* in burn wounds.

S4:7

THE PMRB HISTIDINE KINASE CONTROLS EXPRESSION OF THE QSEBC TWO-COMPONENT SYSTEM VIA A COGNATE AND A NON-COGNATE RESPONSE REGULATOR: CROSS-REGULATION OR A FOUR-COMPONENT SYSTEM?

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Although bacterial two-component systems are largely isolated from each other to avoid signal confusion, some flexibility must exist to allow bacteria to respond to thousands of signals, using only the finite number of two-component systems harbored in their genomes. PmrAB and QseBC are two signaling modules that are conserved in many Gram-negative bacteria. PmrAB responds to ferric iron and other signals and directs the expression of genes that are important for LPS modification. QseBC has been reported to respond to epinephrine and norepinephrine and deletion studies have implicated QseBC in the virulence of several pathogens. Uropathogenic E. coli (UPEC) mutants deleted for the QseC sensor are drastically attenuated in murine models of urinary tract infection and this attenuation stems from the constitutive phosphorylation of the QseB response regulator by the PmrB histidine kinase. PmrB phosphorylates the non-cognate QseB at rates that are comparable to QseC-mediated phosphotransfer, but unlike QseC the phosphatase kinetics of PmrB towards QseB are slow. Loss of QseC also leads to the de-regulation of known PmrA-regulated targets. We thus hypothesized that PmrAB and QseBC are physiologically connected. Stimulation of wild-type UPEC with ferric iron, but not epinephrine, induced the expression of qseBC and this signal-dependent activation required both QseB and PmrA. Biochemical and in vivo analyses revealed that QseB and PmrA directly control transcription of the qseBC operon. While QseB activates qseBC expression, PmrA exerts a positive or negative effect on qseBC transcription depending on its concentration relative to QseB. When PmrA and QseB are in a 1:1 or 2:1 ratio, PmrA assists QseB binding to the qseBC promoter and serves as a transcription enhancer. When PmrA is found in higher concentrations in the cell it occludes QseB binding and inhibits qseBC expression. Parallel mutagenesis studies revealed that abrogating the kinase activity of the QseC sensor did not alter its ability to rescue the qseC deletion phenotype. Thus, while the QseC phosphatase function is crucial for regulating QseB activity, the kinase function of QseC is dispensable, possibly due to the overlapping kinase activity of PmrB. Taken together, our findings show that in UPEC QseBC and PmrAB are physiologically connected via non-partner interactions that impact target gene expression. We are now investigating the global effects of QseBC-PmrAB interactions in response to signals.

S5:1

OPENING LINES OF COMMUNICATION: QUORUM SENSING AT THE INTERSECTION OF SYNTHETIC BIOLOGY AND MICROELECTRONICS

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Synthetic biology provides a means for articulating concepts into new products and products. Its toolbox is extensive, including the ability to create synthetic genomes and tailor their regulation. Early successes augmented the cell's biosynthetic capacity and rewired its regulation, transforming our ability to produce products ranging from small molecules to fully functional therapeutic proteins at high yield. Also, the theoretical formalisms of metabolic engineering provided a basis for optimally routing its biochemical flux. These activities focused largely on the cell's intracellular biochemical network and relied less on molecular cues from its immediate surroundings. The emergence of quorum sensing (QS) as a model for signal transduction has enabled a

reexamination of metabolic flux and regulation by hardwiring population-scale biological function to extracellular cues. Regulatory and fabrication modules are feasible, owing to a few relatively simple QS signal transduction cascades. QS provides a context for entirely new products and processes that consider the individual or small populations of cells. E. coli were engineered to swim towards and interrogate receptor density on nearby cancer cells, and based on threshold levels, induce heterologous protein synthesis. Indeed, new opportunities are emerging by understanding and manipulating such communication networks that exist between cells, however there are limited means for actuating and control. By connecting biological systems and their molecular based communication networks to those of microfabricated devices, new synergies are envisioned. We are developing methods to interrogate QS and other biological signaling phenomena by connecting to microfabricated devices enabling bi-directional communication and control. Some examples will be discussed, including a new redox capacitor that enables storage and readout of bioelectric functions.

S5:2

A SYSTEMS ANALYSIS OF DUAL SIGNALING CONTROL OF CONJUGATIVE DRUG RESISTANCE TRANSFER IN ENTEROCOCCUS FAECALIS

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Enterococcus faecalis, a major causative agent of hospital-acquired infections, is resistant to many known antibiotics. Its ability to laterally transfer conjugative plasmids encoding antibiotic resistance and virulence determinants to other pathogens in the environment poses a serious therapeutic concern. Two antagonistic signaling peptides controls the transfer of tetracycline-resistance plasmid pCF10: a peptide pheromone produced by plasmid-free recipient cells triggers the conjugative transfer in plasmid-containing donors; and an inhibitor peptide encoded in the plasmid and produced by donor cells serves to modulate donor response in accordance with the relative abundance of donors and recipients. The dual signaling system allows the donor to calibrate its response to the probability of successful transfer and economize its resources. A multi-scale mathematical model was developed to include intracellular molecular mechanisms as well as population level interactions between donors and recipients. Kinetic parameters for the model were estimated from literature values and augmented by RNA-Seq data and binding constant measurements using surface plasmon resonance and isothermal titration calorimetry. The model was further verified by comparing the model-simulated dynamic response to experimentally measured transcript profiles. While a calibrated response by donor cells can avoid wasteful induction of conjugative transfer system, a delay of transfer may imperil cell survival under some conditions. We hypothesize that a mechanism that allows for heterogeneous response by donor cells will safeguard against such peril. Simulation of the induced expression profiles using the model suggested plasmid copy number distribution in donor cells and stochastic behavior as possible sources of the heterogeneity in donor response to the dual signals. We are currently using newly developed fluorescent reporter fusion constructs to further examine and refine the model.

S5:3

A MODULAR VIEW ON CELL DENSITY ENCODING SCHEMES IN BACTERIAL QUORUM SENSING

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Nature has evolved and exploits a rich spectrum of architecturally diverse quorum sensing systems across and even within species. This

diversity comprises both the way how cell density information is encoded into a concentration of signaling molecules as well as how this information is decoded into a cellular response. Here we focus on the encoding process and investigate theoretically how differences in signal transport, signal modification and site of signal detection shape the encoding function and affect the sensitivity and the noise characteristics of the cell-density-encoding process. We find that different network modules are capable of implementing both fairly basic as well as more complex encoding schemes, whose qualitative characteristics vary with cell density and are linked to network architecture, providing the basis for a hierarchical classification scheme. We exploit the tight relationship between encoding behavior and network architecture to constrain the network topology of partially characterized natural systems, and verify one such prediction by showing experimentally that Vibrio harveyi is capable of importing Autoinducer 2. The framework developed here may serve to guide the reverse engineering of natural systems and generally facilitates a better understanding of the complexities arising in the quorum-sensing process due to variations in the physical organization of the encoder network module.

S5:4

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S5:5

ELECTROCHEMICAL QUANTIFICATION OF SECONDARY METABOLITES PRODUCED BY 3D-PRINTED BACTERIAL AGGREGATES

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Bacteria are regularly found in nature as dense aggregates of ~10^1-10^5 cells. These communities are highly organized, and the spatial configuration within a cluster and between neighboring microcolonies can influence how cells sense small molecules to interact with the environment and each other. Although it is clear that spatial relationships may impact how microbes perceive their surroundings, studying the molecular processes that govern these interactions in a relevant context to nature remains challenging due to the technical difficulties associated with manipulating small populations. Micro-3D printing is a lithographic technique capable of arranging bacteria *in situ* by printing protein-based walls around individual cells or small populations. This 3D-printing strategy can organize bacteria in complex arrangements to investigate how spatial and environmental parameters influence social behaviors within small populations. Here, we introduce a combined approach, where micro-3D printing and scanning electrochemical microscopy (SECM) are coupled to create a quantitative spatial map of pyocyanin, a quorum sensing (QS)-regulated redox-active metabolite produced by *Pseudomonas aeruginosa*, in real time within groups of $<10^5$ cells. We describe the process of merging these technologies for the specific application of measuring pyocyanin production as a proxy for QS activity within *P. aeruginosa* aggregates. We show that QS occurs within clusters as small as 500 cells, and characterize the requirements for neighboring communities to sense and respond to one another. These studies establish the value of this novel approach for determining how spatial parameters influence bacteria.

S5:6

QUORUM SENSING OF PSEUDOMONAS PUTIDA ISOF

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Quorum sensing (QS) regulates cooperative behaviour in a number of bacterial species. It is increasingly recognized that environmental conditions influence quorum sensing systems in many species. Some experimental setups,

e.g. batch cultures, inherently cause changes of the environmental conditions as depletion of nutrients and accumulation of waste products. Alternatively, approaches based on chemostats or microfluidic devices have been used, the latter focussing on attached, growth of attached colonies instead of plankton. A comparison of the outcome of such different approaches will support the understanding of purpose and functionality of the signalling. We thus conducted a series of studies investigating the dynamics of acyl homoserine lactone signal production and degradation in *Pseudomonas putida* IsoF. The main hypothesis was that the QS system works differently under changing environmental conditions resp. life styles (plankton versus biofilm). a) The bacteria were grown in batch cultures until their entry into the stationary phase. b) The behaviour under more continuous conditions was investigated under chemostat conditions using different dilution rates. Time courses of cell density and concentrations of acyl homoserine lactones resp. their degradation products are analysed both in chemostat and batch culture experiments. c) Induction behaviour of the QS system in cells growing in surface-attached microcolonies was analysed under flow chamber conditions. The dynamics of QS controlled GFP fluorescence in the cells resp colonies was measured. The values of quorum sensing parameters were estimated by fitting the data to mathematical models. The planktonic studies a) and b) revealed a surprising constancy with respect to quorum sensing parameters, i.e. non-induced and induced signal production rates per cell, and signal threshold. Modelling indicated that the determined parameters values also fit to the induction dynamics in microcolonies in study c). Thus, the QS system is rather robust to changing environments, at least in the investigated range. Interestingly, the flow chamber experiments indicated that communication is focussed rather within colonies than between colonies, raising the question to which degree all cells in planktonic populations communicate with each other.

S6:1

SYPF, AN UNUSUAL TWO-COMPONENT REGULATOR OF BIOFILM FORMATION AND COLONIZATION

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Biofilm formation and colonization by *Vibrio fischeri* depends on the expression of the *syp* (symbiosis polysaccharide) locus, a set of 18 genes that encode proteins involved in synthesizing, modifying, and exporting the polysaccharide component of the matrix. A complex phosphorelay comprised of multiple two-component regulators dictates whether biofilm formation occurs. In culture, biofilm formation depends on overexpression of one or another of these regulators. Specifically, the unlinked sensor kinase RscS induces *syp* transcription in a mechanism that depends on the presence of SypG, the response regulator that serves as the direct transcriptional activator. Data to date suggest that RscS activates SypG and inactivates SypE, a second response regulator whose default activity is to inhibit biofilm formation at a level below *syp* transcription. Alternatively, biofilm formation can be induced by overexpression of SypF*, a mutant version of the hybrid sensor kinase SypF with increased activity. Here, we probed the specific role of SypF in biofilm formation. We found that the ability of RscS to induce biofilm formation depends upon the presence of SypF, suggesting that SypF functions at or below RscS. Indeed, our data indicate that SypF functions between RscS and the two response regulators, SypG and SypE, to promote biofilm formation. Although wild-type SypF can function as a canonical hybrid sensor kinase *in vitro*, the first two of its three conserved sites of phosphorylation are not required for the ability of SypF to promote RscS-induced biofilm formation. Instead, only the C-terminal HPt domain of SypF is crucial for this phenotype, a result that suggests that the two sensor kinase proteins

participate in an atypical phosphotranfer reaction. To determine the physiological relevance of these findings, we assessed the role of SypF in host colonization, which does not require overexpression of regulators. Deletion of the *sypF* gene severely impaired colonization, but this defect could be complemented by a wild-type copy of the *sypF* gene present in single copy in the chromosome. The colonization defect could also be complemented by a *sypF* derivative that expressed the HPt domain alone. Together, these findings indicate that RscS and SypF interact in an atypical way to control biofilm formation and host colonization by *V. fischeri*.

S6:2

INTERACTION BETWEEN PSEUDOMONAS FLUORESCENS PF0-1 AND PEDOBACTER SP. V48 INDUCES SOCIAL MOTILITY

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Soil bacteria live in multispecies communities in which interactions influence physiology and behavior. Here we describe "social motility" across a hard surface, a phenotype which is apparent during interaction between Pseudomonas fluorescens Pf0-1 and Pedobacter sp. V48, but not displayed by either bacterium alone. Social motility is observable only when the bacteria are grown close together, suggesting a contact-dependent cell-cell interaction is necessary. Transcriptome analysis reveals numerous gene expression changes in both species during interaction. The social motility represents a convenient phenotype with which to dissect the basis of interaction between P. fluorescens and Pedobacter. We have used reciprocal transposon mutagenesis screens and experimental evolution to explore the mechanistic basis of social motility and the contact-dependent interaction which drives it. Transposon mutagenesis has yielded three classes of mutants: non-motile, slow moving, and fast moving when interacting with the wild-type partner. In P. fluorescens, we isolated five mutants which cause reduced social motility, two of which have insertions in genes associated with Type 6 Secretion. Among 10 mutants leading to increased social motility, seven are required for flagellum or pilus assembly. Of note in Pedobacter, two null mutants have insertions in predicted polyketide synthase genes, while six fast mutants have insertions in genes for polysaccharide or LPS production. Experimental evolution has yielded spontaneous mutants in sectors migrating rapidly from the main mixed colony. Evolved Pedobacter and P. fluorescens strains conferring increased social motility have growth defects in monoculture, suggesting a fitness tradeoff. Phenotypes of evolved Pedobacter strains include increased mucoidy and production of a putative surfactant. Mucoid strains have point mutations in the same polysaccharide locus identified by transposon mutagenesis. The isolation of transposon and spontaneous mutants of both species which impact social motility indicates both species contribute to the trait, and coupled with transcriptome data, suggests a two-way communication which is partially dependent on a P. fluorescens T6SS. Our data suggest that a signal from P. fluorescens triggers production of a polysaccharide and a surfactant which both contribute to social motility, but the mechanism by which Pedobacter influences P. fluorescens remains unknown.

S6:3

MANIPULATING THE INTERSPECIES QUORUM SENSING SIGNAL AI-2 IN THE MOUSE GUT

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The mammalian gastrointestinal tract harbours a diverse and complex resident bacterial

community, called the commensal microbiota, which interacts with the host and have been implicated in many physiological processes of great importance to host health. The ability of bacteria to communicate within and between species through small signalling molecules to regulate behaviours at the community level is well-established. We hypothesise that such communication, known as quorum sensing, plays a role in the inter-species interactions occurring in the establishment and maintenance of the gut microbiota. The quorum sensing signal autoinducer-2 (AI-2) is a strong candidate for this communication because it can mediate inter-species quorum sensing and is produced by many members of the commensal microbiota. We have previously shown that the enteric bacterium *Escherichia coli* has a mechanism to sequester and process AI-2 produced by a variety of species present in the environment and thus can influence AI-2-dependent bacterial behaviours in these neighbouring bacteria. We are using engineered *E. coli* mutants to manipulate AI-2 levels in the mouse gut and determine the effect of this manipulation within the antibiotic-treated microbiota. Our results showed that increasing AI-2 following antibiotic-induced gut dysbiosis had a measurable effect in the most abundant bacterial Phyla present in the mouse gut. These Phyla are known to be important for protection against pathogens, chemical agents, and inflammatory bowel diseases.

S6:4

A PLANT-RESPONSIVE LUXR HOMOLOG IN A COTTONWOOD TREE ROOT ENDOPHYTE

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We are interested in the root microbiome of the fast-growing Eastern cottonwood tree, *Populus deltoides*. We have shown that quorum sensing signal synthase (*luxI* homologs) and receptor (*luxR* homologs) genes are prevalent in Proteobacteria isolated from *Populus* roots. Interestingly, many of these isolates encode an orphan or solo LuxR homolog, which is closely related to OryR from the rice pathogen *Xanthomonas oryzae*. OryR does not respond to acyl-homoserine lactone quorum sensing signals, instead it detects an unknown plant compound. We discovered an OryR homolog in an endophyte isolated from *Populus* roots, *Pseudomonas sp.* GM79. We created a reporter that was responsive to the GM79 OryR homolog and used it to show that, like the *X. oryzae* OryR, its activity increased in the presence of plant leaf macerates, but it was not influenced by acyl-homoserine lactones. We examined the genomic region surrounding the GM79 plant-responsive *oryR* homolog and found genes annotated as peptidases and genes coding for a putative ABC-type peptide transporter. The *X. oryzae oryR* is also flanked by a peptidase gene and genes annotated as amino acid transporters. Strains with mutations in the coding for the putative peptidases showed increased responses to plant macerates. A strain with a mutation in a gene coding for the putative ABC-type peptide transporter did not respond to plant leaf macerates. We hypothesize that the plant signal(s) enters the bacterial cells by activate transport and that the peptidases affect the activity of the signal. We purified the two peptidases and demonstrated they are most active against N-terminal proline and alanine dipeptides, suggesting that the signal may be a proline and/or alanine-containing peptide. We have partially purified active signal(s) and the purified material can be partially inactivated by one of the peptidases. We believe that a better understanding of these OryR-type LuxR homologs is of general importance as they occur in dozens of bacterial species that are associated with economically important plants.

S7:1

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S7:2

THE EVOLUTIONARY ORIGINS OF QUORUM SENSING

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Quorum sensing (QS) communication in bacteria has been heavily researched owing to its important role in regulating virulence in many species. However, we know little regarding the evolutionary origins of QS systems: how does QS initially evolve and what did it evolve to regulate? The evolutionary emergence of signalling systems presents a chicken and egg problem as a functioning signalling system requires both the production of signals and response to the signals of others: why signal if no one can respond, and why respond if no one is signalling? One solution to this paradox is if secreted functional molecules first serve as cues for behavioural responses in others, and are then subsequently co-opted ('ritualised') as true signal molecules. We use a phylogenetic comparative analysis across the diversity of QS systems in bacteria to show that most quorum signals appear to have initially been extracellular public goods, suggesting that QS initially evolves by co-opting public goods to regulate the expression of other traits. Furthermore, using a combination of a phylogenetic comparative analysis and a meta-analysis of transcriptomic studies of diverse QS systems we show that the primary evolutionary function of QS is to regulate the production of secreted public goods. Overall, our analyses suggest that QS evolved via the co-opting of secreted public goods to regulate other secretions coded in the genome.

S7:3

THE EVOLUTION OF QUORUM SENSING IN A NATURALLY CO-EVOVLED HOST-PATHOGEN MODEL

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Bacteria can engage in multicellular behaviour via quorum sensing (QS), whereby bacteria monitor population density through the secretion of small, diffusible signal molecules. However, the evolutionary forces that maintain QS have rarely been investigated in naturally co-evolved host-pathogen systems. We investigated the evolutionary ecology of the PlcR-PapR QS system in Bacillus thuringiensis, in which the PlcR regulon controls the production of various extracellular proteins, often involved in virulence, in response to an autoinduced heptapeptide signal PapR. We tested the hypothesis that both signal production and PlcR regulated gene expression are social traits, and measured the invasion of isogenic mutants at varying pathogen doses and mutant frequencies. Productivity in host and infection success was positively correlated with the abundance of wild type in inocula. However, mutants could not outcompete wild type bacteria in vivo. Experiments with homogenized insects indicate that mutants can outcompete wild type bacteria in homogeneous environment. Microscopic observation of insect sections with fluorescent QS cells showed that, in the midgut, bacteria population was founded on isolated patches of 1 to 3 individual cells 24 hours post ingestion. However, a mixed population consisted of wild type and mutants was evident 48 hours post ingestion. The results suggested that spatial structure and population bottleneck imposed by the midgut barriers limited invasion of QS mutants. The results confirm that clinical interventions to

prevent QS might be beneficial, as diverse virulence factors are often regulated by QS. If QS regulated virulence factors are required for an essential part of infection, inhibiting these behaviours can be helpful.

S7:4

EXPLOITING COMBINATORIAL SIGNALLING - A NEW TYPE OF CHEATING STRATEGY IN PSEUDOMONAS AERUGINOSA

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The notion of exploiting sociality and social traits in bacteria has received wide attention in recent years in the context of evolutionary dynamics and understanding the evolution of virulence, and ultimately infection. A recurring question in the literature focuses around how do bacteria in natural environments maintain social traits when cheats have a higher relative fitness? One way to achieve this is to link the acquisition of private goods to the same system that produces public goods. We recently showed that the opportunistic pathogen Pseudomonas aeruginosa can sense and respond combinatorially and in some cases synergistically to two signal molecules. By producing and responding to multiple signals, bacteria can use quorum sensing (QS) to correctly determine a combination of population density and other environmental factors. Correctly responding to a particular environment allows bacteria to maximise their fitness whilst maintaing social traits. However this introduces an interesting conundrum that potentially allows bacteria to cheat while still producing a certain level of public goods. By losing the synergistic response to a combination of molecules, an individual would still be able to survive during the restrictions levied through private goods metabolism and would have a higher relative fitness during public goods production as they benefit from those in the population producing public goods at the higher synergistic level. We tested whether such signal synergy mutants arise during a long term selection experiment in which P. aeruginosa PAO1 was cycled between a private and public goods media. We found that individuals arose that lost the ability to respond synergistically to signal molecules, and we examined the relative fitness of these in competition experiments with the ancestor strain that maintains combinatorial signalling. Our work therefore shows that by exploiting combinatorial signalling, P. aeruginosa can overcome metabolic incentives to cooperate. More generally, we show that there are different types of cheating strategy, and we add to the growing body of evidence that a number of factors are required to work together to control social cheating in natural environments.

S7:5

FACULTATIVE CHEATING DRIVES THE MAINTENANCE OF QUORUM-SENSING PHEROTYPE DIVERSITY IN STRUCTURED POPULATIONS

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Bacteria utilize quorum-sensing (QS) to regulate the secretion of other molecules, which serve as public goods, benefiting all members of the community. In sufficiently well mixed conditions, such behavior is susceptible to invasion by QS reception “cheater” mutants that avoid the costs of quorum-response but benefit from the response of others. In wild isolates, QS reception mutants are rare, suggesting that either QS drives the production of private goods or that cooperation is maintained by the high relatedness of cooperating cells. In contrast, many bacterial species show high intra-specific allelic divergence of their QS locus, forming multiple pherotypes where a signal from one pherotype specifically activates its cognate receptor but not the receptor of another pherotype. Often, these pherotypes show a phylogenetic distribution indicating their horizontal transfer (HGT) within the species. It remains unclear what evolutionary forces select for pherotypes divergence, its

maintenance and its horizontal spread. Here we combine bioinformatics, mathematical modeling and experimentation to demonstrate that facultative cheating between pherotypes can select for rapid horizontal gene transfer even under conditions where relatedness between cells is high enough to prevent the spread of cheaters. We show that the *comQXP* QS system of *B. subtilis* has a cooperative function in swarming and that under these conditions, a minority pherotype invades into the majority pherotype. Importantly, we show that selection for the minority pherotype occurs also in spatially structured populations that prevent the invasion of receptor mutants. Our results therefore support a social role for QS and illustrate the complexity of evolutionary dynamics of bacterial social system.

Poster Abstracts

1

MECHANISM FOR THE SPECIFIC TARGETING OF METHYLTRANSFERASES TO CHEMORECEPTORS IN PSEUDMONAS AERUGINOSA PAO1

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Bacteria constantly sense and adapt to changing environmental conditions to assure survival. This important function is primarily mediated by one-component systems, two-component systems, and chemosensory pathways. Core proteins of chemosensory pathways are the CheA sensor kinase, CheW coupling protein, CheY response regulator, CheR methyltransferase, CheB methylesterase, and chemoreceptors. Methyltransferases of the CheR family and methylesterases of the CheB family control chemoreceptor methylation, and this dynamic posttranslational modification is necessary for proper chemotaxis of bacteria. Studies with enterobacteria that contain a single CheR and CheB show that, in addition to binding at the methylation site, some chemoreceptors bind CheR or CheB through additional high-affinity sites at distinct pentapeptide sequences in the chemoreceptors. Pseudomonas aeruginosa PAO1 has five gene clusters encoding chemosensory signaling proteins that assemble into four chemosensory pathways, termed Che, Che2, Wsp, and Chp. Apart from the 26 chemoreceptor genes of P. aeruginosa this strain has four CheR paralogous methyltransferases (CheR1, CheR2, CheR3, and WspC). Three of these chemoreceptors have C-terminal extensions with terminaal pentapeptides of sequence GWEEF, EVELF or GVEQA. We produced the four CheR methyltransferases of P. aeruginosa as purified recombinant proteins, and their functionality was validated using microcalorimetric titrations with the methylation susbrate SAM and the methylation product SAH (Garcia-Fontana, Corral Lugo, & Krell, 2014). The titration of the four CheR paralogues with any of the three above mentioned pentapeptides revealed a single interaction, namely that between CheR2 and the GWEEF pentapeptide, which was derived from the McpB chemoreceptor. Interestingly, the genes encoding CheR2 and McpB are vicinal on the genome and form both part of the che2 chemosensory pathway. This interaction was also detected for the titration of full-length McpB with CheR2. In vitro assay shows that McpB is exclusively methylated by CheR2. Deletion of the terminal pentapeptide from McpB abolished both, the interaction and methylation of McpB. When clustered according to protein sequence, bacterial CheR proteins form two distinct families-those that bind pentapeptide: containing chemoreceptors and those that do not. These two families are distinguished by an insertion of three amino acids in the β-subdomain of CheR. Deletion of this insertion in CheR2 prevented its interaction with and methylation of McpB. Data suggest that the CheR2-McpB interaction is a strict requirement for any methylation activity. Pentapeptide-containing chemoreceptors are common to many bacteria species; thus, these short, distinct motifs may enable the specific assembly of signaling complexes that mediate different responses.

2

EVOLUTION OF RESISTANCE TO A LAST-RESORT ANTIBIOTIC IN STAPHYLOCCOCUS AUREUS VIA BACTERIAL COMPETITION

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Antibiotic resistance is a key medical concern, with antibiotic use likely being an important cause. However, here we describe an alterna-

tive route to clinically-relevant antibiotic resistance that occurs solely due to competitive interactions between bacterial cells. We consistently observe that isolates of Methicillin-resistant Staphylococcus aureus diversify spontaneously into two distinct, sequentially arising strains. The first evolved strain outgrows the parent strain via secretion of surfactants and a toxic bacteriocin. The second is resistant to the bacteriocin. Importantly, this second strain is also resistant to intermediate levels of vancomycin. This so-called VISA (vancomycin-intermediate S. aureus) phenotype is seen in many hard-to-treat clinical isolates. This strain diversification also occurs during in vivo infection in a mouse model, consistent with the fact that both coevolved phenotypes resemble strains commonly found in clinic. Our study shows how competition between coevolving bacterial strains can generate antibiotic resistance and recapitulate key clinical phenotypes.

3

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE RAFT-ASSOCIATED PROTEIN FLOTILLIN FROM THE BACTERIUM STAPHYLOCOCCUS AUREUS

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Bacterial membranes contain discrete microdomains that are structurally and functionally similar to the lipid rafts of eukaryotes. An important structural component of the lipid rafts is the membrane-bound protein Fotillin, which is a chaperone responsible for the recruitment of other proteins to lipid rafts, thereby facilitating raft-harbored protein interactions. The ease with which bacteria can be genetically modified offers a tractable model to study the physiological role of Flotilllin within the lipid rafts, which has complicated studies in their eukaryotic counterparts. Here we present a structural and functional characterization of Flotillin protein FloA from the human pathogen Staphylococcus aureus. We have performed a number of biochemistry and cell biology approaches to ultimately understand the influence of FloA in the activation of the diverse signal transduction pathways that are related to the development of staphylococcal infections. Further characterization of these interactions led us to find sophisticated regulatory mechanisms in signaling transduction, which should directly influence an infection process. Overall, the discovery of lipid rafts in bacteria reveals an unexpected level of sophistication in signal transduction and membrane organization that is unprecedented in bacteria and shows that bacteria as more complex organisms than previously appreciated.

4

A NOVEL FUNCTION TO A KNOWN COMPOUND: HOW *PSEUDOMONAS PROTEGENS* INHIBITS BIOFILM FORMATION IN *BACILLUS SUBTILIS*

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Interspecies signaling has redefined our understanding of how bacteria live and survive in the world. Here, we focus on an interaction between *Bacillus subtilis* and *Pseudomonas protegens*, two common soil bacteria known to occupy the same plant-root niche in the natural environment. *P. protegens* was identified in a co-culture screen designed to discover soil bacteria that inhibit biofilm formation in *B. subtilis*. This screen used a *B. subtilis* reporter strain in which a biofilm-specific promoter drives the transcription of proteins that inhibit the transcription of the yellow fluorescent protein. Thus, *B. subtilis* cells that are not producing biofilm can be easily identified by

their fluorescence. In order to explore how *P. protegens* inhibited *B. subtilis* biofilm gene expression, we used its conditioned medium to perform a series of biological assays and chemical fractionations. In combination with analytical LC-MS and NMR, we identified the compound responsible for the observed biological activity as 2,4-diacetylphloroglucinol (DAPG). DAPG is a known compound secreted by *P. protegens*; however, it has not previously been shown to inhibit biofilm formation in any bacterium. To determine how DAPG affects biofilm formation in *B. subtilis*, we characterized its effect on pellicle formation both phenotypically and with flow cytometry using transcriptional reporters for various developmental processes in *B. subtilis*. This approach allowed us to quantify *B. subtilis* cellular development at the single-cell level, and examine the heterogeneity of the cells present in pellicles and biofilms in response to DAPG. We are currently exploring the genetic pathway through which DAPG acts in *B. subtilis*, as well implementing an in vivo model to observe these bacterial interactions on plant roots. By understanding how DAPG inhibits biofilm formation in *B. subtilis*, we will gain insights into how bacterial interspecies signaling can impact processes important to both plant and human health.

5

LOND IS REQUIRED FOR REGULATED SECRETION OF THE SIGNALLING PROTEASE POPC IN MYXOCOCCUS XANTHUS

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The intercellular C-signal plays a fundamental role in fruiting body morphogenesis in *M. xanthus*. It induces and coordinates three elementary processes involved in fruiting body formation: rippling, aggregation and sporulation. It is also required for the expression of developmental genes that are turned on after 6h of starvation. The C-signal is a 17 kDa cell-surface associated protein (p17) generated by N-terminal proteolytic processing of a 25kDa precursor protein (p25), encoded by *csgA* gene. p25 is present in both vegetative and starving cells, however it is only cleaved to generate p17 during starvation. The molecular mechanism underlying the regulated proteolysis of p25 is based on regulated secretion of the subtilisin-like serine protease PopC. PopC is retained within the cytoplasm of vegetative cells and is slowly secreted only during starvation thus restricting p25 cleavage to starving cells. Recent work has defined the regulatory mechanism by which PopC secretion is activated. PopC secretion is controlled post-translationally by a cascade involving PopD and (p)ppGpp synthase RelA. During vegetative growth, PopC and PopD form a cytoplasmic complex, which blocks PopC secretion. In response to starvation, RelA is activated and induces the proteolytic degradation of PopD leading to release of PopC for secretion. Regulated proteolysis of PopD appeared to require ATP-dependent metalloprotease activity with some evidence indicating integral membrane AAA+ protease $FtsH^{D}$. Recently, we have isolated a collection of non-developing mutants affected in PopC secretion and thus in generation of active C-signal. Complete genome sequencing revealed point mutations in LonD, another ATP dependent metalloprotease. In our current studies, we are investigating the role of LonD in PopC secretion and in development of *Myxococcus xanthus* in general.

6

MOLECULAR BASIS OF TRAA RECOGNITION THAT GOVERNS OUTER MEMBRANE EXCHANGE AND SOCIAL BEHAVIORS IN MYXOBACTERIA

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Kin recognition plays a key role in how free-living microbes identify sibling cells to mediate beneficial social behaviors. *Myxococ-*

cus xanthus, a soil-dwelling bacterium, serves as an important model system for studying complex multicellular interactions in bacteria. Previously we described a process whereby myxobacteria transiently fuse and exchange their outer membrane (OM) proteins and lipids. Two genetic determinants required for OM exchange are called TraA and TraB. Studies of OM exchange among *M. xanthus* environmental isolates suggested that TraA functions as a homophilic cell surface receptor to identify sibling cells to engage in OM exchange. Here cellular "goods" are shared among individuals that carry the same or very similar *traA* alleles. To elucidate the molecular mechanism of TraA recognition, we sought to identify key subregions and amino acids within TraA which govern specificity. To do this we created a series of chimeric *traA* alleles within the polymorphic region (PA14-like domain) that determines specificity. Recognition between the chimeric alleles and parental alleles were then tested by an extracellular complementation assay whereby motility mutants can be rescued by OM transfer of deficient motility proteins. Strikingly, from these chimeric and subsequent site directed mutagenesis studies, one particular amino acid position was shown to govern cell-cell specificity among a sub-group of *traA* alleles. In these studies, recognition could be switched by simply changing a single amino acid. These findings support our model that kin recognition in OM exchange is governed by TraA homotypic interactions between adjacent cells. Additionally, these findings provide a foundation for predicting uncharacterized TraA recognition groups and for engineering new recognition groups *de novo*.

7

RNA-SEQ-BASED TRANSCRIPTOME ANALYSIS OF QUORUM SENSING EFFECTING ON STAPHYLOCOCCUS AUREUS BIOFILMS

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Background: Expression of most virulence factors in Staphylococcus aureus is controlled by the accessory gene regulator (agr) locus, which encodes a two-component signaling pathway whose active ligand is a bacterial quorum sensing peptide (autoinducing peptide [AIP]) encoded by agr. The agr locus consists of four genes agrBDCA. A polymorphism in the amino acid sequence of AIP and of its corresponding receptor (AgrC) divides S. aureus strains into four major groups, agr-(I-IV). All agr-(II-III) isolates were defective in agrDCA and consequently they do not have a functional AIP. In addition, agr-III strains are able to transcribe icaR, sarA, and rsbU at the late- and post-exponential phases. agr-(I-IV) variants have functional agr-locus. Recent reports indicated that all glycopeptides intermediate S. aureus strains (GISA) examined belonging to agr-II were defective for agr function. In contrast, these strains were strong biofilm producers. In this work, we confirm that S. aureus ATCC25923, a quality control strain in antimicrobial susceptibility test, is capable of forming biofilm in vitro, which belongs to agr-III strain. Therefore, these findings lead to a hypothesis that S. aureus may have an enhanced ability of forming a thick biofilm due to agr-locus inactivation, which is unrelated to resistance to antibiotics. **Methods:** To investigate the possible mechanisms of methicillin-susceptible S. aureus biofilm formation, firstly, we used crystal violet staining assays and SEM images to measure the impacts of two compounds (ursolic acid and resveratrol, which could inhibit MRSA biofilm) on S. aureus ATCC25923 biofilm. Next, we used

high-throughout Illumina sequencing of cDNA (Illumina RNA-seq) to study the differentially expressed genes of S. aureus ATCC25923 biofilm by addition of two compounds and compared with differentially expressed genes of MRSA biofilm supplemented with the same compounds as previously described by our group. **Results and Discussion:** Crystal violet staining assays and SEM images showed that S. aureus ATCC25923 biofilm is thicker than MRSA's and its biofilm growth is faster. However, only ursolic acid could inhibit its biofilm formation. RNA-seq transcriptome analysis indicated that the MSSA may have an enhanced ability of forming a thick biofilm due to agr-locus inactivation, which is unrelated to resistance to antibiotics. In addition, the data showed that once sensitive strain formed biofilm, such sensitive strain would become drug-resistant strain. Meanwhile, sensitive strains biofilms resistance to antibiotics may be higher than some drug-resistant strains biofilms due to agr-locus inactivation. Therefore, the infection from antimicrobial sensitive clinical S. aureus can not be ignored. Further studies are necessary to identify the correlation mechanism of other clinical S. aureus biofilm formation and agr-regulated quorum sensing system.

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MEMBRANE VESICLE FORMATION VIA "HOLIN-ENDOLYSIN" SYSTEM IN *PSEUDOMONAS AERUGINOSA* PAO1

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Bacteria can adapt to surrounding environment and communicate with other cells using various cellular materials. Membrane vesicles (MVs) are naturally produced in Gram-negative bacteria, and considered to deliver cellular contents including proteins, DNA and signaling molecules to prokaryotic and eukaryotic cells. Although their biological functions of MVs in the bacterial communities have become recognized to date, how MV formation is regulated is poorly understood. The opportunistic pathogen, *Pseudomonas aeruginosa* is one of the well-known bacteria in the study of MVs. In this bacterium, the *Pseudomonas* quinolone signal (PQS), quorum-sensing signal of *P. aeruginosa*, is reported to be one of the major factors that induce MV formation in previous studies (Whitely *et al*. 2005, Tashiro *et al*. 2010). In addition, we found that *P. aeruginosa* produces MVs under anaerobic condition and MVs formation is induced through SOS response, which is relative to repairing DNA damage (Toyofuku *et al*. 2013). Further studies revealed that the production of pyocin, which is induced by SOS response, is involved in MV formation. In this study, we examined how pyocin production induces MV formation. As a result, the mutant of *PA0614* and *PA0629*, which encode putative holin and endolysin, respectively, formed less MVs than WT. Furthermore, MV production increased by the expression of *PA0614* and *PA0629* in *Escherichia coli*, however, no increase of MV production was observed by *PA0629* that carried a point mutation in the catalytic site. Hence, holin-lysin system, the system considered to be involved in bacteriophage or bacteriocin release by the degradation of the peptidoglycan, is considered to be involved in MV formation. Generally, Holin-lysin system is known to be widely conserved in bacteria. Therefore, we suggest that this mechanism is possibly a common MV formation mechanism in bacteria.

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TOXIN YAFQ AND PHOSPHODIESTERASE DOSP INCREASE PERSISTER CELL FORMATION BY REDUCING INDOLE SIGNALING

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Persister cells survive stresses by slowing metabolism. Since toxins of toxin/antitoxin (TA) systems increase persister cell formation, we investigated the influence of toxin YafQ of the

YafQ/DinJ E. coli TA system on persister cell formation. Under stress, toxin YafQ alters metabolism by cleaving transcripts with in-frame 5'-AAA-G/A-3' sites. We found that production of YafQ increased persister cell formation dramatically with multiple antibiotics, and through proteomics, found that YafQ reduced tryptophanase levels (TnaA mRNA has 16 putative YafQ cleavage sites). Consistently, TnaA mRNA levels were also reduced by YafQ. Tryptophanase is activated in the stationary phase by the stationary-phase sigma factor RpoS, which was also reduced dramatically upon production of YafQ. Tryptophanase converts tryptophan into indole, and as expected, indole levels were reduced by the production of YafQ. Corroborating the effect of YafQ on persistence, addition of indole reduced persistence. Furthermore, persistence increased upon deleting tnaA, and persistence decreased upon adding tryptophan to the medium to increase indole levels. Also, YafQ production had a much smaller effect on persistence in a strain unable to produce indole. Therefore, YafQ increases persistence by reducing indole, and TA systems are related to cell signaling. Furthermore, given that the population of persisters increases in biofilms and that c-di-GMP is an intracellular signal that increases biofilm formation, we sought to determine whether c-di-GMP has a role in bacterial persistence. By examining the effect of 30 genes from E. coli, including diguanylate cyclases that synthesize c-di-GMP and phosphodiesterases that breakdown c-di-GMP, we determined that DosP (direct oxygen sensing phosphodiesterase) increases persistence by over a thousand fold. Using both transcriptomic and proteomic approaches, we determined that DosP increases persistence by decreasing tryptophanase activity and thus indole. Despite the role of DosP as a c-di-GMP phosphodiesterase, the decrease in tryptophanase activity was found to be a result of cyclic adenosine monophosphate (cAMP) phosphodiesterase activity. Corroborating this result, the reduction of cAMP via CpdA, a cAMP-specific phosphodiesterase, increased persistence and reduced indole levels similarly to DosP. Therefore, phosphodiesterase DosP increases persistence by reducing the interkingdom signal indole via reduction of the global regulator cAMP. Hence, two distinct methods were used to show that the interkingdom signal indole is inversely related to persistence. We previously demonstrated that indole (i) is primarily active at low temperatures whereas AI-2 is the primary signal in the GI tract, (ii) reduces the pathogenicity of cells that do not synthesize it, (iii) influences biofilm formation, (iv) and tightens epithelial cell junctions in the GI tract.

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EFFECT OF C-DI-GMP ON BIOFILM FORMATION AND MOTILITY OF CAMPYLOBACTER JEJUNI

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Cyclic Diguanosine Monophosphate (c-di-GMP) is a naturally occurring small molecule that acts as an intracellular second messenger regulating important signalling systems in bacteria, including motility and biofilm formation. The role of c-di-GMP in Campylobacter jejuni is unknown. In this study, the biofilm inhibition assay was used along with Confocal Laser Scanning Microscopy (CLSM) to investigate the effect of different concentrations of extracellular c-di-GMP on C. jejuni. We found that extracellular c-di-GMP significantly reduced (>50%) biofilm formation compared to the untreated control with no effect on growth. We also found that c-di-GMP promotes chemotactic motility as judged by a plate-based swarm assay. This observation may indicate a role in the transition between the sessile and motile lifestyle of C. jejuni. These results suggest that c-di-GMP inhibits biofilm formation by modulating the transition between biofilm to planktonic forms.

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QUANTITATIVE IN VIVO FUNCTIONAL ASSAYS SUGGEST HOW A TRANSMEMBRANE HISTIDINE KINASE KINC BECOMES ACTIVE TO INDUCE CANNIBALISM AND BIOFILM FORMATION IN BACILLUS SUBTILIS

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Upon starvation, Bacillus subtilis cells differentiate into different subsets, undergoing cannibalism, biofilm formation, or sporulation. These processes require a multiple component phosphorelay, wherein the master regulator Spo0A is activated upon phosphorylation by one or a combination of five histidine kinases (KinA-KinE) via two intermediate phosphotransferases, Spo0F and Spo0B. While KinA and KinB are known to be primarily responsible for sporulation, KinC has been demonstrated to contribute for triggering biofilm formation and cannibalism. At present, it remains unknown how KinC becomes active. In this study, we established an in vivo quantitative assay system for KinC activity using an IPTG-inducible expression system. Using this system, we found that the N-terminal transmembrane domain is dispensable but the PAS domain is needed for the kinase activity. Second, an in vivo chemical cross-linking experiment demonstrated that the soluble and functional KinC (KinCΔTM1+2) forms a tetramer. Third, genetic experiments reveal that KinC activity and the membrane localization are independent of both the lipid raft marker proteins FloTA and cytoplasmic potassium concentration, which were previously shown to be required for the kinase activity. Finally, we demonstrated that KinC controls cannibalism and biofilm formation in a manner dependent on phosphorelay. Based on these results, we propose a revised model in which KinC becomes active by forming a homotetramer via the N-terminal PAS domain, but its activity is independent of both lipid raft and the potassium leakage, which was previously suggested to be induced by surfactin, a cyclic lipopeptide antibiotic.

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EVIDENCE THAT AUTOPHOSPHORYLATION OF THE MAJOR SPORULATION KINASE IN BACILLUS SUBTILIS OCCURS IN TRANS

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Entry into sporulation in Bacillus subtilis is governed by a multi-component phosphorelay, a complex version of two-component system which includes five histidine kinases (KinA-KinE), two phosphotransferases (Spo0F and Spo0B), and a response regulator (Spo0A). Among five histidine kinases, KinA is known as the major sporulation kinase, where it is autophosphorylated with ATP upon starvation and then transfers phosphate group to the downstream components (Spo0F, Spo0B and Spo0A) in a His-Asp-His-Asp signaling pathway. The recent results of the computational approach suggest that the autophosphorylation reaction of KinA occurs within the same protomer in the homodimer in a cis fashion. However, no direct experimental data is available to support this notion. Our recent study demonstrated that KinA forms a homotetramer, not dimer, mediated by the N-terminal domain, as a functional unit. Furthermore, when the N-terminal domain was overexpressed in the wild-type strain cultured under starvation conditions, sporulation was impaired. This impairment of sporulation can be explained by forming non-functional heterotetramer of KinA, resulting in the reduced level of Spo0A~P. These results suggest that autophosphorylation of KinA occurs in a transphosphorylation manner. To test this hypothesis and provide direct evidence whether the autophosphorylation of KinA occurs in a cis- or a trans-fashion, we generated a series of strains expressing homogeneous or heterogeneous protein complexes consisting of various combinations of

the phospho-accepting histidine point mutant protein and the catalytic ATP-binding domain point mutant protein. Each of the purified homo- and heterotetramers was incubated with [γ-32P]ATP in vitro and the reaction mixtures were analyzed on SDS-PAGE followed by autoradiography. The results suggested that ATP initially binds to one or more protomers within the tetramer complex and then the γ-phosphate is transmitted to another in a trans fashion. We further found the ATP-binding deficient point mutant is complemented in vivo by the phosphorylation deficient point mutant. Taken together, these in vivo and in vitro results reinforce evidence that KinA autophosphorylation occurs in a trans fashion.

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A THREE-DIMENSIONAL HYDRODYNAMIC MODEL FOR BIOFILMS COUPLED WITH QUORUM SENSING

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Biofilms are microorganism, where bacteria stay together, embedded in glue-like extracellular polymeric substances (EPS). It is observed that bacteria in biofilms communicate and cooperate by sensing the density of signaling molecules, which phenomenon is common known as quorum sensing. Recently, we have developed a 3d hydrodynamic model to account for the bacterial cell's quorum sensing ability by classifying the bacterial cells into downgraded and upgraded quorum sensing cells, as well as non quorum sensing cells, in which quorum sensing molecule is introduced to regulate the quorum sensing activity. We implement the model in full three dimension in space to arrive at a numerical solver studying biofilm development under quorum sensing regulation, as well as hydrodynamic effects on quorum sensing induction. Our model predicts that quorum sensing regulation contributes to heterogeneous structure development during biofilm formation. Besides, our model have shown that QS regulation is beneficial for the biofilm development in the long run, but may not of benefit in a short time window. In addition, our model suggests that the induction of quorum sensing in biofilm is sensitive to hydrodynamic stress.

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THE ROLE OF VIBRIO CHOLERAE QRR1-4 SRNAS IN MAINTAINING COMMUNITY STABILITY THROUGH QUORUM SENSING

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Vibrio cholerae (Vc), the causative agent of the acute diarrheal disease cholera, utilizes a highly regulated quorum sensing (QS) circuit to control the expression of many genes including those involved in virulence and biofilm formation. The switch between low cell density (LCD) and high cell density (HCD) gene expression in Vc is assumed to center on the reciprocal production of two transcriptional regulators AphA and HapR. At LCD, four small RNAs (sRNAs) Qrr1-4 are made and they activate and repress the expression of AphA and HapR, respectively. At HCD, Qrr1-4 are not made, thus, AphA expression is repressed and production of HapR is activated. Aside from virulence and biofilm formation, QS is believed to be important for maintaining the stability of a bacterial community through synchronizing gene expression according to cell density. However, the genes that are important for maintaining community stability and the mechanism of QS control are not well defined. We hypothesize that, because QS is employed to monitor cell density and species complexity, QS is likely involved in coping with stress caused by overpopulation and resource deprivation. Therefore, there are additional roles for QS in adapting to the fluctuations in the environment that occur when exogenous nutrients are consumed and metabolized. To test this idea, we studied how different Vc QS mutants behave in the presence of metabolic stresses. We show that a genetically LCD-locked Vc mutant constantly

producing Qrr1-4 is sensitive to accumulation of by-products of carbohydrate metabolism, while a genetically HCD-locked Vc mutant producing no Qrr1-4 is not. By testing mutants defective in different downstream QS pathway components, we show that Qrr1-4 sRNAs, but not AphA nor HapR, contribute to the hypersensitivity to carbohydrate metabolism. Our findings suggest Vc enlists an sRNA-dependent, HCD-specific gene expression program to resolve metabolic stress. We further tested the idea that the ability to withstand the accumulation of toxic by-products is essential for maintaining a stable community of Vc, and observed that the inability to withstand such toxicity can cause an entire population to collapse over time. Uncovering the mechanism of QS control of population stability during resource deprivation will help us to better understand the role that cell signaling plays in cell survival when faced with other potential stress conditions.

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ACTIVATION OF GAB CLUSTER TRANSCRIPTION IN BACILLUS THURINGIENSIS BY Γ-AMINOBUTYRIC ACID OR SUCCINIC SEMIALDEHYDE IS MEDIATED BY THE SIGMA 54-DEPENDENT ACTIVATOR GABR

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Bacillus thuringiensis GabR is a Sigma 54 (encoded by sigL)-dependent activators that has three typical domains, an N-terminal regulatory domain Per-ARNT-Sim (PAS), a central AAA+ (ATPases associated with different cellular activities) domain and a C-terminal helix-turn-helix (HTH) DNA binding domain. GabR positively regulates the expression of the gab gene cluster, which is responsible for the γ-aminobutyric acid (GABA) shunt. Purified GabR was found to specifically bind to a repeat region that mapped 58 bp upstream of the start codon of the gabT gene. The specific signal factors GABA and succinic semialdehyde (SSA) activated gabT expression. GABA- and SSA-inducible gabT transcription was controlled by Sigma 54 and activated by GabR, but the induction of gabT transcription by GABA and SSA is not the result of Sigma 54 or GabR induced expression. Deletion of the PAS domain of GabR resulted in increased gabT transcription activity, both in the presence or absence of GABA. It suggests that the PAS domain of GabR acts as a signal sensor domain that in the presence of GABA and SSA relieves GabR enhancer transcriptional activity.

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MEMBRANE VESICLE TRAFFICKING OF A LONG-CHAIN ACYL-HOMOSERINE LACTONE, C16-HSL

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N-Acyl-homoserine lactone (AHL) is one of the most common classes of signals produced in Gram-negative bacteria. The acyl side chain of the AHLs varies in length that confers the specificity to the signal. Paracoccus denitrificans is a Gram-negative bacterium capable of thriving under aerobic and anoxic conditions. This bacterium has been reported to produce a long chain acyl-homoserine lactone (C16-HSL) that is predicted to be highly hydrophobic. A previous report has suggested that the majority of the C16-HSL produced in P. denitrificans is associated with the cell membrane (Schaefer et al., J. bac. (2002)), questioning how the signal is excreted to the extracellular environment. Here we show that in a Paracoccus denitrificans closely related strain, Paracoccus sp. N11 that we isolated from an activated sludge, C16-HSL is packed in membrane vesicles (MV). Paracoccus sp. N11 produced MVs under normal culture conditions that were examined by transmission

electron microscopy. C16-HSL production in N11 strain and its presence in MVs were confirmed by MS analysis. Our results demonstrate that once packed in MVs, C16-HSL is able to freely diffuse in aqueous environments while C16-HSL alone is absorbed to hydrophobic surfaces. Interestingly, MVs were also isolated from activated sludge, suggesting their active roles in the natural environment. MVs have been shown to carry Pseudomonas quinolone signal (PQS) in Pseudomonas aeruginosa (Mashburn et al., Nature (2005)), and our results strengthen the role of MVs in cell-cell communication, and further imply that other hydrophobic long-chain AHLs may be carried by MVs.

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ANTHRANILATE, AN INTERMEDIATE OF TRYPTOPHAN METABOLISM ANTAGONIZES BIOFILM FORMATION AND INDOLE SIGNALING IN PSEUDOMONAS AERUGINOSA

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Anthranilate and indole are aromatic compounds that are alternatively produced from tryptophan degradation according to bacterial species. P. aeruginosa produces anthranilate from tryptophan degradation. While the effects of tryptophan and indole on the biofilm formation of bacteria have been reported, the influence of anthranilate on biofilm formation was not addressed. In this study, we investigated the anthranilate effects on the P. aeruginosa biofilm formation. While indole enhances and accelerates the biofilm formation of P. aeruginosa throughout development, the anthranilate effect on biofilm formation was differentially exerted depending on the developmental stage and the presence of shear force. Anthranilate a bit accelerated the initial attachment of P. aeruginosa at the early stage of biofilm development and appeared to build more biofilm without shear force, but with shear force, it dampened the maturation of mushroom structure and crumbled biofilm at late stage, making flat biofilm. Anthranilate was able to crumble the pre-formed biofilm and extracellular polymeric substance (EPS) staining also showed the biofilm-crumbling effect of anthranilate. To investigate the interplay of anthranilate with indole in the biofilm formation, we co-treated anthranilate with indole and found that the addition of anthranilate abolished the biofilm-enhancing effect of indole. Interestingly, the anthranilate degradation pathway was synergistically activated by co-treatment of anthranilate and indole. HPLC analysis shows that the anthranilate accumulation in P. aeruginosa decreased by the indole-treatment, demonstrating that the indole-activation of the anthranilate degradation pathway reduced the anthranilate level. Based on these results, we suggest two points: 1) anthranilate has a crumbling effect on biofilm formation of P. aeruginosa and can be a promising anti-biofilm agent. 2) the biofilm-enhancing effect of indole may come from the reduction of anthranilate level.

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THE ORNITHINE LIPID METABOLISM INFLUENCES THE BIOFILM FORMATION AND VIRULENCE OF PSEUDOMONAS AERUGINOSA BY MODULATING QUORUM SENSING

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Ornithine lipids (OLs) are a component of bacterial membrane. OLs are widely found in outer membrane of many gram-negative bacteria, but not detected in Eukarya and Archaea. Pseudomonas aeruginosa, an opportunistic pathogen has olsBA genes that constitute an operon to function the ornithine lipid biosynthesis. olsBA encodes acyltransferases and works in two steps for the OL biosynthesis, in which OlsB transfers an acyl group to ornithine to make lyso-ornithine lipid and OlsA

converts the lyso-ornithine lipid into ornithine lipid by another acyl-group transfer. OLs are reported to increase in phosphorus-free culture condition and olsBA operon of P. aeruginosa is induced in phosphate-limiting condition. While OLs were suggested to reduce the toxic effect of endotoxin probably functioning as an antagonist, a recently study showed that the mutation of this operon had no effect on the virulence of P. aeruginosa. In this study we found that the overexpression of olsBA operon modulated some virulence related-phenotypes of P. aeruginosa, including quorum sensing regulation, biofilm formation, and motility. We found that the overexpression of this operon modulates quorum sensing by reducing the quorum sensing signal production. Interestingly, the olsBA-overexpressing P. aeruginosa cells induced calcium release of animal cells, implying that it may modulate the physiology of host cells.

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BACTERIAL QUORUM SENSING AND METABOLIC SLOWING IN A COOPERATIVE POPULATION

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Quorum sensing regulates bacterial social behaviors, such as virulence, motility, biofilm formation, and toxin production, in response to cell density. Acyl-homoserine lactone-mediated quorum sensing controls cooperativity of individual cells in many species of *Proteobacteria*. These cooperative activities are costly at an individual level but provide benefits to the group. One long-standing question is whether quorum sensing controls nutrient acquisition and contributes to maintain homeostatic primary metabolism of individuals in a cooperative population. In crowded but cooperative populations, quorum sensing might coordinate nutrient utilization and homeostatic primary metabolism of individual cells. We show that the QS-dependent LysR-type transcriptional regulator QsmR functions to downregulate glucose uptake, substrate level and oxidative phosphorylation, and *de novo* nucleotide biosynthesis in the rice pathogen *Burkholderia glumae*. Systematic analysis of core primary metabolite levels showed that quorum sensing deficiency caused perturbation of nutrient acquisition and energy and nucleotide metabolism of individuals within the group. The quorum sensing mutants grew faster than the wild type at early exponential stage in Luria-Bertani medium and outcompeted the wild type in co-culture. Quorum sensing-mediated metabolic slowing of individuals indicates that quorum sensing functions as a metabolic switch to ensure efficient energy and resource utilization of individuals in crowded environments.

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STIGMERGIC BEHAVIOUR LEADS TO THE SELF-ORGANISATION OF BACTERIAL BIOFILMS

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Introduction: Many bacterial pathogens have the capacity to actively expand their biofilm communities via complex multi-cellular behaviours. We have observed that when the biofilms of Pseudomonas aeruginosa are cultured at the interstitial surface between a coverslip and solidified nutrient media, the resulting biofilms are characterised by an extensive pattern of interconnected trails that emerges as a consequence of the active expansion of these communities.
Aim: To identify the factors governing emergent pattern formation during P. aeruginosa biofilm expansion. **Experimental methods:** Bacterial biofilms were cultured at the interstitial space between solidified growth media and a glass coverslip. Biofilm expansion was observed using phase contrast time-lapse

microscopy and the topography of the underlying media was imaged using atomic force microscopy (AFM) and 3D optical profilometry after the cells where removed by washing the samples with water. **Results:** Our observations have revealed that during the migration of P. aeruginosa biofilms, aggregates of cells at the advancing edge forge furrows as they migrate across the semi-solid media. The formation of a series of interconnecting furrows and the reinforcing effect of cells traversing these furrows leads to extensive remodelling of the substratum. Our analyses indicate that whilst the furrows are shallow relative to the height of the bacterial cells, this appears to be sufficient to confine cells within the furrows. The generation and maintenance of the interconnected furrow network therefore accounts for the extensive large-scale patterning that is characteristic of these bacterial biofilms. **Conclusion:** Our observations indicate that self-organised pattern formation during biofilm expansion across semi-solid media is a stigmergic phenomenon. The concept of stigmergy describes processes of self-organised group behaviours that occur through indirect communication via persistent cues in the environment left by individuals that influence the behavior of other individuals of the group at a later point in time. Stigmergy has been extensively examined in higher organisms and non-living systems. We propose that stigmergy can be included in the repertoire of systems used by bacteria to coordinate complex multicellular behaviours.

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COORDINATION OF KEY PHYSIOLOGICAL OUTPUTS DURING QUORUM SENSING IN THE PHYTOPATHOGEN PANTOEA STEWARTII

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Pantoea stewartii subsp. *stewartii* is a Gram-negative proteobacterium that infects corn plants causing wilt disease. Quorum sensing (QS) controls exopolysaccharide production, the secretion of which during the late stages of infection blocks water transport in the xylem. The key master QS regulator in *P. stewartii* is EsaR. At low cell densities EsaR represses or activates expression of a number of genes in the absence of its acyl homoserine lactone (AHL) ligand. At high cell densities binding of AHL inactivates EsaR leading to deactivation or derepression of its direct targets. Prior work has demonstrated that the QS response orchestrates three major physiological responses in *P. stewartii*: capsule and cell envelope biosynthesis, surface motility and adhesion, and stress response. It is hypothesized that there is coordinate expression of these outputs through feed-forward and feed-back regulation modulated by the regulators RcsA, LrhA and UspA present downstream in the QS regulon. RNA-Seq is being used to examine the physiological impact of deleting the genes encoding these three regulators. In addition, the role of these and other QS-controlled genes is being tested in plant virulence assays to establish a better understanding of the key factors necessary for the pathogenesis of this model xylem-dwelling bacterium. Preliminary findings support the existence of multi-layered regulatory control of virulence during QS in *P. stewartii.*

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DIFFERENTIAL RNA-SEQ ANALYSIS OF VIBRIO CHOLERAE IDENTIFIES THE VQMR SRNA AS A REGULATOR OF COLLECTIVE BEHAVIORS

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The causative agent of cholera is Vibrio cholerae which colonizes the small intestine and causes profuse diarrheal symptoms. Regulation of V. cholerae virulence mainly occurs through cell-to-cell signaling, i.e. Quorum Sensing

(QS). In this study we performed differential RNA-sequencing (dRNA-seq) analyses of wild-type and low-cell-density locked V. cholerae. Our analyses identified a total of 7641 transcriptional start sites (TSS) at all conditions and ~40% of these TSSs initiated antisense transcripts. We further identified 107 non-coding transcripts and genome-wide TSS mapping in combination with phylogenetic comparisons allowed the re-annotation of ~130 genes on both chromosomes of V. cholerae. Our analyses also revealed expression of the previously unidentified VqmR sRNA upstream of the vqmA gene. Transposon mutagenesis identified VqmA as the transcriptional activator of VqmR and microarray-based target gene searches revealed eight mRNA targets of VqmR. We confirmed direct regulation by VqmR for all target genes including the mRNAs of the rtxBA toxin genes and the vpsT transcriptional regulator as well as additional mRNAs relevant for the chemotaxis, siderophore uptake and metabolism. In summary, our data provide a high-density map of the V. cholerae transcriptome and highlight the importance post-transcriptional control for collective behavior of this major human pathogen.

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DEFINING THE REGULATORY MECHANISMS OF NATURAL COMPETENCE IN VIBRIO CHOLERAE

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In the waterborne human pathogen Vibrio cholerae, quorum sensing mediates several critical behaviors such as virulence, biofilm formation, and natural competence. Natural competence enables V. cholerae to horizontally acquire genetic material through DNA uptake and recombination. Synthesis of the components of the DNA uptake apparatus is controlled by several transcriptional regulators that respond to specific environmental cues. HapR, the master regulator of quorum sensing, is active at high cell density and induces expression of QstR in response to autoinducers. Transcription factor TfoX is expressed in the presence of environmental chitin. CytR (cytidine repressor) and CRP (cAMP receptor protein) are active during nucleoside and carbon starvation, respectively. Although each of these regulators is required for natural competence, the mechanism by which each promotes transcription of competence apparatus genes is currently unknown. Through a combination of genomic analyses, genetic screens, and gene reporter assays we show that CytR and CRP positively regulate expression of multiple competence apparatus genes, such as comEA, pilA, vc0857, as well as the competence regulator qstR. Our expanded analysis revealed distinct classes of competence gene promoters; one class that requires only TfoX, another that needs CytR as well, and finally a third class that also requires QstR (and therefore HapR). Screening of > 150,000 transposon mutants identified no negative regulators of competence, but an on-going complementary screen for positive regulators has revealed several putative targets that might serve as activators of the competence pathway. It has been hypothesized that the coupling of quorum sensing with genetic control of natural competence promotes transfer of beneficial mutations, by coordinating the timing of gene expression to favor acquisition of extracellular DNA provided by 'kin'. A deeper insight into the regulatory mechanisms of natural competence in V. cholerae will enable a better understanding of the way in which cell to cell communication in many bacteria enables rapid evolution by promoting horizontal gene transfer.

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PHRA CONTROLS POPULATION HETEROGENEITY IN B. SUBTILIS BY INTERCELLULAR SIGNALING

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Quorum sensing is commonly associated with the coordination of a synchronized homogeneous population-wide response. In contrast, still relatively little is known about whether and how intercellular signaling contributes to the development of phenotypic diversity in the population. Sporulation in Bacillus subtilis has emerged as a model system for studying heterogeneous population development. Sporulation is controlled by a complex circuitry that involves extracellular signaling peptides from the Phr-family. Phrs are produced by an export-import circuit and have been suggested to serve in quorum sensing or alternatively, to implement an autocrine-like time-delay circuit on the level of the individual cell. Here, we analyzed the effects of perturbed PhrA-signaling on population development by using quantitative fluorescence timelapse microscopy assays. Our data demonstrates that PhrA can serve in global cellular communication: it functions as a diffusive signaling molecule that causes a concentration dependent population-level response by adjusting the ratio of sporulating and non-sporulating cells. One can mimic the effects of external PhrA stimulations by varying the size of the sending and receiving populations -or their ratio- during development respectively. Thus, PhrA signaling exhibits some traits that are characteristic for quorum sensing. However, rather than coordinating a homogeneous population response, PhrA seems to act as a potent regulator of population diversity.

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THE TONB-DEPENDENT RECEPTOR OAR AND THE ASSOCIATED LIPOPROTEIN MLPA ARE IMPORTANT FOR SECRETION OF A SIGNALING PROTEASE IN *MYXOCOCCUS XANTHUS*

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In response to starvation cells of *Myxococcus xanthus* initiates a developmental program that culminates in the formation of spore-filled fruiting bodies. The intercellular C-signal is essential for fruiting body formation and sporulation and acts as a morphogen to induce distinct responses at discrete thresholds during fruiting body formation. The C-signal is a cell surface-exposed protein. Consistently, C-signal transmission involves a contact-dependent mechanism in which C-signal on one cell interacts with a receptor on a neighboring cell. The C-signal is generated by proteolytic cleavage of a 25 kDa precursor protein (p25) by the secreted protease PopC. Cleavage of p25 by PopC generates a 17 kDa protein (p17), which is the bona fide C-signal. p25 as well as p17 are anchored in the outer membrane. PopC as well as p25 accumulate in vegetative cells; however, PopC only cleaves p25 in starving cells. We have previously shown the restriction of p25 cleavage by PopC in starving cells relies on regulated secretion of PopC: In vegetative cells, PopC is complexed by the PopD protein and PopC secretion is blocked; in response to starvation, and in a RelA-dependent manner, PopD is degraded and PopC secretion is induced. Once secreted, PopC is able to cleave p25 at the cell surface giving rise to the formation of p17. The mechanism underlying PopC secretion remains an open question. Here we report the identification of two proteins important for PopC secretion, the TonB-dependent receptor Oar and the lipoprotein MlpA. Oar and MlpA mutually stabilize each other, and secretion of PopC during development is strongly impaired in an *oar* mutant. Further detailed results will be presented.

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KIN DISCRIMINATION IN BACILLUS SUBTILIS

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Individuals of many animal species have remarkable abilities to behave differently at first encounter towards non related groups. Bacillus subtilis, a soil dwelling bacterium, which shows diverse social behaviors including quorum sensing, biofilm formation and swarming, also responds differently to nonrelated groups. For example, genetic difference at the comQXPA quorum sensing locus results in selective induction of QS response only in isolates of the same pherotype (communication specificity group) that carry genetically similar QS loci. Here we address another, possibly more general kin discrimination mechanism among swarms of highly related isolates of B. subtilis inhabiting soil at micrometer distances. In addition we explore interdependences between their genetic / ecological distance, pherotype association and ability to discriminate between kin and non- kin. We show for the first time that B. subtilis indeed possess a remarkable kin discrimination mechanism, which manifests in the formation of a clearly visible boundary line between swarms growing on a semi-solid agar media. Frequency of kin discrimination increased with genetic distance among strains and was always detected when strains were ecologically distinct. The boundary line, however, was never visible between two clonal swarms and was significantly less frequent among relatives that shared the ecotype and/or pherotype. Using MALDI imaging we investigated ions found in the boundary line and with transposon mutagenesis we identified potential genetic determinants that may contribute to this kin discrimination phenomenon during swarming or surface growth. Our results demonstrate a novel social behavior of B. subtilis that contributes significantly to the understanding of links between relatedness, ecology and intraspecific social interactions.

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THE PRODUCTION OF OUTER MEMBRANE VESICLES IN QUORUM SENSING MUTANTS OF *BURKHOLDERIA GLUMAE*

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The rice pathogen, *Burkholderia glumae* possesses one LuxI-R type quorum sensing system, TofI-R. The complex of C8-HSL and its cognate receptor, TofR, activates expression of toxin, flagella genes and an IclR type transcriptional regulator gene, *qsmR*. During growth in Luria-Bertani medium, *B. glumae* faces ammonia-mediated alkaline toxicity as a result of amino acid catabolism. In the wild-type, quorum sensing activates oxalate biosynthesis genes to avoid alkaline toxicity. However, quorum sensing mutants do not produce oxalate, which causes cell death at stationary phase. Quorum sensing mutants secreted abundant outer membrane vesicles (OMVs) into culture supernatant whereas the wild-type did not produce OMVs during growth. We purified OMVs from quorum sensing mutants using density-gradient sedimentation technique. Four membrane proteins (type VI secretion system component protein, ABC transporter protein, outer membrane protein, phage integrase family protein) and three periplasmic proteins (protease Do, lipoprotein, transglutaminase) were identified from the OMVs with high confidence by LC-MS/MS analyses. To answer the question why quorum sensing mutants produce OMVs into environment, we hypothesized that the vesiculation in quorum sensing mutants might give a short-term protection against envelope stress. According to metabolome analyses in *B. glumae*, certain metabolites such as glutamate were accumulated higher in quorum sensing mutants than in the wild-type in exponential phase. This indicated that abnormal concen-

tration of specific metabolites might cause a turgor pressure problem to bacterial envelope. We propose that OMVs of *B. glumae* might be induced to relieve turgor stress caused by imbalanced metabolism. Proteome profiles and physiological nature of *B. glumae* OMVs provide a basis on further study to determine how quorum sensing is related to producing OMVs and what biological functions and properties of OMVs are.

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A NEW ROLE FOR SELF RECOGNITION IN BACTERIAL POPULATIONS: ASSESSING LOCAL ENVIRONMENTS

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Swarming populations of the bacterium *Proteus mirabilis* can recognize foreign isolates of the same species, often resulting in a macroscopic boundary. In mixed swarms, self-recognition behavior can also be observed by the dominance of one strain at the expanding edges, while the other is inhibited to the swarm interior. In the model *P. mirabilis* strain BB2000, self recognition is partially mediated by an Ids system that requires an active type VI secretion (T6S) system. Intriguingly, not all cells in a clonal swarm appear to express Ids proteins at any given time. Moreover, *ids* deletion mutants are temporally inhibited in coswarms and form boundaries against the wild-type parent, despite the Ids proteins having no measured lethal activity. Given the complexity of the Ids system, we have queried whether *ids* expression may govern individual cell fates within clonal and mixed swarms. Using quantitative fluorescence microscopy and physiological analysis, we have begun to dissect the role of Ids-mediated self recognition by surveying both an entire swarm and the life-cycles of individual cells. Our primary input is to alter the initial ratios of BB2000 to an Ids-deficient mutant strain. First, we found that initial thresholds of *ids* expressing self cells are required for inhibition. Furthermore, a minimal number of mutant nonself cells are also needed: high self-to-nonself ratios result in inhibition of neither strain. Second, using single-cell analysis of *ids* promoter activity using a plasmid-based fluorescence reporter, we confirmed that expression of the *ids* system is affected by the social environment: the *ids* promoter is upregulated by the presence of nonself. Finally, using *in situ* single-cell fluorescence microscopy of active swarms, we have begun to analyze cell fates throughout a complete swarm cycle, chronicling the role of Ids-mediated self recognition in population growth and migration. Here, we present evidence that BB2000 uses the Ids system to assess and potentially communicate about its local social environment, raising the tantalizing possibility of similar signaling events in other social bacteria with non-lethal T6S-associated factors.

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OVEREXPRESSION OF A SMALL GENE IN THE *DEV*-OPERON CAN INHIBIT *MYXOCOCCUS XANTHUS* DEVELOPMENT

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When cells of *Myxococcus xanthus* are starved of nutrients they aggregate and from fruiting bodies, in which rod-shaped cells morph into rounded cells, eventually forming spores. Positional information is transmitted by C-signaling via transcription factor FruA, while starvation information may be transmitted via MrpC. MrpC and FruA bind cooperatively to the promoter regions of gene loci such as the *dev* operon, and regulate transcription. The *dev* operon regulates expression of *fruA*, and of other downstream loci such as *exo* and *nfs*, which are required for spore morphogenesis and cell shape maintenance. Mutants of *dev* operon genes such as *devS*, *devR* and *devT* are developmentally impaired, and qPCR analysis showed that *devS* and *devR* negatively autoregulate P_{dev} expression 10-fold. The first gene of the *dev* operon, *MXAN7266*, is predicted to code for a

40-residue peptide, and it was shown to be expressed in a *lacZ*-translational fusion. Deletion of DNA spanning -38 to +19 of the *dev* promoter, or a clean deletion of *MXAN7266*, resulted in no change in the developmental phenotype of wild type DK1622 but restored normal development in a *devS*, *devR* or *devT* mutant. The *devS MXAN7266* double mutant (Δ*devS* Δ*MXAN7266*) was successfully complemented with a fragment of P_{dev} containing *MXAN7266*. We propose that MXAN7266 negatively regulates expression of genes important for sporulation, and is countered by DevS, DevR, and DevT. Co-development of Δ*devS* (which overexpresses *MXAN7266*), with the wild type, did not affect development of either strain. This indicates that MXAN7266 is likely not secreted extracellularly, but acts in a cell-autonomous manner. To test whether *MXAN7266* codes for a protein or an RNA product, a single thymidine was added between the original +54 and +55 DNA bases in the reading frame of *MXAN7266* in a Δ*devS* background. If *MXAN7266* codes for a protein product, this would result in the creation of a stop codon within its reading frame, whereas the structure of an RNA molecule might not be significantly altered by an additional nucleic acid base. The developmental phenotype of the resultant *MXAN7266*-stop codon mutant in Δ*devS* background functionally resembled that of Δ*devS* Δ*MXAN7266*. This indicates that *MXAN7266* likely codes for a protein, rather than for a small RNA product. Relative P_{dev} transcript level in Δ*devS* Δ*MXAN7266* was about 5-fold higher than wild type, but lower than the 10-fold increase observed in Δ*devS*. This suggests that MXAN7266 may have a positive regulatory effect on the *dev*-promoter, and DevS may act in part by inhibiting *MXAN7266* expression. Expression from P_{nfs} and P_{exo} were respectively three to fifteen fold lower in Δ*devS* and Δ*devS* Δ*MXAN7266*, suggesting that DevS and MXAN7266 positively regulate expression of *exo* and *nfs*. Taken together, our results suggest that further investigation of MXAN7266 is key to understanding regulation of the *dev* operon, and its role in sporulation.

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ASSOCIATION OF ANTIBIOTIC RESISTANCE AND CAPSULE SWITCHING IN THE EMERGENCE OF VACCINE ESCAPE RECOMBINANT STREPTOCOCCUS PNEUMONIAE 7B CLONE IN ABSENCE OF PNEUMOCOCCAL VACCINATION

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Background: Antibiotics act as cell to cell communication molecules that induce competence for genetic transformation and fratricide in Streptococcus pneumoniae to acquire exogenous DNA resulting genomic and phenotypic diversity. **Aims & Methods:** Studied capsule switching, seroconversion and genomic homology among antibiotic-resistant pneumococci from children with invasive diseases by antibiotic resistance, serotyping, MLST and eBURST analysis in Bangladesh where pneumococcal vaccines are not used routinely. **Results:** Among 136 pneumococci, 11 common serogroups (77% of invasive-isolates) were 6, 14, 19, 5, 12, 1, 7, 45, 2, 9 and 23 (in descending order), where as 11 most common serogroups of colonized-isolates comprising 78% were 6, 19, 14, 23, 9, 7, 13, 15, 21, 22 and 37. 71% were antibiotic resistant, MDR in 12%. Macrolide resistance was detected in six isolates (four 7B, one 9V and one 18C). Four 7B isolates were MDR; three had ST 1553 and one ST 1586, a single locus variant of ST 1553. 9V isolate were MDR and ST 1553 indicating genomic homology with 7B and capsule switching where prevalent MDR 9V acquired 7B capsule resulting in seroconversion (9V 7B). eBURST analysis of MLST 1586 and 1553, and available pneumococcal MLST database (www.mlst.net) indicated that ST 1553 and ST 1586 were not closely associated with any other clone. **Conclusion:** Thus, newly emerged vaccine escape recombinant MDR pneumococcus 7B originated by 7B caps locus acquisition by 9V preferably by antibiotic-induced cell-cell signaling and

caused invasive diseases. To our knowledge, this unique pneumococcal clonal complex was detected for the first time.

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CONFLICTING MESSAGES: SIGNALS FROM THE EXTRACELLULAR MATRIX MAINTAIN MOTILITY WITHIN THE BIOFILM

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Bacteria in nature are usually found in complex multicellular structures, termed biofilms, which protect them from numerous environmental threats. The biofilm structure is the end result of collective behavior of different cell types in a coordinated fashion, achieved by the accurate regulation of dedicated genetic programs. So far, it was well established that in the single-cell level once a bacterial cell commits to the biofilm state it represses motility both transcriptionally and post-translationally. However, at the population level, we found a motile cell sub-population that is tightly retained inside the biofilm of Bacillus subtilis, enabling fast occupation of new niches, as well as invasion of neighboring colonies of foreign bacterial species. This dogma-breaking phenomenon depends on a unique signal originating in the extracellular matrix (ECM) that surrounds the cells in the biofilm, maintaining a motile cell sub-population. This ECM-based signal acts to induce motility, as mutants deficient in ECM production show lower numbers of motile cells. When the ECM extracted from a wild type biofilm was externally added to the ECM mutants, motility level was restored. In particular, TasA, the proteinaceous component of B. subtilis biofilm, appears to be a specific chemical signal that strongly induces bacterial motility. Another line of evidence for the importance of maintaining motility in the biofilm emerges from the highly frequent evolution of hypermotile suppressors in ΔtasA mutant colonies. We suggest that in the absence of ECM-based induction of motility, a strong selective pressure acts to reinstate motility in the biofilm.

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QUORUM SENSING REGULATION OF ANTIMICROBIALS PROTECTS PUBLIC-GOODS COOPERATION FROM EXPLOITATION BY OTHER BACTERIAL SPECIES

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Quorum sensing (QS) is a cell-cell communication system that can regulate cooperative behaviors in bacterial populations. *Pseudomonas aeruginosa* has a complex QS system that regulates the production of several secreted products, including proteases, which are shared among the whole population. Such products are termed "public goods." We, and others, have previously shown that QS in *P. aeruginosa* can serve as a model to study public goods cooperation. *P. aeruginosa* requires the production of a QS-regulated secreted protease, elastase, to grow when casein is the sole carbon and energy source. In this setting, QS mutant "social cheaters" arise that avail themselves of the carbon liberated by the elastase produced by the QS-intact cooperators. Other bacterial species may also reap the benefits of the nutrients liberated by elastase, and these may be viewed as another type of cheater. *P. aeruginosa* QS also controls antimicrobials -- such as rhamnolipids, phenazines, hydrogen cyanide, and quinolones -- that can inhibit growth of other bacteria. We asked if QS-produced antimicrobials prevent invasion or "cheating" by other species against *P. aeruginosa*. As a competitor strain, we used *Burkholderia multivorans* strain AMT0468-1, which exhibits similar growth kinetics as *P. aeruginosa* PAO1 in nutrient-rich conditions. We observed that *P. aeruginosa* inhibits growth of *B. multivorans* in nutrient-rich conditions, and that this is due to the combined effects of

three QS-produced antimicrobials; phenazine, hydrogen cyanide and rhamnolipids. In casein media, where growth requires QS-controlled elastase, wild-type *P. aeruginosa* similarly out-competed *B. multivorans*. Our strain of *B. multivorans* did not grow to high density alone in casein media, likely because it does not produce the necessary protease required to liberate nutrients from casein. Strikingly, however, *B. multivorans* grew well in co-culture with an antimicrobial-deficient *P. aeruginosa* mutant and could invade the *P. aeruginosa* population even from a very low starting density. This result shows that QS-controlled antimicrobials protect the *P. aeruginosa* population from cheating by *Burkholderia*. These findings add to the increasing body of work supporting the view that QS is important for competition in mixed-species environments. Furthermore, the results show that by co-regulating proteases and antimicrobials, QS may protect and stabilize the cooperative production of proteases.

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EVOLUTIONARY PRESSURE ON THE PSEUDOMONAS AERUGINOSA QUORUM SENSING REGULON

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The opportunistic pathogen *Pseudomonas aeruginosa* regulates the expression of dozens of genes via two acyl-homoserine lactone (AHL)-responsive quorum-sensing transcription factors, LasR and RhlR. Many quorum-controlled genes code for "public goods," which are shared among the population. Thus quorum sensing can be a cooperative behavior. Experiments show this leaves *P. aeruginosa* quorum sensing systems susceptible to "social cheating" by LasR mutants. Such cheaters avail themselves of public goods, but do not incur the metabolic cost of quorum sensing and therefore can outcompete cooperators. In one human disease, cystic fibrosis, patients are chronically colonized with *P. aeruginosa* and LasR mutants accumulate over time. We have also observed CF isolates with intact quorum systems have small quorum-sensing regulons as compared to environmental isolates. We tested the hypothesis that, when quorum-sensing-regulated factors are required for growth of *P. aeruginosa*, the quorum regulon will be adaptively reduced. We grew *P. aeruginosa* on casein and adenosine, a condition in which quorum sensing is required for growth and emergence of LasR mutants is constrained. After over 1000 generations of growth, the frequency of LasR mutants remained below 1%. We developed a method to assess the quorum sensing regulon on a population level by growing evolved populations in the presence of absence of an AHL-degrading enzyme and using RNA-seq to identify genes that were affected by signal. After 160 days (roughly 900 generations), the number of quorum-activated genes was reduced by 40% as compared to the progenitor. Our data suggest that when bacteria grow under conditions that require quorum sensing, there is a relatively rapid reduction in the number of genes directly or indirectly regulated by quorum-sensing transcription factors. These data also imply that the large quorum regulon in environmental isolates of *P. aeruginosa* reflects varied and numerous selective factors. Finally, our results suggest LasR mutants and isolates with relatively small quorum sensing regulons may reflect environmental conditions where this bacterium depends on quorum-regulated factors for either growth or survival.

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QS SYSTEMS CAN ACCUMULATE AS SELFISH GENETIC ELEMENTS

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The Rap-Phr family of quorum sensing systems is found in the Bacillus subtilis and cereus groups. The peptide signal of this system, which is encoded by the phr gene, de-represses

the activity of the Rap receptor. In the absence of signal, most studied Rap receptors repress the activity of either the Spo0A or ComA response regulators. A typical B. subtilis strain contains 8 paralogs of this system, where each paralog encodes for a different signaling peptide which specifically interact with its cognate receptor but no other (non-orphan) receptors. In addition, all rap-phr systems are also similarly regulated transcriptionally. It is therefore unclear what drives the evolutionary accumulation of these redundant systems. Here we demonstrate both experimentally and theoretically that the accumulation of rap-phr systems might be the result of a cheating behavior. In a co-culture where only part but not all of the population had acquired an additional rap-phr system, there will be less of the novel phr signal than the one which is produced by the entire population. Thus, leading to less derepression of the cognate novel rap gene which results in decreased cooperative behavior (i.e. cheating). Therefore we suggest that the accumulation of rap-phr systems is not the result of added group benefit but rather due to their selfish element like behavior.

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PSEUDOMONAS AERUGINOSA QUORUM SENSING: COOPERATOR POLICING OF SOCIAL CHEATERS

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There are two acyl-homoserine lactone quorum sensing (QS) circuits in *P. aeruginosa*, LasR-I and RhlR-I. LasR-I activates RhlR-I and thus serves as a master QS regulator. Together, these circuits activate hundreds of genes, many of which code for production of secreted or excreted factors. These factors are called "public goods" and can be shared among individuals in the group. *P. aeruginosa* requires QS-activated secreted proteases for growth on casein as the sole carbon and energy source, and over time LasR mutants emerge and invade populations of LasR-intact cooperators. These mutants are "social cheaters" that reap the benefit of the cooperator-produced proteases without being burdened by the metabolic costs of public goods production. We and others have observed that these social cheaters come to an equilibrium with cooperators and do not cause the population to collapse, as would occur if the number of cooperators fell below the quorum threshold. Here we probe the molecular basis of the equilibrium. We hypothesized that cooperators can police cheaters by intoxicating them. RhlR activates genes coding for production of several reactive toxic compounds, including peroxides and hydrogen cyanide. We believe RhlR also induces immunity to these factors. We reasoned that RhlR-activated genes allow *P. aeruginosa* cooperators to police cheaters, and that $LasR^+$, $RhlR^-$ cooperators are "policing mutants." Policing mutants will not have a capacity to limit invasion by $LasR^-$, $RhlR^-$ cheaters and we expect population crashes for failure to achieve a quorum. This prediction was borne out by experiments where $LasR^-$, $RhlR^-$ social cheaters rapidly arose to high frequencies and caused a population crash. We then showed that policing involves cyanide production by wildtype *P. aeruginosa* by following social evolution in an $HcnC^-$ cooperator, which cannot synthesize hydrogen cyanide. Like the $RhlR^-$ cooperators, populations of $HcnC^-$ cooperators were overrun by LasR mutants. To test the hypothesis that wildtype *P. aeruginosa* and $LasR^-$ cheaters coexist by virtue of RhlR-activated toxin secretion, and not a direct cell-cell interaction, we used dialysis membranes to separate $LasR^+$, $RhlR^-$ cooperator populations from either $LasR^+$, $RhlR^+$ or $HcnC^-$ cooperators. Over 48 hours, the cell yields of $LasR^+$, $RhlR^-$ cooperators with the wildtype were two logs lower than when they were when grown with the $HcnC^-$ cooperators. Our studies support the notion that groups of cooperating *P. aeruginosa* can police social cheaters and we provide a plausible mechanism for this phenomenon.

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QUORUM SENSING PROMOTES DEFENSE AND SELF-PRESERVATION DURING INTERSPECIES COMPETITION

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Many Proteobacteria use acyl-homoserine lactone mediated quorum sensing to control the production of public goods such as antibiotics or proteases whose benefits are shared among all of the cooperating members of the population. Because quorum sensing systems regulate public goods, they may be susceptible to invasion by social cheaters, such as a quorum-defective mutant, which can benefit from the public goods but do not incur the cost of producing them. In some species quorum sensing also controls production of efflux pumps and other factors that may protect the cells from antimicrobials produced by other species; these are not believed to be shared among all of the members of the population making them private goods. Quorum regulation of such defensive strategies may be important for protecting a niche or competing for limited resources in multispecies bacterial communities. To investigate the importance of quorum sensing in defense during inter-species competition, we used a model of competition between two soil saprophytes, Chromobacterium violaceum and Burkholderia thailandensis, that we previously described. We demonstrate that C. violaceum quorum sensing mutants are more sensitive than wild type to a potent bactobolin antibiotic produced by B. thailandensis. C. violaceum quorum sensing mutants have a growth advantage over their quorum-intact parent in laboratory growth media. However in co-culture with B. thailandensis, quorum-intact C. violaceum outcompetes the quorum-defective cells. This result was dependent on the production of bactobolin by B. thailandensis. Our results suggest that quorum sensing is important for defense during interspecies competition and that the defensive strategies are likely private because they protect only the producing cells. During growth in mixed microbial communities, quorum sensing-controlled defense strategies may serve to restrain the emergence of quorum-defective social cheaters.

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ACYL-HSL QUORUM SENSING CONTROL OF AN EXTRACELLULAR PRODUCT IN A SYNTHETIC SYSTEM

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In *Pseudomonas aeruginosa*, quorum sensing (QS) controls and coordinates the production of many secreted and excreted products, for example proteases, which can be shared by all members of a community. QS allows a production delay of these products until cells are at a sufficient density to benefit from them. The *P. aeruginosa* LasR-LasI QS system is at the top of a QS-signaling cascade. LasI is a 3-oxo-C12-HSL synthase and LasR is a 3-oxo-C12-HSL responsive transcriptional activator. We aim to study the costs and benefits of QS control of extracellular products in a simplified synthetic recombinant *Escherichia coli* system. To do this we have developed a recombinant *E. coli* enterochelin mutant (*entF*) with chromosomal copies of *lasR, lasI,* and a *lasI*-promoter-controlled *entF.* The *E. coli entF* mutant does not grow in iron-restricted medium, whereas the genetically engineered QS *E. coli* and the wildtype grow to similar densities. The *entF* mutant can obtain iron and grow together with the wildtype or the QS controlled *entF* strain on iron-restricted medium. However, the Ent$^-$ freeloaders do not increase in frequency when grown together with enterochelin-producing strains. We are now in position to compare the costs and benefits of QS vs constitutive control of an extracellular product during growth of *E. coli* growing under a variety of conditions.

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A DESIGN PRINCIPLE OF QUORUM SESNING REGULATION

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Quorum sensing (QS) signaling systems typically regulate cooperative traits that are costly to the individual cell but beneficial to the population. A common network structure of QS systems is a positive feedback loop that is manifested by QS dependent activation of the QS signal. The prevalent view is that regulation by QS is advantageous because QS activates cooperative behaviors only in dense populations, in which they are beneficial. In addition, the positive feedback structure is typically regarded as a mechanism that sharpens the threshold-like behavior of QS response. Other aspects, relating to the social nature of cooperative traits and the possibility of exploitation by cheaters, are often overlooked. In our work, we ask how the regulatory structure of cooperative traits affects the evolutionary stability of cooperation. In this framework, we use mathematical modeling to show that QS regulation is beneficial since it grants a better resistance against cheaters in a structured population. It does so by allowing cooperators to sense nearby signaling mutants, and to reduce cooperative effort accordingly. Moreover, we demonstrate that unlike regular QS regulated cooperators that are susceptible to cheating by receptor mutants, a positive feedback on the QS signal provides resistance against these mutants. More generally, our results provide a condition for the evolutionary stability of cooperation, which generalizes Hamilton's rule for cases of regulated cooperation. Furthermore, we are testing our predictions in a synthetic system of feedback regulated cooperative behavior in Bacillus subtilis. Preliminary experimental results will be presented.

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A QUORUM SENSING MOLECULE PQS, PRIVATISES IRON FOR PSEUDOMONAS AERUGINOSA

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In addition to signalling, quorum sensing molecules often have secondary properties. In *Pseudomonas aeruginosa*, the *Pseudomonas* quinolone signal (PQS) binds iron with a high affinity causing an iron starvation response. This PQS:iron complex can then be used as an iron source to support the growth of *P. aeruginosa* via the production of iron scavenging siderophores. Here we investigate the possibility that the production of PQS enhances the fitness of *P. aeruginosa* when in competition for iron with other species. We present a simple evolutionary model for PQS production which predicts that (a) PQS increases competitive ability in an iron-dependent manner and (b) that high PQS production necessitates correspondingly high investment in iron scavenging siderophores. We present experimental support for both predictions. Firstly, a meta-analysis of previous work as well as new experiments, indicate that the iron binding property of PQS reduces competitor growth, favouring *P. aeruginosa* in competition. Secondly we show that PQS production and siderophore production are positively correlated in natural isolates of *P. aeruginosa* indicating that PQS induced iron sequestration must be accompanied by investment in iron acquisition. Finally we show that PQS, whilst beneficial for competition with other species, can result in intensified competition with con-specific cheats that do not invest in iron acquisition. Our results are suggestive of the possibility that PQS acts to privatise iron, reducing its availability to other species. Such investment in the future availability of nutrients presents novel behavioural strategy to overcome heterospecific competition.

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EVOLUTIONARY TRICKS TO CONSTRAIN QS SIGNALLING IN BACILLUS SUBTILIS

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Quorum sensing (QS) signals serve as spatiotemporal triggers of cooperation among microbes, however evolutionary stability of signalling has not received much experimental insight. Bacillus subtilis uses evolutionary ancient ComQXPA QS system where the peptide signal ComX undergoes posttranslational isoprenylation by ComQ. At critical concentration ComX activates its cognate receptor ComP which thorough transcriptional activator ComA triggers expression of lipopeptide antibiotic surfactin (srfABCD) and genetic competence for transformation. Multiple sequence alignment of comQXPA loci from soil isolates confirmed that signal linked- (comQ and comX) and receptor-encoding genes (comP) coevolved. We asked to which extent signalling and response to ComQXPA QS are co-dependent and how signal deficient cells with functional ComP respond to ComX produced by the wild type (WT) in homogeneous environment. We monitored QS response of the WT (srfA-cfp) and the signal-deficient (ΔcomQ; srfA-yfp) strains in 1:1 co-culture by fluorescence microscopy and the response of these strains in monocultures supplemented with ComX. We discovered that the mutant overly responds to ComX, which leads to increased production of surfactin and increased genetic competence. We find also that surfactin promotes the DNA release for intraspecific genetic exchange. Paradoxically therefore, non-signalling mutants act as both hypercooperators - because they share surfactin more generously than the wild type despite the high fitness costs of its production; and as hypercheats - because they take up more exogenous DNA than WT under selective conditions. Consequently, the mutants cannot invade but they have a window of increased opportunity to revert into WT. Our results indicate that the ComQ dependent intracellular link couples prudent QS response with QS signalling, providing constrains on signal production. Evolutionary stability of signalling in B. subtilis could be then explained by direct benefits, because the signal serves as a coercion to non-senders while the sender is equipped with the negative feedback regulation of the QS response. In addition, signal deficiency of B. subtilis in the presence of WT simultaneously translates into decreased fitness and increased recombination rate. This supports long-standing hypothesis on fitness associated recombination as a way to maximize the adaptive potential of genetic competence within species.

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NON-SOCIAL ADAPTATION DEFERS A TRAGEDY OF THE COMMONS IN QUORUM-SENSING BACTERIAL POPULATIONS

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In a process termed quorum sensing (QS), the opportunistic pathogen *Pseudomonas aeruginosa* uses diffusible signaling molecules to regulate the expression of numerous secreted factors or public goods that are shared within the population. But not all cells respond to QS signals. These social cheaters typically harbor a mutation in the QS receptor gene *lasR* and exploit the public goods produced by cooperators. Here we show that non-social adaptation under growth conditions that require QS-dependent public goods increases tolerance to cheating and defers a tragedy of the commons. The underlying mutation is in the transcriptional repressor gene *psdR*. This mutation has no effect on public goods expression but instead increases individual fitness by derepressing growth-limiting intracellular metabolism.

Even though *psdR* mutant populations remain susceptible to invasion by isogenic *psdR lasR* cheaters, they are able to bear a higher cheater-load, and they are completely resistant to invasion by *lasR* cheaters with functional *psdR*. Mutations in *psdR* also sustain cooperation at wild-type levels when paired with certain partial loss-of-function *lasR* mutations. Targeted sequencing of multiple evolved isolates revealed that mutations in *psdR* arise before mutations in *lasR*, and rapidly sweep through the population. Our results indicate that strong selection for QS can lead to adaptations in non-social, intracellular traits that increase the fitness of cooperating individuals and thereby contribute to population-wide maintenance of QS and associated cooperative behaviors.

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SELF-PRODUCED PEL AND PSL ARE ONLY OF DIRECT BENEFIT IN PSEUDOMONAS AERUGINOSA BIOFILMS

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Pseudomonas aeruginosa is known to assume a surface attached lifestyle and grow as multicellular communities known as biofilms during chronic infection. These sessile cells are surrounded by a hydrated matrix of polysaccharides, of which PEL and PSL are the main structural constituents of non-mucoid strains. These self produced extracellular polysaccharides act as signals during biofilm formation (indirectly increasing c-di-GMP levels), however it is unclear as to wether the effects of PEL and PSL are exploitable by social cheating. We carried out in vitro selection experiments using plastic beads to allow biofilm formation of PEL/PSL producers and non-producers in both high- and low-related environments (high and low strain diversity, respectively). The ratio of producers and non-producers were assessed at each successive selection round. A planktonic control population (absence of plastic beads) was also tested. Our results show that PEL and PSL provide a direct fitness benefit in biofilm populations whilst having zero benefit (or cost) in planktonic populations. Furthermore, it was evident that the production of both PEL and PSL was not exploitable by non-producing "cheaters" due to the loss of non-producers in low-related populations after four and six rounds of selection, respectively. Additionally, the loss of PEL non-producers two selection rounds prior to PSL non-producers suggests this particular extracellular polysaccharide is less socially exploitable by cheaters than the latter. Our results suggest that non-producing mutants would not survive in the environment (i.e. during chronic infection), or that their frequency would be extremely low. Furthermore, it is possible that PEL and PSL production are non-social due to the positive feedback regulatory circuit which would directly benefit producing cells more.

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THROMBOCYTOPENIA AND PROLONGED PROTHROMBIN TIME IN NEONATAL SEPTICEMIA

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Septicemia in neonates refers to generalized bacterial infection documented by positive blood culture in the first 28days of life and is one of the leading causes of neonatal mortality in sub-Sahara Africa. Thrombocytopenia in newborns is a result of increased platelet consumption; sepsis was found to be the most common risk factor. The objective of the study was to determine if there are organism-specific platelet responses among the 2 groups of bacterial agents: Gram-positive and Gram-negative bacteria, and also to examine the association of platelet count and prothrombin time with neonatal septicemia. 232 blood samples were collected for this study. The blood culture was performed using Bactec

9050, an instrumented blood culture system. The platelet count and prothrombin time were performed using Abacus Junior5 hematology analyzer and i-STAT 1 analyzer respectively. Of the 231 neonates hospitalised with clinical sepsis, blood culture reports were positive in 51 cases (21.4%). Klebsiella spp. (35.3%) and Staphylococcus aureus (27.5%) were the most common Gram-negative and Gram-positive isolates respectively. Thrombocytopenia was observed in 30(58.8%) of the neonates with septicemia. Of the 9(17.6%) patients with severe thrombocytopenia, seven (77.8%) had Klebsiella spp. septicemia. Out of the 21(63.6%) of thrombocytopenia produced by Gram-negative isolate, 17(80.9) had increased prothrombin time. In conclusion, Gram-negative organisms showed the highest cases of severe thrombocytopenia and prolonged PT. This study has helped to establish a disturbance in hemostatic systems in neonates with septicemia. Further studies, however, may be required to assess other hemostasis parameters in order to understand their interaction with the infectious organisms in neonates.

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NEW SIGNALING PATHWAYS AND METABOLITES FOR HOST:PATHOGEN COMMUNICATION DURING GASTROINTESTINAL INFECTIONS

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Background: Precise and regulated activation and production of bacterial virulence factors during gastrointestinal infection is a critical determinant for diarrhea. Signaling molecules, receptors, downstream regulators and in vivo spatiotemporal expression kinetics for several virulence factors, and subsequent host responses to infections are also not properly understood and defined. These processes can serve as targets for prophylactic and therapeutic interventions against infectious diseases. **Findings:** We report discovery of several bacteria two-component signaling pathways (TCS) and their cognate signals (host metabolites), which regulate toxin production in Vibrio cholerae and other enteric bacteria by mutually exclusive and novel non-canonical mechanisms. We also report two novel high-throughput and systems-biology portable assays to measure host and bacterial RNA expression profiles and metabolites of infected mice, and generate a "molecular signature" of diarrheal diseases. We first utilized high-throughput screening to identify bacterial TCS pathways regulating cholera toxin production, followed by genomics, digital gene-expression technology, RNA-Seq, metabolomics, proteomics and animal models to decipher their detailed mechanism. One TCS pathway acts a phosphorylation-mediated switch between bacterial virulence gene expression and host metabolism. It activates bacterial toxin production during hypoxia via a non-phosphorylated response regulator, and represses host metabolic process detrimental to pathogenesis in it's phosphorylated form. A second TCS pathway senses host potassium levels, and activates toxin production via tyrosine phosphorylation of its response regulator. Thus, this pathway can switch between aspartate and tyrosine phosphorylation of its cognate response regulator to modulate bacterial virulence and pathogenesis. Using sequencing and 2D LC/ESI/MS/MS proteomics, I identify protein-interacting partners and DNA binding sites of these TCS. By characterizing a battery of locked, null, constitutively actively genetic point mutants under standard laboratory conditions, followed by profiling spatiotemporal kinetics of host immunity and metabolism regulated by these pathway using digital gene-expression technology and high-throughput metabolomics in a mouse model, I confirm phenotypes and mechanisms of these signaling modalities. **Significance:** My data integrates bacterial signaling and host responses in vivo to identify a new landscape of host-pathogen interactions, metabolites and mechanisms for infectious diseases. I also report two novel RNA and metabolic profiling approaches that

can be ported into systems-biology platforms, which will have wide utility in microbiology research.

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MICROBIAL INTERACTIONS AND EVOLUTION IN CHRONIC CYSTIC FIBROSIS INFECTIONS

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Chronic cystic fibrosis (CF) airway infections provide opportunities for fundamental investigations related to microbial evolutionary dynamics, diversity, and interactions within a natural polymicrobial ecosystem. Patients with CF are predisposed to airway infections from a wide range of microbial species of which Pseudomonas aeruginosa is the major contributor to patient morbidity and mortality. It is well-established that diverse factors such as the host defense, antibiotic treatment in the clinic, and a heterogeneous distribution of nutrients drive P. aeruginosa evolutionary adaptation to the lung environment. However, whether interactions with other infecting microbes is also an evolutionary driver is not well understood. To begin to explore the relationship between evolution and microbial interactions, we have focused on two distinct P. aeruginosa lineages (called "DK1" and "DK2") that have transmitted among and evolved in CF patients in Denmark during the last 40 years. Although most patients are infected with either clone-type, DK1 and DK2 have also been co-existing in many patients for extended periods. Here, we focused on a single patient with a mixed infection containing both clone types. To investigate interactions between DK1 and DK2 we sequenced the genomes of isolates sampled from the patient over 15 years. Surprisingly, we identified isolates with mosaic genomes: These isolates (which we call "DK1/2") had DK2-based genomes but containing regions of DK1 DNA acquired by horizontal gene transfer and recombination. DK2 isolates are sensitive towards R5 pyocins produced by other P. aeruginosa lineages. We show that the transferred regions provide enhanced R5 pyocin resistance to DK1/2. Our data suggest that the within-host genetic interactions between co-infecting DK1 and DK2 strains could be driven by super-infections with R5 producing genotypes. To more systematically explore interactions between DK1 and DK2, we are mapping phenotypic interactions between 100 DK1 and DK2 isolates sampled from multiple patients. For that purpose we are currently performing a pairwise screening on agar surfaces using differentially fluorescent-tagged strains to assess neutral, negative or positive effect. Our results point towards an unexplored area for novel interference treatment strategies in relation to microbe-microbe interactions.

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WITHIN-HOST EVOLUTION OF PSEUDOMONAS AERUGINOSA TOWARD IRON ACQUISITION FROM HEMOGLOBIN IN POLYMICROBIAL CF INFECTIONS

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Bacterial pathogens require iron to survive and colonize a human host but their access to free iron is often limited by iron-withholding process where free iron is bound by proteins such as hemoglobin. Although most pathogens have developed tactics to acquire iron from host proteins, little is known about how evolutionary processes modulate bacterial iron acquisition systems in chronic, polymicrobial infections where interspecies competition for limited iron could be an evolutionary driver. To begin to address this issue, we use chronic airway infections in patients with cystic fibrosis (CF) as a model to investigate evolutionary adaptation to an iron-limited environment in a polymicrobial context. Here, we investigate the within-host evolution of the transmissible P. aeruginosa DK2 lineage sampled from (CF) airway infections over a period of

several decades. We find a positive selection for promoter mutations in P. aeruginosa DK2 leading to increased expression of the phu (Pseudomonas heme utilization) system. By mimicking conditions of the CF airways in vitro, we experimentally demonstrate that increased expression of phuR confers a growth advantage in the presence of hemoglobin, thus suggesting that P. aeruginosa evolves towards iron acquisition from hemoglobin. We also find similar adaptive mutations in the genomes of two additional P. aeruginosa lineages ruling out the specificity of these mutations to this particular lineage. Furthermore, in all three lineages phuR promoter mutations coincide with the loss of pyoverdine production, suggesting that within-host adaptation towards heme utilization is coupled to the loss of pyoverdine production. We hypothesize that this particular adaptation in P. aeruginosa DK2 has an impact on interspecies interaction with other members of the CF polymicrobial community capable of heme utilization. We are currently testing this hypothesis by exploring competition for iron from hemoglobin between P. aeruginosa DK2 and Staphylococcus aureus that are frequently co-isolated from CF infections.

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INNATE IMMUNE DYSFUNCTION IS ASSOCIATED WITH ELEVATED A20 EXPRESSION DURING INFLUENZA A VIRUS/S. PNEUMONIAE COINFECTION IN MICE

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Both, influenza A virus and S. pneumoniae are a leading cause of morbidity and mortality worldwide. However, most influenza-related mortality is not due to the viral infection alone but rather secondary bacterial infections, mainly caused by S. pneumoniae. The mechanisms driving virulent influenza coinfection are poorly defined, making it difficult to develop effective therapeutic strategies. This study investigates signaling events evoked by influenza infections affecting the innate immune response and cellular clearance mechanisms in the lung. Using an in vivo model of subsequent infections with influenza A virus and S. pneumoniae via the intratracheal infection route we show that sublethal influenza infections clearly predispose for severe pneumococcal infections even at low bacterial doses. Coinfected C57BL/6 mice are more susceptible to pneumococcal infection than single-infected mice, resulting in drastically less survival and earlier development of pneumonia and bacteremia. Despite an upregulation of the endogenous TLR inhibitor A20 at 24 hours post secondary infection in lungs of coinfected mice, cytokine analyses show a significant increase of proinflammatory cytokines and increased numbers of neutrophils in the airways of co-infected compared to single-infected mice. This enhanced inflammation associated with tissue damage contributes to the severity of secondary bacterial pneumonia suggesting that influenza-induced A20 is either not sufficient to suppress the inflammation or is dysfunctional.

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REGULATORY CHARACTERISTICS OF VIBRIO VULNIFICUS GBPA, ENCODING N-ACETYL GLUCOSAMINE BINDING PROTEIN AND ESSENTIAL FOR PATHOGENESIS

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Mucin glycoprotein is a major component of mucus layer that is the major site of entry for most pathogens and serves as the initiation surfaces for host-microbe interactions. Vibrio vulnificus, a model human enteric pathogen, is

a causative agent of fatal septicemia and utilizes the mucin to survive and cause disease in host. From RNA-seq analysis of V. vulnificus grown with mucin-containing media, numerous genes which were induced by mucin were identified. Among them, a gbpA gene encoding an N-acetyl glucosamine binding protein GbpA, a homologue of V. choelrae GbpA, was selected and further studied. The influence of global regulatory proteins on the expression of gbpA was examined, and quorum sensing regulator SmcR and Fe-S cluster regulator IscR were found to downregulate and upregulate the gbpA expression at the transcriptional level, respectively. Primer extension analysis revealed that the transcription of gbpA begins at a single site. Direct bindings of SmcR and IscR to PgbpA were demonstrated by EMSA. The binding sites for SmcR and IscR were mapped based on a deletion analysis of the PgbpA and confirmed by DNase I protection assays. A mutational analysis demonstrated that GbpA contributes to the ability of adherence to mucin-secreting HT-29 MTX cells as well as mucin. In addition, the gbpA mutant exhibited reduced intestinal colonization and virulence in mice. The combined results proposed a model in which SmcR and IscR cooperate for precise control of the expression level of GbpA during infection.

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A MOVING TARGET: ACQUISITION OF A CRYPTIC MEGAPLASMID RESULTS IN SENSITIVITY TO UNKNOWN INHIBITORY AGENT

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It is well known that microbes can alter surrounding environments as well as interact with their host and fellow microorganisms. Therefore, to better understand complexes such as the microbiome, it is necessary to understand the dynamics of microbe-microbe interactions and potential drivers of microbial communities. The Pseudomonas genus consists of diverse microorganisms that can be readily isolated from most environments allowing for numerous interactions with hosts and other bacteria. Pseudomonas syringae pv. lachrymans 107 contains a 1Mb cryptic megaplasmid that can be easily transferred across Pseudomonas species. We have yet to discover a clear benefit to acquiring the pMP, but strains that have recently acquired the megaplasmid demonstrate lowered growth rates, reduced biofilm production, decreased antibiotic resistance, and decreased thermotolerance. Despite these costs the pMP is stably maintained within the recipient strain, potentially due to high rates of transfer. One intriguing and, to our knowledge, unprecedented cost of megaplasmid acquisition is the sensitivity to an unknown agent produced by various Pseudomonas species at stationary phase that inhibits growth specifically of pMP strains. Our goal is to better understand how acquisition of a large, self-transmissible plasmid with such a cost could shift interactions within bacterial communities and further alter evolutionary dynamics in natural environments. With increased knowledge of this unknown inhibitory agent, it is possible that an interesting microbial community dynamic where pathogens forced to uptake the pMP could be targeted and treated with extracted supernatant leading to novel forms of agricultural and medical treatment exists.

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PSEUDOMONAS AERUGINOSA QUORUM SENSING IN CHRONIC WOUND INFECTIONS

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Pseudomonas aeruginosa is one of the most frequent causes of chronic wound infections. The production of most virulence factors that contribute to the pathogenesis of P. aeruginosa is regulated by cell to cell signaling, or quorum sensing (QS). We have previously demon-

strated that QS is essential to the pathogenesis of P. aeruginosa in burn wound infections and quorum signals have also been detected in ischemic wounds; however, little has been elucidated about the role of QS in the chronic wound environment. To determine the extent of QS activity in P. aeruginosa-infected chronic wounds we: used LCMS to detect QS signals in murine chronic wound tissue; used fluorescent QS 'reporter' strains and confocal microscopy to indirectly visualize the expression of P. aeruginosa QS genes in situ; analyzed the global expression of P. aeruginosa in vivo with RNAseq technology; and compared the infection sequela of wild-type P. aeruginosa versus a QS mutant in mouse chronic wounds. Our data indicate that QS is active early in murine chronic wound infections, but quickly tapers off. We also observed that a QS mutant strain of P. aeruginosa was significantly less tolerant to gentamicin treatment in situ compared to a wild-type strain, and that the tolerance of the wild-type was reduced after early treatment with a QS inhibitor. Taken together, our data indicate that QS may be important in the formation of biofilms early in chronic wound infections, which results in increased antibiotic tolerance.

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WHOLE GENOME SEQUENCING ENABLES CHARACTERIZATION OF LUXI HOMOLOGUE OF BURKHOLDERIA CEPACIA GG4

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Background: Quorum sensing is a mechanism for regulating proteobacterial gene expression in response to changes in cell density. Such cell-to-cell communication regulates a diverse array of physiological activities, ranging from biofilm formation to swarming motility. By far, the most extensively studied QS molecules is N-acyl homoserine lactone (AHL). These signalling molecules are produced by LuxI homologue or commonly known as AHL synthase. Recently, the whole-genome sequencing of B. cepacia strain GG4, isolated from rhizosphere of ginger, was performed. Preliminary studies showed that the wild type strain was capable of AHL synthesis. We aimed to characterize the putative luxI homologue and postulated similar AHL profile by the recombinant AHL synthase. **Methods:** The genome of strain GG4 was analyzed with RAST server and BLAST2GO for gene predictions and annotations. From the annotated genome, the putative luxI homologue, burI, was identified and cloned into pET28a for overexpression in E. coli BL21. The recombinant BurI protein was purified and the production of AHL was characterized using liquid chromatography mass spectrometry (LC-MS) **Results: I**n silico analysis of the 6.6 Mb genome of strain GG4 revealed the abundance of genes coding for carbohydrate, fats and protein metabolism, which suggests that the bacterium is well adapted to nutrient-rich rhizosphere environment. The burI gene which encodes the 25 kDa putative AHL synthase was overexpressed in E. coli. The protein sequence has a high degree of homology with AHL synthases from other Burkholderia strains. LC-MS analysis of the culture supernatant showed the presence of 3-oxo-hexanoylhomoserine lactone, N-octanoylhomoserine lactone, 3-hydroxy-octanoylhomoserine lactone and N-nonanoyl-homoserine lactone. To our knowledge, strain GG4 is the first soil isolate among Burkholderia strains to synthesize N-nonanoylhomoserine lactone. The results also indicates that BurI is indeed the AHL synthase as the AHL profile was found to be similar to its wild type strain. **Conclusion:** In this present study, the genome of strain GG2 is a significant addition of the genomic data from the Burkholderia genus and will therefore be a valuable resource for future investigation. We also reported the production of AHL by E. coli harboring the recombinant BurI was indeed similar to the wild type. This finding represents the initial step in elucidating the role and the mechanism of the autoinducer system possessed by strain GG4.

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RECOMBINATION DRIVES PSEUDOMONAS AERUGINOSA DIVERSITY DURING CYSTIC FIBROSIS INFECTION

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During chronic bacterial infection, evolution and diversification of founding clones facilitates adaptation to in vivo growth and the appearance of antibiotic resistance. The Cystic Fibrosis (CF) lung harbors a complex, polymicrobial ecosystem, in which Pseudomonas aeruginosa is capable of sustaining chronic infections, which are highly resistant to multiple antibiotics. It is now well established that founder P. aeruginosa populations evolve over many years of chronic CF infection, leading to high levels of temporal phenotypic and genetic diversity within a single patient. Recent studies examining diversity within P. aeruginosa populations isolated from the CF lung have described significant variation in antibiotic susceptibility profiles in isolates, which vary in morphological appearance. However, no study has conducted a detailed examination of a single, morphologically homogeneous population of P. aeruginosa, nor has any study provided a comprehensive map at the genome level of phenotypic variation within a CF lung population. We address this gap in understanding of how diversity evolves in the CF lung by investigating the phenotypic and genotypic diversity of 44 morphologically identical Liverpool epidemic strain B58 P. aeruginosa 'variants' taken from a single CF patient sputum sample at a single snapshot in time. Comprehensive phenotypic analysis of these variants reveals large variances and trade-offs in both growth and the production of virulence factors and quorum sensing (QS) signals. Whole genome analysis indicates high levels of intra-variant diversity and that recombination and not spontaneous mutation is the dominant driver of diversity in this population. Furthermore, phenotypic differences between variants are statistically associated with distinct recombination events rather than linked to mutations in known genes. Our results support a number of important and previously unobserved findings: (i) the significant role of recombination in driving phenotypic and genetic diversification during in vivo chronic respiratory infection; (ii) the potential impact of in vivo diversity on patient-to-patient transmission studies; (iii) the complexity of in vivo genetic mutations and their resulting effects on phenotype.

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DOSE-DEPENDENT EFFECTS OF BACILLUS ON COLONIC BACTERIAL COMMUNITY IN PIGLETS CHALLENGED WITH ESCHERICHIA COLI, AS REVEALED BY DEEP 16S RRNA SEQUENCING

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Probiotic could be a promising alternative to antibiotics for the prevention of enteric infections; however, further information on the underlying mechanisms is required. Stable commensal gut microbial community contributes to block pathogenic action in infection. Understanding probiotic effects on bacterial community in gut is essential to realize the full benefits and consequences of in-feed probiotics. In this study, we defined the lumenal bacterial community from the colon, and characterized the dose effects (3.9×10^9 CFU/d or 7.8×10^9 CFU/d) of in-feed *Bacillus* mixture (*Bacillus subtilis* and *Bacillus licheniformis*) on the community in an F4 (K88)-positive Enterotoxigenic *Escherichia coli* infection model of F4 receptor-negative piglets enteritis (n = 6) using culture-independent Illumina

high-through 16S rRNA V4 gene sequencing. After quality filter, 376,600 - 1,230,800 reads were used for further analysis. 347 - 516 operational taxonomic unit (OTU) were obtained. We found that the colonic bacterial community, regardless of the different treatment, was mainly composed of members of the phyla Bacteroidetes, Spirochetes, Firmicutes, Tenericutes and Proteobacteria, presenting on > 90% of bacteria species. The collateral effects on the microbiota of *Bacillus*-fed animals caused divergence from that of control animals, with notable changes being increases in the relative abundance of *lactobacillus* and *Bifidobacteria* populations and decreases in *Escherichia coli* populations. *Bacillus* mixture increased the community diversity as determined by shannon index. High dose of *Bacillus* mixture increased the number of class Bacilli and decreased the number of class Mollicutes and Thermoplasmata. We suggested that up- and down- regulation of specific bacterial species may be involved in colonization against *Escherichia coli* providing a potential therapeutic approach through specific manipulation of the intestinal microbiome. Also, it adds further suggest a threshold reference for the use of probiotics in clinical practice. **Key words:** Bacillus, Microbial community, DNA sequencing, piglets

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HIGH FH BINDING CAPABILITY OF CLINICAL ISOLATES OF STREPTOCOCCUS PNEUMONIAE SEROTYPE 6B IS ASSOCIATED WITH THE PRESENCE OF AN ADDITIONAL COPY OF PSPC

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Pneumococcal surface protein C (PspC), a major virulence factor of Streptococcus pneumoniae mediates evasion of the host immune system by binding to human complement regulatory protein factor H (FH). Binding studies using different clinical isolates of serotype 6B strains showed high FH binding by few isolates compared to other strains, including TIGR4 and D39. Subsequent whole genome sequence analyses revealed the presence of two different pspC genes in the clinical isolates, pspC6.9 encoding a choline binding version and pspC9.4 encoding a cell wall anchored LPXTG protein. Interestingly, PspC9.4 displayed an overall low sequence homology to various PspC proteins which are known to interact with FH. Further investigations showed that PspC9.4 binds human FH through its amino terminal domain and a 9 amino acid motif present in the N terminal domain is directly involved in this interaction. Interaction kinetics between recombinant His-PspC9.438-434 and FH using surface plasmon resonance displayed a significantly higher binding affinity in comparison to other PspC family members. We here show that high FH binding capability of clinical isolates of serotype 6B strains is associated with the presence of an additional copy of PspC, pspC9.4.

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THE SIALIC ACID N-ACETYL NEURAMINIC ACID ACTS AS A HUMAN-SPECIFIC SIGNAL TO ENHANCE VIRULENCE IN STREPTOCOCCUS PNEUMONIAE TIGR4

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Streptococcus pneumoniae is a pathogen known to cause severe diseases like pneumonia or septicemia. When entering the host S. pneumoniae encounters a mucin-layer decorated with sialic acids. In most animals N-acetylneuraminic acid (Neu5Ac) is converted to N-glycolylneuraminic acid (Neu5Gc) by the cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH). A mutation in the CMAH gene, exclusively found in humans, leads to a loss of synthesis of Neu5Gc and to

an overproduction of Neu5Ac. In this study we are investigating the role of sialic acids as signaling molecules in host specificity and pathogenesis. We observed an increase in transcription and enzyme activity of the pneumococcal neuraminidase A (NanA) in response to the sialic acid Neu5Ac as compared to Neu5Gc. After intranasal challenge, NanA mediates an earlier development of pneumonia, septicemia and lower survival rates in CMAH-/- as compared to C57BL/6 wild-type mice. Here, we show that S. pneumoniae is able to decrease chemokine levels and neutrophil recruitment in the lungs of CMAH-/- mice. In conclusion, S. pneumoniae leads to increased virulence in response to Neu5Ac, mainly abundant in humans, and is able to alter the innate immune response.

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CONTRIBUTION OF HOST MONOSACCHARIDES TO BIOFILM FORMATION DURING STREPTOCOCCUS PNEUMONIAE NASOPHARYNGEAL COLONIZATION

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Streptococcus pneumoniae is an important etiologic agent causing otitis media, pneumonia, meningitis, and bacteremia. Asymptomatic nasopharyngeal colonization is a requisite step in the development of invasive disease, and is mediated by formation of immunoquiescent pneumococcal biofilms. Multiple investigators have now suggested that pneumococcal neuraminidase (NanA) plays an important role during colonization. Within the nutrient sparse nasopharynx, neuraminidase may act to cleave sialic acid (SA) from host epithelial cells. The utilization of SA may act as an important nutritional signal for colonizing bacteria. Herein, we examine the contribution of neuraminidase and SA, as well as other carbohydrate sources, to biofilm formation by S. pneumoniae. In vivo biofilm formation was examined using scanning electron microscopy of septal epithelia isolated from colonized mice. Production of neuraminidase (responsible for cleavage of SA from host cells) was assayed for a number of clinical isolate strains using a standard fluorometric assay. Streptococcus pneumoniae biofilms were grown in the presence and absence of various carbohydrate sources on polystyrene plates or glass slides and stained to determine levels of biofilm formation and overall biofilm architecture. A mutant deficient in NanA was not attenuated in an in vitro biofilm model, but displayed a complete inability to form biofilms within the nasopharynx. Clinical isolate strains exhibited a wide range of neuraminidase enzymatic activity, potentially contributing to the varying abilities of these strains to form biofilms in vivo. Both free SA and mucin-bound SA were able to increase biofilm formation in vitro in a modest fashion at low concentrations. The increase in biofilm formation in the presence of mucin-bound SA was abrogated in a neuraminidase deficient mutant. Conversely, addition of glucose and other preferred sugars was markedly inhibitory to biofilm formation. This data suggests that site-specific nutrient availability may have a profound affect on colonization by influencing the bacterial mode of growth. Therefore, carbohydrate availability may act as an important signal during pneumococcal biofilm formation and dispersal.

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BACTERIAL FIGHT-AND-FLIGHT RESPONSES ENHANCE VIRULENCE IN A POLYMICROBIAL INFECTION

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The oral pathogen Aggregatibacter actinomycetemcomitans (Aa) resides in infection sites with many microbes including commensal streptococci such as Streptococcus gordonii (Sg). During infection, Sg promotes the viru-

lence of Aa by producing its preferred carbon source, L-lactate, a phenomenon referred to as cross-feeding. However, as with many streptococci, Sg also produces high levels of the antimicrobial hydrogen peroxide (H2O2), leading to the question of how Aa deals with this potent antimicrobial during co-infection. Here we demonstrate that Aa possesses two complementary responses to H2O2: a detoxification or "fight" response mediated by catalase (KatA) and a dispersion or "flight" response mediated by Dispersin B (DspB), an enzyme that dissolves Aa biofilms. Using a murine abscess infection model, we show that both of these responses are required for Sg to promote Aa virulence. While the role of KatA is to detoxify H2O2 during co-infection, three-dimensional (3D) spatial analysis of mixed infections revealed that DspB is required for Aa to spatially organize itself at an optimal distance (>4 μm) from Sg, which we propose allows cross-feeding yet reduces exposure to inhibitory levels of H2O2. In addition, these behaviors benefit not only Aa but also Sg, suggesting that "fight" and "flight" stimulate the fitness of the community. These results reveal that an antimicrobial produced by a human commensal bacterium enhances the virulence of a pathogenic bacterium by modulating its spatial location in the infection site.

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QUORUM SENSING ENHANCEMENT OF THE STRESS RESPONSE PROMOTES RESISTANCE TO QUORUM QUENCHING AND PREVENTS SOCIAL CHEATING

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Quorum sensing (QS) coordinates the expression of virulence factors and allows bacteria to counteract the immune response, partly by increasing their tolerance to the oxidative stress generated by immune cells. Despite the recognized role of QS in enhancing the oxidative stress response, the consequences of this relationship for the bacterial ecology remain unexplored. In this work we demonstrate that QS increases resistance also to osmotic, thermal and heavy metal stress.Furthermore a QS-deficient lasR rhlR mutant is unable to exert a robust response against H2O2 as it has less induction of catalase and NADPH-producing dehydrogenases. Phenotypic microarrays revealed that the mutant is very sensitive to several toxic compounds. As the anti-oxidative enzymes are private goods not shared by the population, only the individuals that produce them benefit from their action. Based on this premise, we show that in mixed populations of wild-type and the mexR mutant (resistant to the QS inhibitor furanone C-30), treatment with C-30 and H2O2 increases the proportion of mexR mutants; hence, oxidative stress selects resistance to QS compounds. In addition, oxidative stress alone strongly selects for strains with active QS systems that are able to exert a robust anti oxidative response and thereby decreases the proportion of QS cheaters in cultures that are otherwise prone to invasion by cheats. As in natural environments stress is omnipresent, it is likely that this QS enhancement of stress tolerance allows cells to counteract QS inhibition and invasions by social cheaters, therefore having a broad impact in bacterial ecology.

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RESISTANCE AGAINST QUORUM QUENCHERS IN PSEUDOMONAS AERUGINOSA CLINICAL ISOLATES

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Resistance against Quorum quenchers in Pseudomonas aeruginosa clinical isolates. Quorum quenching is a promising alternative to combat infections of several bacterial pathogens including Pseudomonas aeruginosa, one of the advantages of these approach in comparison with the utilization of antibiotics is that interfering with cell-cell communication attenuates virulence without affecting growth (at least in rich culture medium); hence it was postulated that the selection of clones resistant to quorum quenching will be minimum or even absent, due the lack of a selective pressure that favor the spreading of the resistant clones; however, since in addition to control the expression of virulence traits quorum sensing also controls the expression of some private goods (such as the enzymes necessary to catabolize adenosine); conditions in which a strong selective pressure exists can be readily achieved. Recently, we demonstrated that when using adenosine as sole carbon source the selection of clones resistant against the canonical quorum quencher, the brominated C-30 furanone was possible, the selected clones were mutants that over express the multidrug efflux pump MexAB-OmpR, moreover we also discovered that clinical isolates with similar mutations are also able to resist C-30 inhibition (of adenosine based growth), even if those strains were not pre treated by the compound (1, 3). Our recent work was focused in the characterization of several P. aeruginosa clinical strains isolated from several sources, including blood, urine and catheter tips, and of strains isolated from cystic fibrosis patients, among those strains we found some isolates resistant against C-30 and some of them also resistant against another quorum quencher 5 fluorouracil (2) and we are currently working in the characterization of those strains in order to understand the subjacent resistant mechanisms, preliminary work suggest that in addition to efflux other resistant mechanisms are also involved, since C-30 resistant strains that are sensitive to antibiotics (hence the over-expression of efflux pumps is not expected) were also identified. References 1. García-Contreras, R., T. Maeda, and T. K. Wood. 2013. Resistance to quorum-quenching compounds. Appl Environ Microbiol 79:6840-6. 2. García-Contreras, R., M. Martinez-Vazquez, N. Velazquez Guadarrama, A. G. Villegas Paneda, T. Hashimoto, T. Maeda, H. Quezada, and T. K. Wood. 2013. Resistance to the quorum-quenching compounds brominated furanone C-30 and 5-fluorouracil in Pseudomonas aeruginosa clinical isolates. Pathog Dis 68:8-11. 3. Maeda, T., R. García-Contreras, M. Pu, L. Sheng, L. R. Garcia, M. Tomas, and T. K. Wood. 2012. Quorum quenching quandary: resistance to antivirulence compounds. ISME J 6:493-501.

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INTERFERENCE OF BACTERIAL ENERGY GENERATION AND QUORUM-SENSING REGULATOR FOLDING BY INDOLE

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Indole has received great attention because of its extensive effects on various biological functions in the bacterial population, such as biofilm formation, antibiotic resistance and virulence. Indole has been also reported to function as an intercellular signal, like QS signals, in bacterial communities. We demonstrated that many genes involved in energy generation and chaperones are highly expressed under indole treatment in both Pseudomonas putida and Acinetobacter oleivorans. P. putida hslU mutant study partially supported that chaperone is important for protecting cells under indole. Subsequent biochemical analyses have shown that indole increases the NADH/NAD(+) ratio and decreases the adenosine triphosphate (ATP) concentration inside cells, due to membrane perturbation and higher expression of TCA cycle genes. This

energy reduction leads to a reduction in cell size and an enhancement of biofilm formation in P. putida. Interestingly, our in vitro protein-refolding assay using malate dehydrogenase with purified GroEL/GroES demonstrated that indole interferes with protein folding. Indole enhanced P. putida biofilm formation and inhibited swimming motility, which were not observed when AHL was already bound to the QS regulator, thereby suggesting that the quorum sensing regulator PpoR-AHL complex masks the effects of indole. Quorum sensing (QS)-dependent biofilm formation and motility were controlled by AqsR in A. oleivorans. QS-controlled phenotypes appeared to be inhibited by indole and the aqsR mutant had the same phenotypes. We demonstrated that the turnover rate of AqsR became more rapid without AHL signal. The addition of exogenous indole decreased the expression of two AqsR-targeted genes. The overexpression of AqsR in Escherichia coli was impossible with the indole treatment. Surprisingly, our [(35)S] methionine pulse-labelling data demonstrated that the stability and folding of AqsR protein decreased under indole without changing aqsR mRNA expression in E. coli. Indole also resulted in a loss of TraR-dependent traG expression in an Agrobacterium tumefaciens indicator strain. However, when indole was added after incubation with exogenous AHL, indole could not inhibit the TraR-dependent expression of the traG promoter. When P. aeruginosa and Chromobacterium violaceum were tested to extend this conclusion, QS-dependent production of pyocyanin and violacein was inhibited by indole without reducing the QS signal production under indole treatment. Here, we provided evidence showing that the indole effect on QS-controlled bacterial phenotypes is due to reduction of energy generation and inhibited QS regulator folding.

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IDENTIFICATION AND CHARACTERIZATION OF THE *N*-ACYLHOMOSERINE LACTONE-DEGRADING GENE FROM THE COAGULASE-NEGATIVE STAPHYLOCOCCI (CNS)

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Many gram-negative bacteria have a quorum-sensing system and produce *N*-acylhomoserine lactones (AHLs) that they use them as a quorum-sensing signal molecule. AHL-degrading genes have been cloned and characterized from various bacteria. Coagulase-negative staphylococci (CNS) are present on the skin of animals and considered to be less virulent. In this study, the novel AHL-degrading gene was screened from the genome sequences of CNS species. In previous study, we have identified a novel AHL-lactonase gene (*ahlS*) from potato leaf-associated *Solibacillus silvestris*. The *ahlS* homologues were present in the genome of CNS strains, which were *Staphylococcus carnosus*, *S. haemolyticus*, and *S. saprophyticus*. To check the AHL-degrading activity, these three CNS strains were cultivated in LB medium containing 10 µM C6-HSL or C10-HSL. After incubation for 9 h, AHLs were completely degraded by these strains. The *ahlS* genes on the genome of CNS strains were amplified by PCR and cloned into the pHSG398 vector. *Escherichia coli* harboring the *ahlS*-expressing plasmids showed AHL-degrading activity. Therefore, it was revealed the *ahlS* homolohues on the genome of CNS strains function as AHL-degrading gene. We also isolated another AHL-degrading CNS strain, *Staphylococcus sciuri* StLB252, from the plant surface. To amplify the internal region of the *ahlS* homolog from StLB252, degenerate PCR primers were designed based on the *ahlS* gene from *S. haemolyticus*. The *ahlS* gene on the genome of StLB252 was successfully amplified by

PCR and showed AHL-degrading activity as well as the *ahlS* genes from other CNS strains. These results demonstrated that the *ahlS* genes are widely conserved in AHL-degrading CNS strains.

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EXTRACELLULAR POLYSACCHARIDE ALGINATE INTERFERE WITH CELL-CELL SIGNALING

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Most bacteria exist as communities such as biofilms in the environment, and bacteria use signaling molecule to communicate with each other in such kind of bacterial communities. It has been reported that bacteria detect their population by sensing the concentration of the signaling molecules and regulate the gene expression of the community, which is called quorum sensing. Bacteria use quorum sensing to control the group behavior. Interestingly, the frequency of spontaneous mutation in biofilms is very high, and various spontaneous mutants emerge in biofilms. However, the interaction between the mutants and the wild-type strain are still not fully understood. Therefore, the aim of this study is to investigate the cell-cell communication of spontaneous mutants. We chose the mucoid mutant of *Pseudomonas aeruginosa* as our research object, which is well known to dominate in *P. aeruginosa* biofilms in the lung of cystic fibrosis patients and overproduce extracellular polysaccharide alginate. In this study, we focused on the alginate overproduction phenotype of mucoid mutant, and examined if alginate interfere the signaling response of mucoid mutants. We compared how the mucoid mutant and nonmucoid strain response to PQS. Also, the PQS response in a nonmucoid strain was tested in the presence and absence of exogenous alginate. We found that alginate may interfere the response to PQS. The results suggested that mucoid mutant is not able to response to PQS. Our research imply that mucoid mutant may act as an uncooperative strain to group behavior in bacterial community because of its poor response ability to PQS.

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THE INTERFERENCE OF THE INTRACELLULAR SIGNALING MOLECULE C-DI-GMP ON CELL-TO-CELL COMMUNICATION

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Biofilm dispersion and cell-to-cell communication are highly related. Nitric oxide (NO) is one of the cues that has been shown to induce biofilm dispersion in many bacteria including *Pseudomonas aeruginosa*. Therefore the accumulation of NO inside biofilms is important in determining the timing of dispersion. Still how the accumulation of NO is controlled is undetermined. We demonstrated one of the communication signal *Pseudomonas* Quinolone signal (PQS) in *P. aeruginosa* induces NO-mediated biofilm dispersion. A delay in dispersion of a Δ*pqsA* mutant of *P. aeuginosa* PAO1, that could not produce PQS was observed compared to the parent strain. In this process, PQS promotes *nirS* gene expression during denitrification and accumulate Nitric Oxide. However, no significant difference of *nirS* expression is detected in presence or absence of PQS when cultured planktonically. Due to the difference of *nirS* gene expression between biofilm and planktonic cells, we considered that intracellular second messenger, c-di-GMP is involved. Here we analyzed the effect of c-di-GMP on cell-to-cell communication and demonstrate that c-di-GMP interferes PQS quorum sensing mediated regulation of denitrification gene.In this study, we construct *wspF* deletion mutant which can overproduce c-di-GMP and thus could mimic the biofilm state of cells. Besides, we deleted the *pel* and *psl* genes to prevent

aggregating of cells in liquid shaking culture as is used in other previous study. Hence, we used Δ*pel*Δ*psl* and Δ*pel*Δ*psl*Δ*wspF* strains to compare denitrification gene expression with or without PQS. Expression of *nirS* gene increased when PQS was added in the c-di-GMP overproducing strain Δ*pel*Δ*psl*Δ*wspF*, but not in the Δ*pel*Δ*psl* strain. No significant difference in the expression of *norB* gene, which encodes NO reductase was observed among the strains tested. Our data suggest that PQS promotes accumulation of NO by upregulating *nirS* gene expression in the presence of c-di-GMP. Our data further imply that c-di-GMP modulates PQS regulon.

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AMIE, A NOVEL *N*-ACYLHOMOSERINE LACTONE BELONGING TO THE AMIDASE FAMILY, FROM THE ACTIVATED SLUDGE ISOLATE *ACINETOBACTER* SP. OOI24

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A number of gram-negative bacteria use *N*-acylhomoserine lactones (AHLs) as quorum-sensing signal molecules. AHL-degrading genes have been cloned from various microorganisms and used to repress gene expression via AHL-mediated quorum sensing. In previous study, we have reported that *Acinetobacter* strains, which isolated from activated sludge, have AHL-degrading activity. For cloning of novel AHL-degrading gene, pUC118-based genomic library of *Acinetobacter* sp. strain Ooi24 was prepared. When approximately 10,000 clones were screened, one positive clone showed AHL-degrading activity and contained seven ORFs. Because sequencing analysis revealed that the fourth ORF, designated *amiE*, encoded the hydrolase family, we next determined whether *amiE* works as an AHL-degrading gene. To determine whether AmiE functions as an AHL-degrading enzyme, we used HPLC to analyze the structure of 3OC10-HSL digested by AmiE. HPLC analysis revealed that AmiE functions as an AHL acylase, which hydrolyzes the amide bond of AHL. AmiE showed homology with a member of amidases (EC 3.5.1.4) but not with any known AHL acylase enzymes. To analyze the substrate specificity of AmiE, we mixed a cell suspension of *Escherichia coli* harboring *amiE* gene with a wide range of AHLs. AmiE degraded C8-HSL, C10-HSL, and C12-HSL and showed the highest degrading activity against C10-HSL, but it did not degrade C6-HSL. These results suggested that AmiE has activity similar to those of known AHL acylases. An amino acid sequence of AmiE from Ooi24 showed greater than 99% identities with uncharacterized proteins from *Acinetobacter ursingii* CIP 107286 and *Acinetobacter* sp. CIP 102129 but it was not found in the genome sequences of other *Acinetobacter* strains. The presence of transposase-like genes around the *amiE* genes of these three *Acinetobacter* strains suggests that *amiE* was transferred by a putative transposon. Furthermore, the expression of AmiE in *Pseudomonas aeruginosa* PAO1 reduced AHL production and elastase activity, which was regulated by AHL-mediated quorum sensing.

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A SMALL-MOLECULE INHIBITOR OF SMCR, A QOURUM SENSING MASTER REGULATOR, IN VIBRIO VULNIFICUS

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Bacterial cell-to-cell communication known as quorum sensing (QS) is involved in diverse physiological processes including biofilm development and pathogenesis. An opportunistic human pathogen Vibrio vulnificus also has a well conserved QS system consisting of the homologues of V. harveyi Autoinducer-2 (AI-2) signaling components. Among them, SmcR, a homologue of LuxR, plays a critical role in regulation of various QS regulon as a master transcriptional regulator. Previously, we determined the crystal structure of SmcR and

revealed that SmcR belongs to the TetR family protein possessing putative ligand-binding pockets. To identify the small molecules which inhibit the function of SmcR, we performed high-throughput screening of 8844 molecules at 20 µM concentration using Escherichia coli dual plasmid system containing SmcR-expressing and SmcR-dependent reporter plasmids. As a result, eight molecules were screened out and verified. Among them, a chemical 377B6 exhibiting the most effective SmcR-inhibition activity was selected as a SmcR inhibitor. Effect of 377B6 on biofilm development of V. vulnificus was assessed using an assay based on crystal violet staining at different time points. 377B6 significantly inhibited biofilm detachment of wild type V. vulnificus to the smcR mutant level. Furthermore, to determine the influence of 377B6 on the expression of SmcR-dependent and -independent genes, the transcriptomic changes induced by 377B6 in V. vulnificus biofilm cells were analyzed by RNA-sequencing. In conclusion, the combined results suggested that this chemical could be a novel lead compound to interfere the QS of V. vulnificus.

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QUORUM QUENCHING N- ACYL HOMOSERINE LACTONASE FROM SOIL ISOLATE LYSINIBACILLUS SP. GS50

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Quorum sensing is an example of cell-to-cell communication in bacteria and depends on the production, secretion and response to small, diffusible signal molecule called autoinducer which is Acyl Homoserine Lactone (AHL) in Gram negative bacteria. Lysinibacillus sp. Gs50 isolated form soil was found to be the best among 97 isolates screened for their AHL degradation ability but not able to utilise it as sole carbon source for growth. AHL-degrading enzymes such as lactonase has been widely characterized in Bacillus species but has not been yet reported in Lysinibacillus sp. Isolate Lysinibacillus sp.Gs50 treated C6-AHL could be restored under acidic conditions indicated the presence of putative AHL lactonase in it. The isolate was able to degrade AHL of various acyl chain lengths. Furthermore, the enzyme was found to be localised into the membrane of the bacteria. To identify the gene responsible for AHL degradation, primers were designed against hypothetical protein Bsph_3377 [Lysinibacillus sphaericus C3-41] (NCBI Reference Sequence: YP_001698999.1) for the putative AHL lactonase .Using these primers gene was amplified and cloned into expression vector pET22b(+) which resulted in the expression of 35kDa size protein. The soft rot attenuation studies on potato by recombinant E.coli BL21 against the plant pathogen Pectobacterium carotovorum carotovorum BR1 (PccBR1, laboratory isolate) and Mass spectrum analysis to ascertain the mechanism of the enzyme are being conducted. Thus, this is the first report of the quorum quenching N-acyl homoserine lactonase from Lysinibacillus sp. and its application to quorum quenching as biocontrol mechanism against plant pathogen Pectobacterium carotovorum carotovorum BR1, the causal agent of soft rot.

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DEGRADATION OF N-ACYLHOMOSERINE LACTONE QUORUM SENSING SIGNALING MOLECULES BY FISH-ASSOCIATED FLAVOBACTERIUM ISOLATES

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Members of the genus *Flavobacterium* are often implicated in aquaculture disease outbreaks worldwide. Their biofilm-forming ability, virulence, recalcitrance to antimicrobial therapy often leads to recurring outbreaks in fish farms and economic losses. Their ability to communicate *via* quorum sensing (QS) and/or to quench QS was investigated in order to understand the role of cell-to-cell communication in *Flavobacterium* pathogenicity and survival.

Twenty-eight *Flavobacterium johnsoniae*-like isolates from South African trout, salmon, eel and fish tank biofilm and six *Flavobacterium* spp. type strains were assessed for acyl-homoserine lactone production (AHL) using *Chromobacterium violaceum* CV026 and VIR07 and *Agrobacterium* A136 biosensor T-streak assays. Quorum quenching (QQ) was assessed using *C. violaceum* CV026 (short-chain AHL) and VIR07 (long-chain AHL) biosensor sandwich assays as well as AHL-degrading assays (C4 – C12 AHLs). QQ was correlated with presence of lactonase gene, *aidC*, using primer sets 1 (PS1) and 2 (PS2) and PCR amplification. No AHL production was observed with all biosensor T-streak assays. QQ was only observed for 17.9% (5/28) of *F. johnsoniae*-like isolates using the short-chain *C. violaceum* CV026 biosensor sandwich assay, while no QQ was observed with the long chain VIR07 system. Both short- and long-chain AHL degradation was observed for all *F. johnsoniae*-like isolates using the AHL degradation assay, however, type strains did not display QQ abilities. The AidC lactonase gene was amplified from 35.7% (10/28) and 10.7% (3/28) of *F. johnsoniae*-like isolates using PS1 and PS2, respectively. While QS has not been observed for *Flavobacterium* species, *F. johnsoniae*-like possess the ability to enzymatically degrade and prevent cell-to-cell signalling of other organisms within their immediate environment. This would allow them to exclude competitors, thus facilitating their survival. The lack of QS might be attributed to limitations of biosensors used and/or use of signalling molecules other than AHLs. While the *aidC* lactonase has been identified in some of the isolates tested, other QQ enzymes (lactonases and//or acylases) originating from diverse sources may be responsible for the observed QQ.

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DECIPHERING THE MOLECULAR STRUCTURE AND FUNCTION OF VIRB4-LIKE ATPASES IN NUCLEOPROTEIN IMPORT AND EXPORT

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Bacterial type IV secretion systems (T4SS) transfer macromolecules like nucleic acids and proteins from bacteria to eukaryotes or between bacterial cells generally by a direct contact dependent communication mechanism. VirB4-like proteins are associated with all T4SSs described to date. These signature ATPases function in assembly of the T4SS channel and biogenesis of extracellular pili in Gram-negative systems. They are also required for translocation of secretion substrates and nucleoprotein uptake during pilus-mediated phage infection. Very little is known about the regulation of VirB4-like ATPases in protein trafficking. It is also not known whether these proteins are involved in recognition of substrates destined for secretion. Our paradigm to study control of VirB4-like ATPases is TraC of the F-like conjugative plasmid RI. TraC was modified with a strep-tag and its function verified by complementation of conjugative transfer of a αtraC derivative of R1. However, purified strep-TraC did not exhibit ATPase activity in vitro. One possible explanation may be that ATPase activity is controlled by oligomeric state. Hexamers of VirB4-like protein TrwK hydrolyze ATP in vitro and ATP binding forces the formation of hexamers. Strep-TraC was incubated with a 20-fold molar excess of non-hydrolysable ATP and analyzed by gel filtration. The chromatograms indicate that strep-TraC behaves as a monomer in solution with and without ATP. The ATPase activity

might be controlled by interaction(s) of TraC with other relaxosome proteins. Possible interaction partners are currently under investigation. Enzymatic activity of VirB4-like ATPases can also be controlled intra-molecularly. The C-terminus of the VirB4-like protein TrwK possesses alpha helices, which were shown to have an auto-inhibitory activity. Secondary structure prediction of TraC shows four conserved alpha helices. The significance of these alpha helices for enzyme regulation is under investigation. Interestingly, truncated versions of TraC, lacking individual alpha helices are unable to complement the function of wild type traC in conjugative transfer and phage infections. To test the hypotheses that TraC is subject to auto-inhibition and that the alpha helices mediate protein-protein interactions, truncated versions of TraC will be purified. The ability of truncated TraC to hydrolyze ATP will be tested with and without other potential binding partners. Also, the purified truncated versions of TraC will be used in protein-protein interaction studies with other relaxosome proteins. Furthermore, these TraC truncated variants are used in phage-infection assays to study nucleoprotein uptake by T4SS.

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A NATURAL PRODUCT-BASED CYSTEINE DERIVATIVE INHIBITS QUORUM SENSING AND INDUCES IRON STARVATION RESPONSE IN PSEUDOMONAS AERUGINOSA

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Pseudomonas aeruginosa is a Gram negative opportunistic pathogen that utilizes multiple quorum sensing (QS) pathways to coordinate group behaviors such as biofilm formation and virulence. Small molecule inhibitors that can antagonize QS are desired as they hold the potential to control bacterial infections while reducing the selective pressure incurred by traditional antibiotics. Previously we identified several cysteine-based derivatives inspired by natural products from the plant Petiveria alliacea, which are capable of antagonizing multiple different quorum sensing circuits. One of these compounds, S-phenyl-L-cysteine sulfoxide (compound 7), was shown to inhibit las-based QS in both E. coli and P. aeruginosa-based reporters as well as reduce biofilm formation and increase Drosophila survival in a P. aeruginosa infection model. In the current work we have performed a global transcriptomic analysis of P. aeruginosa PAO1 when exposed to compound 7 using Illumina-based RNA-sequencing. Interestingly, many genes that were observed to be differentially regulated pertain to secondary metabolism (e.g. anthranilate biosynthesis/degradation) and iron starvation response. Many of these genes have also been shown to be regulated by QS. For example, phnAB (anthranilate biosynthesis) and phzA-G (phenazine biosynthesis) which are positively expressed in the presence of 3-oxo-C12-HSL (the autoinducer for the las pathway) are down-regulated in our experiments. Additionally, we observed that antABC (anthranilate degradation), which is repressed in the presence of 3-oxo-C12-HSL, were amongst the most highly up-regulated genes when exposed to compound 7. Because of the potential anthranilate depletion that may be suggested from these results, there may be less Pseudomonas Quinolone Signal (PQS) produced, as anthranilate is the precursor for PQS biosynthesis. Other highly up-regulated genes include those involved in pyochelin and pyoverdine biosynthesis (two iron-binding siderophores) which suggests an iron starvation response. Although it is well established that QS and iron regulation are intricately intertwined in P. aeruginosa, it is unclear exactly how compound 7 contributes to the observed response. Investigations are currently underway to determine the molecular target of compound 7. It is anticipated that this information will enable the design of more potent QS inhibitors.

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TERPENOID COMPONENTS OF AFRICAN VERNONIA SPECIES AS QUORUM SENSING INHIBITORS

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Despite the widespread use of the genus *Vernonia* in traditional medicine, there is insufficient information on the mechanism/s by which they exert a medicinal effect. Given the increasing incidence of multidrug-resistant pathogens, the medicinal potential of terpenoids from four *Vernonia* species was assessed as natural, plant-based anti-virulence compounds, by inhibition of microbial quorum sensing (QS). The antimicrobial activity of terpenoid components from four *Vernonia* species was assessed against both Gram-positive and Gram-negative bacteria using disc diffusion assays. Inhibition of QS-controlled violacein production in *Chromobacterium violaceum* was quantified using the violacein inhibition assay (extracts ranging from 0-9.5 mg/ml). Qualitative modulation of QS activity and signal synthesis was investigated using the agar diffusion double ring assay and *C. violaceum* and *Agrobacterium tumefaciens* biosensor systems. Varying antimicrobial activity was observed with *V. blumeiodes* extract being the most effective and *V. ambigua* the least effective. Inhibition of QS-controlled violacein production in *Chromobacterium violaceum* was quantified using violacein inhibition assays and was significant in the following order: *V. glaberrima* > *V. ambigua* > *V. blumeiodes* = *V. perrotetii*. Inhibition was concentration-dependent, with the ≥ 90% inhibition being obtained with ≥ 0.6 mg/ml. Qualitative modulation of quorum sensing activity and signal synthesis was investigated using the agar diffusion double ring assay. Both LuxI and LuxR activity were affected by *V. glaberrima* and *V. perrotetii* terpenoids suggesting that they target both quorum sensing signal synthesis and receptor activity, while *V. ambigua* and *V. blumeiodes* terpenoids inhibited signal synthesis (LuxI). *Vernonia* terpenoid components have the potential to be novel therapeutic agents, which might be important in reducing virulence and pathogenicity of drug-resistant bacteria *in vivo*.

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CHIVALRY IN RIVALRY: AN INSIGHT INTO INTERSPECIFIC AND INTRASPECIFIC INTERACTIONS IN SOIL DWELLING BACILLUS CEREUS MSM-S1

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Bacteria have come a long way from being considered as solitary recluses to social organisms. In a natural environment like soil, bacteria communicate with each other using specialized chemical signaling process called quorum sensing (QS). The nature of communication through QS does not always involve co-operative signals rather they often evoke conflicts within and between the species. In spite of extensive research on bacterial interactions, the basic question remains to answer is the detailed molecular mechanisms of signal mediated interference in bacterial interactions. To address the problem, we have investigated the molecular mechanisms involved in soil dwelling Bacillus cereus MSM-S1 during inter- and intraspecific competitive interactions. We show that a single soil bacterium Bacillus cereus MSM-S1 adapt multiple strategies during interspecific and intraspecific interactions. For interspecific interaction, Bacillus cereus MSM-S1 loses the battle against Pseudomonas sp. MSM-M1 by a secreted antibacterial compound of the later in the media. In the case of sibling rivalry between the colonies of Bacillus cereus MSM-S1, the cells stop moving towards each other by inhibition in QS. Our investigation involving Real Time PCR also reveals differential expression of numbers of genes in competing cells of Bacillus cereus colonies and such differentially expressed genes are

involved in spore formation, chemotaxis, flagellar motions, cell division, production of virulence factors and quorum sensing. Interestingly, comparison of cellular protein profiles between interacting and non-interacting edges of Bacillus cereus MSM-S1 colonies show over-expression of a number of proteins and mass spectrometry analyses confirmed that these are associated with bacterial stress response. Therefore, the present study revolves around the ecological and evolutionary significance of understanding the molecular dynamics of signaling network involved in interspecific and intraspecific interactions in bacterial world as the dynamics direct the bacteria to choose between co-operation, co-existence and competition.

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PSEUDOMONAS AERUGINOSA QUORUM SENSING IS ALTERED BY SERUM ALBUMIN

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Chronic wound infections cause high rates of morbidity and mortality in a large patient population and are responsible for a large medical cost burden in the US annually. *Pseudomonas aeruginosa* (PA) is one of the most common bacterial pathogens in chronic wounds, and much of the virulence associated with PA is controlled by a system of cell-cell communication termed quorum sensing, or QS, which involves three distinct regulatory systems (Las, Rhl and PQS). QS can alter the expression of over 5% of PA genes in response to the amount of specific chemical signals, or autoinducers, within an environment. Our lab has previously shown that the blood protein albumin inhibits the ability of PA to lyse *Staphylococcus aureus* and we hypothesize this is due to an inhibition of QS by albumin. In this study, we sought to identify how albumin is altering QS. We observed that QS-controlled exoproducts, such as LasA and LasB, were downregulated when PA was grown in the presence of albumin. We also saw that growth in albumin inhibited the production of the acylated homoserine lactones (AHL) 3OC12-HSL and C4-HSL, but promoted the production of quinolone-related autoinducers. Preliminary binding assays suggest that albumin can bind and sequester AHL-based autoinducers, which represses QS. We are now investigating the physiological relevance of these observations in albumin-rich and albumin-deplete *in vivo* environments.

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EFFECT OF STATIC MAGNETIC FIELD IN THE AUTOAGGREGATION PHENOMENON OF ENTEROPATHOGENIC ESCHERICHIA COLI (EPEC)

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Enteropathogenic Escherichia coli (EPEC) constitute an important cause of infant diarrhea in developing countries. A three-stage EPEC pathogenesis model was described, however, properties as autoaggregation, also are involved in the bacterial pathogenesis. The static magnetic field (SMF), has been used to evaluate its effect on bacteria growth, but little on pathogenicity properties. In this work was analyzed the SMF effect on EPEC autoaggregation, considering time and intensity of ferrite or neodymium magnets in a static model. Strains E coli HB101 and EAEC (enteroaggregative E coli) 49766 (O?:H10), were used as negative and positive controls respectively. Strain E2348/69 EPEC wild type and mutant strains in type III secretion system (T3SS), bundle-forming pilus fimbriae, flagella and EspC (autosecreted protein C) were used to evaluate the magnet effects. An autoaggregation activity is considered positive when autoaggregation index is over 2.5% and negative if the result is less. For autoaggregation assay measure the O.D 600 nm each culture, after each measurement the aliquot was vortexed for 30 s while still in the cuvette and the O.D. 600 nm was measured again. The percent increase

in O.D. 600 nm after vortexing was recorded as a quantitative aggregation index. The results showed that a treatment during 30 min exposure or more with 100 mT reduce significantly the autoaggregation in E2348/69 wild type strain, when it´s compared with untreated strain. The EPEC mutant in T3SS shows similar effects to the observed in the wild type EPEC strain. However, bundle-forming pilus fimbriae, flagella and EspC mutants, the autoaggregation activity is negative and SMF treatment do not induce changes. Although, the specific is not clear the SMF mechanism of action, is possible that it is inducing interference in cell to cell interactions, or regulating the gene expression.

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IDENTIFICATION OF AUTOINDUCING PEPTIDE OF CLOSTRIDIUM PERFRINGENS AND DEVELOPMENT OF ITS INHIBITORS

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Quorum sensing (QS) system of Gram-positive bacteria uses lactone and thiolactone autoinducer peptide (AIP) to modulate expression of certain gene(s), particularly associated with virulence in case of pathogenic bacteria. *C. perfringens* 13 is an anaerobic pathogen, causative agent for gas gangrene (myonecrosis) and produces various toxins, such as α, θ and κ encoded by *plc*, *pfoA*, and *colA*, respectively. It has been found that activation of VirR/VirS system enhances transcription of these toxins which have been implicated in disease process. To identify AIP of the VirR/VirS system, we analyzed culture filtrate of *C. perfringens* 13 by liquid chromatography-mass spectrometry and detected 5- and 6-amino acid residue dehydrated oligopeptides whose molecular weight were correspond to those deduced from amino acid sequence of $AgrD_{Cp}$. The 5- and 6-residue oligopeptides (cyclo(CLWFT) and A-cyclo(CLWFT)) were chemically synthesized and *in vitro* analyzed for their potential to induce QS phenomenon in *agr* mutant *C. perfringens*. The 5-residue oligopeptide showed significantly stronger activity to induce the transcription of perfringolysin O gene (*pfoA*). Furthermore, based on the determined structure of AIP, we designed analogue inhibitors of AIP targeting two component (VirR/VirS) regulatory system of *C. perfringens* 13. As a result, we obtained several thiolactone oligopeptides which are suppressed the transcription of *pfoA* around micromolar concentration. By using those oligopeptides as leading compounds, we will develop an effective antipathogenic drug targeting the cyclic peptide-mediated cell-to-cell communication system of *C. perfringens* 13.

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DEFINING LIGAND SPECIFICITY FOR THE LUXN QUORUM-SENSING RECEPTOR

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Quorum sensing (QS) is a process of cell-cell communication that bacteria use to control collective behaviors. QS relies on the production, release, and receptor-driven detection of extracellular signal molecules called autoinducers. Gram-negative bacteria commonly use acyl homoserine lactones (AHLs) as autoinducers. The bioluminescent marine bacterium Vibrio harveyi relies on N-(3-hydroxybutanoyl)-L-homoserine lactone (3OH-C4 HSL) for QS. The cognate transmembrane histidine kinase receptor, LuxN, is exclusively agonized by 3OH-C4 HSL. AHLs with differing chemical modifications at the C3 position (3-O and 3-H) and those with longer carbon tail lengths (C6 - C12) competitively antagonize LuxN. To identify residues in LuxN governing ligand specificity, we modified AHL structures by chemical synthesis and altered the companion LuxN receptor binding pocket by mutagenesis. We pinpointed His210 as responsible for recognition of the C3 modification, and identified Leu166 as the determinant for chain-

length preference. We also isolated a series of LuxN mutants that are constitutively biased to the agonized state. These mutations reside in the region of the transmembrane domain immediately following the ligand-binding site. We propose that this region acts as the switch that triggers signal transduction. Together, our analyses allowed us to dissect how a histidine sensor kinase differentiates between ligands, and uses that information to regulate kinase activity.

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INTEGRATION OF SIRA/BARA AND LSR REGULATORY SYSTEMS IN SALMONELLA TYPHIMURIUM

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Quorum sensing is a method of bacterial communication developed to regulate gene expression in response to population density. *Salmonella* Typhimurium uses autounducer-2 (AI-2) signaling molecule to regulate the *Salmonella* pathogenicity island-1 (SPI-1) Type 3 Secretion System (T3SS), flagellar gene expression, and biofilm formation. The AI-2 is sensed by the *lsr*-encoded transport cassette and is actively transported into the bacterial cell. Following intracellular phosphorylation, AI-2 negatively regulates the transcriptional repressor protein LsrR, allowing the transcription of *lsr*- and SPI-1 – encoded genes. The global regulatory RNA-binding protein CsrA also regulates the SPI-1 T3SS and is, in turn, regulated through the SirA/BarA-CsrB/C-CsrA regulatory cascade. The interaction between the SirA/BarA two-component system and the *lsr* operon are unknown. The goal of this study was to determine whether SirA/BarA regulates *lsr* expression and AI-2 transport in *Salmonella* Typhimurium. AI-2 production was measured in mutant (*luxS, lsrR, lsrD, sirA, barA, csrA, and csrBC*) and parent (ATCC14028) strains of *Salmonella* Typhimurium using the luminescent response of reporter *V. harveyi* to *Salmonella* AI-2. The β-galactosidase activity of the *lsrR-lacZ* fusion was assayed at OD_{600} = 0.6 in parent and mutant (*sirA*, *barA, csrB*, *csrC*, *csrBC*, and *csrA*) strains. As reported previously, the *lsrD* mutant was impaired in internalizing AI-2, while an *lsrR* mutant was more efficient in AI-2 internalization than the parent strain at two hours of growth. Despite *lsrD* deletion, AI-2 concentration in the extracellular media oscillated over time indicating an alternative transport system. Due to de-repression of the *lsr* operon AI-2 was removed from the extracellular medium faster by the *sirA*, *barA* and *csrBC* mutants, and slower by a *csrA* mutant compared to parent strain at 3h of growth. Mutants in *sirA*, *csrB*, and *csrBC* exhibited decreased *lsrR-lacZ* expression while a *csrA* mutant showed higher expression compared to parent strain. These results imply that LsrR is normally repressed by CsrA, and the SirA/BarA two-component system is involved in *lsr* regulation by controlling *lsrR* expression. Therefore, a SirA-LsrR regulatory cascade could be important in coordinating *Salmonella* AI-2 signaling and virulence.

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DETECTION OF AUTOINDUCERS FROM VEILLONELLA TOBETSUENSIS AND THEIR ROLES IN BIOFILM FORMATION

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Backgrounds: It is suggested that oral Veillonella has important roles in the biofilm formation at early stage. Our previous study demonstrated that the supernatants from V. tobetusensis had inhibiting effects for the biofilm formed by Streptococcus gordonii, but in case of existing bacterial cells of V. tobetsuensis, the biofilm formation was promoted. It was suggested that some factors in supernatants may affect the biofilm formed by S. gordonii. **Purpose:** In this study, autoinducer-1 and autoinducer-2 (AI-1 and AI-2) were focused as one of the factors in the supernatants and tried to detect and purify from the supernatants.

After purification, the effects of autoinducers on biofilm formation were examined. **Method:** The crude AI-1 was extracted by ethyl acetate from the culture supernatants of V. tobetsuensis. Then these extracts were evaporated to dryness after filtration. The residues dissolved in ethanol were used in bioassay and thin layer chromatography using C. violaceum CV026 and R. radiobacter NTL4 (pZLR4) to detect AI-1. AI-2 activities in the supernatants were detected by using Vibrio assay as reported previously. The supernatants were filtered through a 0.2-µm-pore-size filter and subsequently through an Amicon Ultra Centrifugal Filters Ultracel-3K (Millipore). The filtrate were lyophilized, suspended in cold sodium phosphate buffer, and chromatographed on a C18 Sep-Pak reverse-phase column (Waters Co.) according to the manufacturer's instructions. AI-2 activity in the column fractions was followed by monitoring the induction of bioluminescence of Vibrio harveyi BB170 (sensor 1- sensor 2+). After this partial purification, the effects of AI-2 like substance for biofilm formation were examined by using wire method. **Results:** AI-1 was detected in the supernatants from V. tobetsuensis when only R. radiobacter NTL4 (pZLR4) was used. Also, AI-2 like activity was detected strongly in the supernatants from V. tobetsuensis. Partial purification of AI-2 was succeeded and the bioluminescence level was about 10 times compared with that of the supernatants. Furthermore, AI-2 like substance from V. tobetsuensis inhibited the biofilm formed by S. gordonii. **Discussion & Conclusion:** According to prosperity of R. radiobacter NTL4 (pZLR4) and the result of thin layer chromatography, V. tobetsuensis may produce long chain N-acyl homoserine lactone as AI-1. It is the first report that AI-1and AI-2 from V. tobetsuensis has affected the biofilm formation.

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A BIOINFORMATIC SURVEY OF DISTRIBUTION, AMINO ACID CONSERVATION AND PROBABLE FUNCTIONS OF LUXR SOLO REGULATORS IN BACTERIA

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LuxR solo transcriptional regulators contain both an autoinducer binding domain (N-terminal end) and a DNA binding domain (C-terminal end), but are not associated with a N-acyl homoserine lactone (AHL) synthase coding gene in the same genome. Although a few LuxR solos have been characterized, their distributions as well as their role in bacterial signal perception and other processes are poorly understood. In this study we have carried out a systematic survey of distribution of all LuxR transcriptional regulators harboring both domains available in the InterPro database (IPR005143), and identified those lacking a cognate AHL synthase. These proteins were analyzed regarding their distribution, predicted functions for neighboring genes, the presence of AI-2 signaling or endogenous AHL-QS systems. Our preliminary analyses reveal the presence of multiple predicted LuxR solos in many proteobacterial genomes, most of them harboring genes for one or more AHL-QS circuits. Surprisingly, putative LuxR solos were also found in a few non-proteobacterial genomes, which are not reported to have AHL-QS systems. Multiple predicted LuxR solos in the same genome appeared to have different levels of conservation of invariant amino acid residues of autoinducer binding domain questioning their response to AHLs. Finally, presence of LuxR solos in bacteria occupying diverse environments suggests potential ecological functions for these proteins beyond AHL and interkingdom signaling.

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CHARACTERIZATION OF A QUORUM SENSING REGULATED SMALL RNA IN VIBRIO HARVEYI

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At the heart of the *Vibrio harveyi* quorum sensing (QS) circuit lie five homologous small RNAs (sRNAs), Qrr 1-5 (quorum regulatory RNAs). The Qrr sRNAs control the levels of AphA and LuxR, the master low cell density (LCD) and high cell density (HCD) QS regulators, respectively. Hence, the Qrrs are key dictators of whether *V. harveyi* will engage in individual or group behavior. Given the critical role that the Qrr sRNAs play in QS, I am investigating whether there exist additional sRNAs that influence QS. Using high-throughput whole genome microarrays and RNA-sequencing, the Bassler group recently discovered dozens of intergenic sRNAs in *V. harveyi*, which could play roles in QS. I study one of these sRNAs, QsrA (quorum sensing-regulated sRNA A), which exhibits more than 30-fold gene expression change between LCD-locked and HCD-locked *V. harveyi* strains. Northern blot analysis confirmed that QsrA exhibits cell density-dependent production, where it is more highly expressed at HCD than at LCD. Promoter-reporter fusion assays also show that QsrA is directly activated by LuxR and that it is repressed by AphA. Here, I explore whether LuxR and AphA have specific requirements to bind to the QsrA promoter and regulate *qsrA* expression. Additionally, I investigate the biological role of QsrA in *V. harveyi*. Toward this end, I have identified putative QsrA targets by comparing the transcriptomes of wildtype and a *qsrA* knockout *V. harveyi* strain. Preliminary analysis of the most highly regulated targets suggests that QsrA could be involved in the transition from the motile planktonic state to the sessile biofilm state. Using fluorescence and confocal microscopy, I test the effect of QsrA on motility and biofilm formation. Discovery of QsrA supports my thesis that the Qrrs are not the only sRNAs that participate in QS. My characterization of QsrA will elucidate finer details of the *V. harveyi* QS circuit and could lead to novel ways to manipulate QS-mediated behaviors, such as biofilm production and virulence, in *V. harveyi* and possibly other Vibrio species.

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INDOLE SIGNAL INDUCES MULTIDRUG TOLERANCE IN UROPATHOGENIC ESCHERICHIA COLI (UPEC)

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Background: Some species of enterobacteria including Escherichia coli use indole for their social behaviors. TnaA is a synthetic enzyme for indole production from tryptophan, and its expression is activated at high cell density, and also by indole. Therefore, indole production can be auto-regulated like acyl-homoserine lactone quorum sensing signals. We previously studied on indole-mediated social behavior in non-pathogenic E. coli laboratory strain. **Result:** Here, we show roles of indole signal in uropathogenic E. coli (UPEC). We found that indole induces tolerance to several antibiotics including beta-lactams, fluoroquinolones and fosfomycin. The indole-induced multidrug tolerance is attributed to up-regulation of drug efflux genes because UPEC cells grown in the presence of indole had higher expression of drug efflux genes, acrD, mdtEF, yceE and yceL than those in the absence of indole. Wild-type UPEC is able to produce 2 mM of indole at the stationary phase when grown in tryptophan-supplemented medium. We observed expression of these drug efflux genes were activated in a conditioned medium from the tryptophan-supplemented wild-type stationary phase culture but not that from tnaA mutant culture because the tnaA mutant did not produce indole. We also found that induction of acrD but not mdtEF, yceE and yceL requires

two-component signal transduction system (TCS), CpxAR and BaeSR. **Conclusion:** These results suggest that indole acts as a quorum sensing signal instead of acyl-homoserine lactones for UPEC, then induces drug efflux genes through TCS-dependent and independent-pathways, results in increased tolerance to multiple drugs. We presume that indole-type quorum sensing may contribute to an innate bio-defense for UPEC against antibiotic producers in nature.

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STRUCTURE-FUNCTION STUDIES OF GRAM-POSITIVE CELL-CELL SIGNALING AND DEVELOPMENT

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Bacterial quorum sensing is a cell-cell communication process driven by pheromones. At low cell density, in the absence of appreciable amounts of pheromones, bacteria act as individuals. At high cell density, bacteria respond to the pheromones by synchronizing the gene expression of the community. Thus, quorum sensing allows groups of bacteria to coordinate social behaviors including virulence factor expression, motility, biofilm development, bioluminescence, antibiotic production, and genetic competence. Typically,Gram-positive bacteria use oligopeptides as pheromones. Oligopeptide pheromones are synthesized as immature propeptides. The immature pro-peptides are secreted from the cell and subsequently undergo proteolytic maturation. Outside of the cell, the mature peptides can bind to and regulate membrane receptors. Alternatively, the mature peptides can bind to oligopeptide permeases that import the oligopeptides into the cell. Here the oligopeptides bind to and regulate cytosolic receptors. Despite their obvious importance, the mechanistic basis of oligopeptide receptor function and regulation in Gram-positive species is largely unknown. We have used a combination of biophysical (mainly X-ray crystallographic), biochemical, and genetic approaches to determine at the atomic level how Gram-positive quorum-sensing receptors function and how the receptor activities are modulated by oligopeptide pheromones to regulate cell development.

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SELECTIVE INTERACTION OF MEMBRANE VESICLES WITH BACTERIAL CELLS

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Many bacteria secrete membrane vesicles (MVs) ranging from 20 to 200 nm in diameter. MVs contain bacterial important substances including DNA, proteins and low-molecular compounds at high concentrations. Since MVs deliver their content to prokaryotic cells as well as eukaryotic cells, they play an important secretion system for predatory, horizontal DNA transfer and quorum sensing, in microbial communities. However, little is understood the details about the fusion of MVs with bacterial cells. In this study, we aimed to investigate whether MVs selectively interact with specific bacterial cells and obtain new insights into MV-mediated communication between MV-forming bacteria and other microbes. MVs were extracted from each bacterial culture, labeled with a fluorescent reagent FM4-64 and incubated with bacterial cells. After removing freely suspended MVs, interaction of MVs with bacterial cells was quantified as fluorescent intensities associated with bacterial cells using a microtitre plate reader. MVs secreted from Pseudomonas aeruginosa PAO1, which is an opportunistic Gram-negative pathogen, interacted with all bacterial cells tested in this study. The interaction between P. aeruginosa MVs and other bacterial cells were also confirmed through detecting the fluorescence of MVs using flow cytometry analysis, suggesting that MVs derived from P. aeruginosa non-specifically attach or fuse with many kinds of bacterial cells. On the other

hand, MVs secreted from an enterobacterium Buttiauxella agrestis JCM 1090T, which is the most highly MV-forming bacterium tested in this study, interacted with own cells at a high frequency, while did not with other bacterial cells. These results provide striking examples of specific and non-specific interactions of MVs with bacterial cells, and indicate selective properties in MV-mediated cell-cell interaction. Elucidation of mechanisms for interaction of MVs with specific cells would contribute to understanding of the intracellular substance transfer through MVs in heterogeneous microbial communities.

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HOST-MICROBE SMALL MOLECULE SIGNALING

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Mammals have coevolved with commensal bacteria, the majority of which reside in the gut. In a process called quorum sensing (QS), bacteria communicate using chemical signal molecules called autoinducers to synchronize group behaviors. Given the size of the commensal population and its intimate contact with intestinal surfaces, it is likely that these bacteria profoundly influence many aspects of intestinal physiology. However, it is not known how mammals perpetuate beneficial relationships with gut symbionts, and we have a limited understanding of the role that QS plays during bacterial gut symbiosis. The goal of this project is to assess the role of quorum sensing (QS) in host-microbial interkingdom signaling. Preliminary studies show that mammalian host tissues produce a mimic activity that acts analogously to AI-2, the universal bacterial QS autoinducer. This signal differs structurally from bacterial AI-2, but can agonize AI-2 receptors to activate QS responses. Mammalian cells produce mimic when co-cultured in the presence of live but not dead bacteria, implicating an active role for bacteria in stimulating the mammalian cells to produce this activity. Ongoing studies will identify the AI-2 mimic and provide evidence that the host can engage in a bi-directional small-molecule conversation with its commensal counterparts.

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A HIERARCHY QUORUM SENSING SIGNALING NETWORK CONTROLS DIVERSE BIOLOGICAL FUNCTIONS IN BURKHOLDERIA CENOCEPACIA

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Many bacterial pathogens produce quorum sensing (QS) signals in regulation of various biological functions. Burkholderia cenocepacia, which is an important opportunistic human bacterial pathogen, employs N-acyl homoserine lactone (AHL) and cis-2-dodecenoic acid (BDSF) QS systems in regulation of bacterial virulence. It is intriguing how these two QS systems act together in regulation of similar biological functions. Our recent studies showed that the two QS systems constitute a hierarchy signaling network in regulation of swarming motility, biofilm formation and virulence in B. cenocepacia. We found that BDSF signal negatively controls the intracellular cyclic dimeric guanosine monophosphate (c-di-GMP) level through a receptor protein RpfR, which contains Per/Arnt/Sim (PAS)-GGDEF-EAL domains. RpfR regulates the same phenotypes as BDSF signal. In addition, the BDSF− mutant phenotypes could be rescued by in trans expression of RpfR, or its EAL domain that functions as a c-di-GMP phosphodiesterase. BDSF signal is shown to bind to the PAS domain of RpfR with a high affinity and stimulates its phosphodiesterase activity through induction of allosteric conformational changes. Interestingly, BDSF system also controls AHL signal production through regulation of the

AHL synthase CepI expression at transcriptional level by modulating the intracellular level of the second messenger c-di-GMP. Moreover, we showed that the BDSF and AHL systems have a cumulative role in regulation of various biological functions, including swarming motility, biofilm formation and bacterial virulence, and exogenous addition of either BDSF or AHL signal molecules could only partially rescue the changed phenotypes of the double deletion mutant defective in BDSF and AHL signal production. In combination, our work presents a unique and widely conserved cis-unsaturated fatty acid type QS signal receptor that directly links the signal perception to c-di-GMP turnover, and establishes a hierarchy QS signaling network in regulation of bacterial physiology in B. cenocepacia.

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PHEROTYPE AND SPECIES-SPECIFIC SIGNAL INTEGRATION AT QUORUM SENSING PROMOTERS

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Quorum sensing is a common regulatory strategy to control the production of common goods. The underlying molecular signaling networks used for quorum sensing show often puzzling complexity. Frequently several signals converge to regulate target gene expression. For example, in Bacillus subtilis strain-specific pherotype signals and species-specific signals converge to regulate the activity of the transcription factor ComA by modulating its phosphorylation state and its ability to bind DNA, respectively. How the different signals are integrated at the level of target promoters is not well understood. Here, we elucidate mechanistic determinants of promoter activation by pherotype and strain-specific signals using a comprehensive set of in vitro DNA binding studies and in vivo studies of promoter activities. We demonstrate that cooperative binding of the transcription factor at the promoter is required for pherotype signaling. Our results indicate that full promoter activity of natural target genes requires a strain-specific quorum, suggesting that maximum investment into the production of common goods is favored in homogenous social situations.

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ACYL-HOMOSERINE LACTONE-INDEPENDENT ACTIVATION OF AN ORPHAN LUXR IN B. THAILANDENSIS AND B. PSEUDOMALLEI

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LuxR family members with no genetically linked acyl-homoserine lactone (acyl-HSL) signal synthase are called solos or orphans. Orphans are found in the genomes of many Proteobacteria. Of those that have been studied, the majority are acyl-HSL-responsive and thought to detect signals produced by acyl-HSL synthases encoded elsewhere in the genome or produced by other bacteria. A subset of orphans do not respond to acyl-HSLs, and instead respond to as-yet unknown signals derived from plants. However all of the plant signal-responsive orphan LuxR family members have changes in two of the nine key residues attributed to many of the LuxR family proteins, both occurring in the acyl-HSL binding domain. We have found an orphan LuxR, BtaR4 in Burkholderia thailandensis, with all nine of the conserved LuxR-family residues that does not require acyl-HSLs to activate its target genes. Previous work demonstrated BtaR4 is required for virulence in C. elegans and we show that BtaR4 directly regulates gene for the virulence factor malleilactone. Although B. thailandensis encodes three acyl-HSL synthases, these are not required for BtaR4 to activate transcription of the mal-

leilactone biosynthetic genes. Instead, BtaR4 activity correlates with its cellular level, which is influenced by the addition of particular growth-inhibitive antibiotics. BtaR4 activation of target genes requires a lux-box motif in the promoter region of the malliolactone operon (mal) promoter, similar to other LuxR-family proteins. BtaR4 is unique to B. thailandensis and two of its close relatives, Burkholderia pseudomallei and Burkholderia mallei. The B. pseudomallei BtaR4 homolog BpsR4 is also important for virulence in C. elegans and regulates a malleilactone-like gene cluster in response to growth-inhibitive antibiotics, similar to BtaR4. It is unclear if BtaR4/BpsR4 activates its target genes in a ligand-independent manner or if it is activated by a ligand that is not an acyl-HSL. Our finding that a conserved orphan LuxR does not require acyl-HSLs to activate its target broadens our current view of the LuxR family.

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TOXIN SYNTHESIS BY CLOSTRIDIUM DIFFICILE IS STRINGENTLY REGULATED THROUGH QUORUM SIGNALING

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Clostridium difficile infection (CDI) is the most common cause of hospital-acquired and antibiotic-associated diarrhea in the United States, with a total treatment cost estimated between 1 to 4.8 billion U.S. dollars annually. C. difficile, a Gram-positive multidrug-resistant pathogen, overpopulates the colon after the native microbiota has been altered by antibiotic therapy. Treatment of CDI is hampered by increased virulence, sporulation, recurrence, and indiscriminate antibiotic use that destroy the colonic microbiota. Pathogenic C. difficile strains produce toxins A and B, which are directly responsible for disease. Despite the enormous public health problem posed by this pathogen, the molecular mechanisms that regulate production of the toxins remained largely unknown until now. Our data reveal that a novel quorum signal accumulates extracellularly during growth and induces toxin production at high cell density when a threshold signal concentration is reached. Using a classic quorum signaling bioassay, we determined that cell-free stationary-phase supernatants collected after 8 h of growth induce premature toxin production in low-density tester cells. This toxin-inducing activity, termed the TI signal, was purified by acetone precipitation, anion exchange, and HPLC. The purified TI signal stimulated elevated toxin production in both low-density hypervirulent and non-hypervirulent clinical C. difficile strains and induced early transcription of the C. difficile toxin genes. The molecular weight of the TI signal was determined by MALDI-TOF/TOF and MS/MS to be 612.37 Da. This is consistent with the structure of a novel 5-residue cyclic peptide composed of Cys, Pro, Trp, and 2 Ile with a thioester linkage between the carboxyl group of the C-terminal Ile and the N-terminal Cys; these five residues are found within the C. difficile agrD1 gene. A synthetic version of the TI signal induced toxin levels similar to that of the purified signal. We constructed a Himar-based transposon vector to generate isogenic mutants defective in toxin production (Tox-). One Tox- mutant contains an insertion in the agrA2 gene that is able to generate the TI signal, but does not respond to the TI signal, and is deficient in toxin synthesis. Response to the TI signal and toxin synthesis were restored by complementation with wild-type C. difficile agrA2. Finally, while testing diarrheal stools for TI signal, we detected the TI signal only in CDI-positive stools but not in CDI-negative stools. This underscores the clinical relevance of the TI signal in C. difficile pathogenesis during infection. These findings provide direct evidence that C. difficile toxin synthesis is regulated by an Agr quorum signaling and offers new avenues for both rapid CDI detection and development of quorum signaling-based non-antibiotic therapies to combat this life-threatening emerging pathogen.

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EXOGENOUS PROTEASE RELEASED SIGNAL PROMOTING CELL AGGREGATION OF A THERMOPHILIC FILAMENTOUS BACTERIUM

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Cell to cell communication in thermophiles has yet to be identified, while mesophiles have been well characterized. Interspecies communication at high temperature possibly works in hot spring microbial mats composed of a variety of bacteria with high cell density. An anoxygenic photosynthetic bacterium Chloroflexus aggregans, widely distributed in hot spring microbial mats at 48-65 °C, has a rapid gliding motility and their filamentous cells dispersed in liquid media collect together to form dense aggregates. We have obtained an isolate closely related to Bacillus licheiformis from the microbial mats dominated by C. aggregans and found that its culture supernatant promoted the aggregation rate of C. aggregans. This promoting effect was suppressed by the addition of a protease inihibitor. The aim of this study is to understand how exogenous proteases lead C. aggregans to promote cell aggregation. It was confirmed that purified protease of B. licheniformis showed the promoting effect of cell aggregation. C. aggregans cells were removed after the protease treatment to obtain the fraction containing degradation products. This fraction had the promoting activity even after its protease activity was suppressed by a protease inhibitor. We hypothesized that the degradation products derived from cell surface of C. aggregans promoted cell aggregation. This promoting activity was heat tolerant at 98°C and effectively suppressed by a peptidase, Chymotrypsin. Gel filtration was conducted to achieve size fractionation. SDS-PAGE analysis showed that active fractions contained bands with around M.W. 3,000. These results suggest that extracellular peptides (M.W. ~3,000) work as a signal affecting the motility of C. aggregans. These peptides were released through degradation of cell surface proteins of C. aggregans by proteases from other bacteria. To our knowledge this is the first report that exogenous protease released signal causing phenotypical change of bacteria. Thermo-stability of peptides may be a character common to intercellular signals in high temperature environments.

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INTEGRATION OF ENVIRONMENTAL SIGNALS AND CELL DENSITY FOR EFFICIENT CELL-CELL COMMUNICATION IN THE BACILLUS CEREUS GROUP

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In Gram-positive bacteria, cell-cell communication relies in part on cytoplasmic sensors regulated by secreted and re-imported signaling peptides. The Bacillus cytoplasmic quorum sensors Rap, NprR, and PlcR were previously identified as the first members of a new protein family called RNPP. Except for the Rap proteins, these RNPP regulators are transcription factors that directly regulate gene expression. Quorum sensing (QS) controls important biological functions in bacteria of the *Bacillus cereus* group. PlcR was first characterized as the main regulator of virulence in *B. cereus* and *Bacillus thuringiensis* (Bt). NprR controls the necrotrophic properties allowing the bacteria to survive in the infected host. A recent report has shown that the global stationary phase regulator CodY, involved in adaptation to poor growth conditions, represses expression of *nprR* by directly binding to its promoter region (1). It has also been shown that CodY is required for expression of virulence genes belonging to the PlcR regulon (2, 3). However the mechanism underlying this regulation has not been reported. Our experiments indicate that CodY does not directly control expression of *plcR*. We showed that CodY regulates expression of the virulence genes via PapR, PlcR's cognate signaling peptide. Using synthetic peptides we demonstrated that PapR is not efficiently re-imported in Δ*codY* cells.

We also showed that CodY positively regulates expression of the gene encoding the oligopeptide-binding protein allowing entry of PapR in the cell. Contrary to what was expected, this gene did not belong to the *opp* operon encoding the pore required for PapR import. Altogether, these data complete our model of quorum sensing during the lifecycle of Bt and indicate that environmental conditions, as well as cell density, are integrated by bacteria to coordinate their behavior throughout the infectious cycle *via* cell-cell communication.
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SMALL RNAS ARE CENTRAL IN INTEGRATING QUORUM-SENSING SIGNALS IN THE PLANT PATHOGEN PECTOBACTERIUM WASABIAE

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Quorum sensing (QS) controls the production of plant cell wall degrading enzymes (PCWDEs), the major virulence factors in plant pathogens from the genus Pectobacterium (formerly Erwinia spp.). In Pectobacterium spp., two cell-cell signaling circuits control the production of PCWDEs: the N-acyl homoserine lactone (AHL) network and the ExpS/ExpA/RsmA network. Unlike in other bacteria where the AHL system directly controls the expression of multiple genes, in Pectobacterium spp., rsmA is the only known target to be controlled by this system. RsmA is a small RNA-binding protein that binds to the mRNAs of multiple genes affecting translation and suppressing virulence. The deleterious effects on virulence production are counteracted by the ExpS/ExpA system (homologous to the GacA-GacS in Pseudomonas spp. and the BarA-UvrY in E. coli) that upon activation initiates the production of the small RNA (sRNA), RsmB, which sequesters RsmA. Thus, Pectobacterium spp., have developed a distinct alternative to control virulence where the two cell-cell signaling networks are intrinsically linked to each other via RsmA. In this study, we demonstrate that the gene coding for RsmB, is also controlled by the AHL system. Using both, biochemical and genetic assays, we also demonstrate that the amount of PCWDEs is dependent on the quantity of RsmB rather than the quantity of RsmA being produced in response to AHLs. These results provide an explanation on how AHLs and the ExpS/ExpA system converge to exquisitely control the activity of RsmA and consequent all the virulence RsmA-targeted genes.

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AN INTEGRATED QUORUM SENSING SYSTEM AND ITS CLINICAL IMPLICATIONS

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Pseudomonas aeruginosa is an opportunistic human pathogen that may cause severe nosocomial infections in hospital patients and cystic fibrosis sufferers. Pathogenesis of *P. aeruginosa* is complex and involves diverse virulence processes and mechanisms. One of the primary virulence regulatory mechanisms is the cell-cell communication system termed quorum sensing (QS). The QS system of *P. aeruginosa* was previously known to be organized in a *las*, *pqs*, *rhl* hierarchy, with the central *las* system governing the entire chain of QS pathways. It is therefore perplexing that *P. aeruginosa* clini-

cal isolates frequently harbor loss-of-functions mutations in *las*. Our recent discovery of IQS provides useful clues in solving this puzzle (1). IQS is a cell-cell communication signal capable of integrating phosphate-depletion stress cue with bacterial quorum information, even in the absence of the central *las* QS system. Given that phosphate limitation is frequently encountered by *P. aeruginosa* during infections of hosts, the biological significance of IQS has been investigated by using over 20 clinical isolates collected from lung and skin wound infections. Consistent with previous reports, our results show that more than half of the clinical isolates have various mutations in the *las* system, which lead to decreased virulence factor productions under phosphate-rich conditions. In contrast, most of the clinical isolates are capable of producing IQS signal under phosphate-depletion conditions and IQS deletion mutants are attenuated in virulence. These findings suggest that IQS could play an important role in clinical infections and more work is warranted to investigate this intriguing QS system.

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SIBLING RIVALRY IN MYXOBACTERIA TRIGGERED BY CELL SURFACE CUES THAT ACTIVATE T6SS

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Myxobacteria are known for their relatively complex social behaviors that involve cell-cell signaling. One of their interesting behaviors is the ability of Myxococcus xanthus to transiently fuse their outer membranes and exchange (OME) contents. To understand the mechanism of OME, we identified two genes, traA and traB, required in donors and recipients for protein transfer. TraA was further shown to be involved in molecular recognition to distinguish between kin and nonkin cells for OME. Self-recognition is determined by a polymorphic region (PA14-like domain) contained in TraA. One OME-dependent behavior we uncovered was the ability of nonmotile cells to inhibit the movement of motile cells when mixed. Interestingly, we found that a few nonmotile cells could control the gliding motility behavior of many cells, for example at a 1:50 ratio, by a Tra-dependent mechanism. To assess the behavior outcome of mixed strains under different conditions they were labeled with fluorescent proteins or antibiotic markers. Surprisingly, our results found that strains with a functional Adventurous gliding motility system were killed by nonmotile sibling cells. Importantly, killing was Tra-dependent. From a panel of test strains a single nonmotile strain was found that did not inhibit swarm expansion and did not kill. Intrigued by this rare behavior, we sequenced the genome of this strain to gain insight into the killing mechanism. From this analysis a number of SNPs and Indels were identified in type VI secretion system genes. Subsequently we disrupted several type T6SS genes in a nonmotile strain and found killing was reduced. Interestingly, when a T6SS mutant was mixed with an A-motile strain that contained a traA mutation, the tables were turned and the nonmotile strain was killed. In summary, our results indicate that OME plays a key role in 'policing' social interactions between cells, which we hypothesize help myxobacteria transition from individual cells into a well-controlled and coherent multicellular community.

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A NOVEL PARADIGM OF INTERBACTERIAL COMMUNICATION OF ANTIBIOTIC RESISTANCE MEDIATED BY CONSERVED SECRETED BACTERIAL LIPOCALINS

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Communication among bacteria in mixed infections may result in increased resistance to antibiotics, thereby leading to therapeutic failures. Recently, small secreted molecules

were implicated in communication of transient resistance to antibiotics. YceI, a small secreted protein produced by the extremely antibiotic resistant Burkholderia cenocepacia, mediated protection of Pseudomonas aeruginosa in co-culture from the lethal action of polymyxin B (PmB). YceI belongs to a large family of conserved bacterial small proteins that share a common tertiary lipocalin fold, but diverge in amino acid sequence homology. Recombinant bacterial lipocalins or "bacteriocalins" (BCNs) from B. cenocepacia bind PmB; however, until now, there has been no direct demonstration of BCN function. We hypothesize that BCNs are involved in the bacterial response to antimicrobial stress through binding antibiotics. Here, we show by deletion mutagenesis that the B. cenocepacia BCN BCAL3311 contributes to resistance against PmB and other amphiphilic antibiotics, such as rifampicin and norfloxacin, but not the hydrophilic gentamicin. The expression of BCAL3311, determined by chromosomal promoter-luxCDABE transcriptional fusion, was stimulated by amphiphilic antibiotics and not by gentamicin. Fluorometric binding assays showed that BCAL3311 has higher binding affinity to the hydrophobic probe Nile Red relative to basic and acidic dyes. The more hydrophobic antibiotics PmB, rifampicin and norfloxacin replaced Nile Red from its complex with BCAL3311 whereas gentamicin and the deacylated PmB nonapeptide did not, further highlighting the interaction of BCAL3311 with hydrophobic moieties. Exogenous BCAL3311 protected clinically relevant pathogens in vitro such as P. aeruginosa, Acinetobacter baumannii, Salmonella typhi and Shigella flexneri from PmB; it also protected P. aeruginosa from the innate immune response of Galleria mellonella larvae in vivo. Heterologous expression of BCNs from P. aeruginosa, Mycobacterium tuberculosis and Staphylococcus aureus USA300 in B. cenocepacia ΔBCAL3311 revealed a common role of these BCNs against PmB and rifampicin based on cfu count and Etest assays respectively. We propose for the first time a model of antibiotic resistance based on physical binding to hydrophobic moieties, revealing a defined function for the conserved BCNs in the interbacterial communication of resistance. This offers a new avenue for targeting antibiotic resistance and its spread among bacteria by developing inhibitors for BCNs.

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NAGC REPRESSION OF TFOX ENHANCES COLONIZATION OF EUPRYMNA SCOLOPES BY VIBRIO FISCHERI

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Gene regulation enables bacteria to adapt to different and frequently unpredictable environments. NagC is a transcriptional repressor conserved in γ-proteobacteria, including *Vibrio fischeri*, that controls the expression of genes involved in *N*-acetyl-glucosamine (GlcNAc) utilization. NagC repression of these genes is released by allosteric inhibition with GlcNAc-6-phosphate, which is the intracellular form of GlcNAc. Previously, we found that a Δ*nagC* mutant is unable to compete with wild-type cells in colonizing juvenile *Euprymna scolopes* squid. Our present study seeks to understand the mechanism underlying the Δ*nagC* colonization defect. A bioinformatics search for NagC-regulated genes based on potential NagC binding sites within the ES114 genome identified 25 genes within 20 operons. One of these genes was *tfoX*, which encodes a primary regulator of natural transformation in *Vibrionaceae*. Natural transformation is a primary mode of horizontal gene transfer among microbes, and, in *Vibrionaceae* members, requires many TfoX-regulated genes including *comEA* and *pilA*, which encode a periplasmic DNA shuttling protein and pseudopilus subunit, respectively. Using genetic and biochemical assays, we found that NagC represses *tfoX* by directly binding to two sites upstream of *tfoX* as well as to a site upstream of *tfoR*, which encodes a small RNA that post-transcriptionally enhances TfoX. Investigation of several genes

whose homologues in other *Vibrionaceae* members are regulated by TfoX revealed that *pilA*, but not *comEA*, is up-regulated in the Δ*nagC* mutant relative to wild-type expression levels. Using single-strain and co-colonization experiments, we have found that deletion of *tfoX* in a Δ*nagC* mutant restores normal squid colonization profiles. In addition, deletion of *pilA* in a Δ*nagC* mutant partially rescues the Δ*nagC* colonization defect. At 48 post-inoculation, we also found that *V. fischeri* represses *tfoX* transcription and translation within the squid light organ. Taken together, our results show that NagC repression of TfoX is important for *V. fischeri* cells to colonize *E. scolopes*. We propose that the pilus involved in natural transformation interferes with host colonization and that NagC repression of TfoX is the underlying mechanism preventing this interference. We also speculate that NagC repression of TfoX may stabilize the squid-vibrio symbiosis through the suppression of natural transformation within host-associated *V. fischeri* populations.

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METABOLIC INTERACTIONS AND DEMOGRAPHIC FEEDBACKS SHAPE THE EMERGENT FUNCTION AND SPATIAL ORGANISATION OF MICROBIAL COMMUNITIES

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Microbes live predominantly in dense and spatially-structured polymicrobial communities. Within these communities, microbial cells excrete a wide range of metabolites, and this sets the stage for interspecific metabolic interactions. The links, however, between metabolic and ecological interactions, and species spatial-organisation are still poorly understood. Here we develop an individual-based model of a two-species surface-attached community where food (resource) is exchanged for detoxification (service) to investigate how metabolic constraints of individual species shape the emergent spatial and functional relationships of species within the community. We find that strong metabolic interdependence drives the emergence of mutualism, robust interspecific mixing, and increased community productivity. These emergent community properties are driven by demographic feedbacks, such that aid from neighbouring cells directly enhances focal cell growth, which in turn feeds back positively to neighbouring helpers. In contrast, we find that weak metabolic interdependence drives conflict and greater interspecific segregation. Together, these results suggest that species spatial and functional relationships represent the net balance of metabolic interdependencies.

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A NOVEL APPLICATION OF NISIN IN INHIBITING THE FORMATION AND MAINTENANCE OF COMPLEX ORAL BIOFILMS BY DISRUPTING THE BACTERIAL COMMUNITY

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The accumulation of oral biofilms on the teeth and gums can lead to the development of caries and periodontal diseases, some of the most common diseases in humans. Nisin is a unique antimicrobial peptide that has a broad-spectrum inhibitory effect on both Gram-positive and Gram-negative bacteria. Nisin has been approved for human use by the World Health Organization (1969) and the Food and Drug Administration (1988). Recently, there has been a growing interest in utilizing nisin's unique bacteriocin properties for clinical use. Our preliminary data suggest that nisin has antibacterial effects against a periodontal pathogen, Treponema denticola and a cariogenic pathogen, Streptococcus mutans. We hypothesize that nisin may be a novel agent that can inhibit the formation and growth of complex oral biofilms by disrupting the mutualistic

relationship of the bacterial community. To determine the effects of nisin on formation and growth of oral biofilms, biofilms were cultured under both static and flowing conditions. To emulate the physiological conditions, human saliva was used to grow and inoculate multi-species biofilms. In addition, a high throughput Bioflux microfluidic system was used to grow and treat biofilms. Biofilms were cultured for different time periods ranging from 24 - 72 hours and post-treated with nisin at multiple time points (30 sec - 10 min) and concentrations (0.05 - 50 ug/ml). Following the nisin treatment, biofilms were stained with a LIVE/DEAD bacterial viability kit. Biofilms were visualized through confocal microscopy and were rendered in 3D to visualize the biofilm killing, biofilm biomass, biofilm surface coverage, average depth of the biofilm, and roughness (quantitative measure of killing). Our results demonstrated that nisin has a unique ability to inhibit the formation and growth of oral biofilms. Nisin at lower concentrations were able to disrupt the biofilm formation and had a significant dispersive effect. Nisin at higher concentrations had a dual effect of inhibiting formation and killing the bacterial population. Our data showed that nisin was able to penetrate and disrupt the complex interactions within the biofilm to prevent its formation and maintenance. In conclusion, we showed that nisin is a novel bacterial peptide that can inhibit and disrupt the growth patterns of multi-species oral biofilms. In addition, nisin has a unique mode action on disrupting the symbiotic relationships of oral biofilm species based on its concentration and duration of treatment. In future studies, we plan on carrying out genome sequencing based community analyses on biofilms in presence and absence of nisin to further evaluate nisin's specific effect within the complex oral microbiome.

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MICROWAVE-ASSISTED HIGH RESOLUTION IMAGING IN THE STUDY OF BACTERIAL COLONY ORGANIZATION OF INDIVIDUAL AND CO-COLONIZING SPECIES

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In the study of chronic polymicrobial infections, such as are common in patients with cystic fibrosis (CF) and of particular interest to our laboratories, one questions whether the multiple opportunists isolated from sputa colonize different zones of the airway or may co-exist in the same zone or biofilm and thereby affect one another's persistence in the human airway. To learn more about the potentially interacting species and contributions of individual members to the community, we employed a microwave-assisted specimen processing method for stabilization of microbial colonies within their extracellular matrices for transmission electron microscopy imaging (TEM). For investigating colony structure and organization, CF sputum outgrowths as well as individual and mixed species colonies of opportunistic pathogens such as *Pseudomonas aeruginosa* (PA), *Staphylococcus aureus* (SA), and *Candida albicans* (CA), were covered with gelatin or agarose and processed with a microwave-assisted paraformaldehyde with ruthenium red fixation strategy. For assessing preservation of enzymatic activities and localization within a colony by this mild approach, acid phosphatase activity (AcPase) of anaerobic pathogen *Clostridium perfringens* (CP) was evaluated with a modified Gomori stain. Intact colonies with stabilized extracellular matrices were imaged at high resolution and immunogenicity was preserved. PA and SA from CF patient samples differed as to how they co-existed, in some cases in distinct zones, and in others within the same matrix. Sputum outgrowths revealed interfaces between PA and both CA and SA. For CP, TEM

with matrix stabilization revealed that the significant cell-wall associated enzymatic activity within the colony structure was provided by a relatively small percentage of the organisms. In conclusion, TEM analysis of bacterial colony biology with matrix stabilization employing microwave-assisted processing permitted high resolution imaging providing valuable insights into the status of activities and associations within individual and mixes species communities. This "snapshot" tool may assist in the understanding, and development of new strategies to combat complex infections.

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RECEPTOR AFFINITY LIMITS EXPLOITATION OF PYOVERDINE PRODUCTION FROM INTERSPECIES CROSS-FEEDING

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In response to iron limitation, many microorganisms secrete siderophores, a diverse group of secondary metabolites that chelate ferric iron from the extracellular medium. This behavior is considered cooperative because siderophores can deliver iron to an individual cell other than the producer, creating potential for exploitation by non-producers. Interestingly, bacteria frequently have systems enabling the uptake of non-native or "xenosiderophores". Considering the prevalence of such cross-feeding, it is unclear how siderophore production remains evolutionarily stable. In this study, we address this question using controlled fitness studies with two *Pseudomonas* species, *P. aeruginosa* PAO1 and *P. protegens* Pf-5, which respectively serve as the siderophore-producing and cross-feeding strain. The primary siderophores of PAO1 and Pf-5 are the pyoverdines PVD_{PAO1} and $PVD_{Pf\text{-}5}$. Our strain selection exploits the ability of Pf-5 to utilize the non-native PVD_{PAO1} through the expression of an outermembrane receptor recently identified as FpvU. In contrast, PAO1 lacks a receptor capable of recognizing $PVD_{Pf\text{-}5}$. We find that pyoverdine-deficient PAO1 and Pf-5 mutants lacking the sigma factor PvdS grow robustly and at equal rates when supplemented with PVD_{PAO1}. However, in co-culture, PAO1 ΔpvdS has a strong fitness advantage relative to Pf-5 ΔpvdS. This result led us to hypothesize FpvU is an inferior receptor for PVD_{PAO1} compared to PAO1's native receptors, FpvA and FpvB. We explored this hypothesis by developing a mathematical model where receptor affinity can be manipulated. An important prediction from this model is that receptor affinity is most important when siderophore concentrations are low. We tested this prediction empirically by measuring fitness at a range of PVD_{PAO1} concentrations. Consistent with the model, we find PAO1 ΔpvdS has the highest relative fitness advantage when PVD_{PAO1} concentrations are low. Our hypothesis will be further validated by measuring receptor affinities directly. These results indicate that differences in receptor affinity influence interspecies competition for siderophores. Specifically, cross-feeding may be successful at high siderophore concentrations. If limiting however, the superior receptor of the producer confers a distinct fitness advantage. This hypothesis provides a simple explanation for the persistence of siderophore production despite the presence of interspecies cross-feeding.

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IN VITRO ASSESSMENT OF ANTIMICROBIAL EFFICACY OF SECONDARY METABOLITES PRODUCED BY LICHEN SP. COLLECTED FROM NORTH WEST HIMALAYAN REGION, INDIA

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Over the last few year microbial pathogens get mostly resistant to various synthetic drugs, available in the global market so, there is a vivid interest in developing a novel drug especially from the natural origin i.e lichens as it produce a great variety of secondary metabolites and most of them are unique. Our investigation deal with the assessment of two different lichen Peltigera sp. (Sample A) and Cladomia sp. (Sample B) extracted in two separate solvent methanol (ME) and water (AQ). Their antimicrobial efficacies were assessed against various pathogenic bacteria and fungal agents. The MIC value of both these samples varied within the range of 0.7-27 mg/ml. Quantitative and qualitative phytochemical analysis such as total phenol, polysaccharide concentration, TBA, reducing capacity, antioxidant and free radical scavenging ability were also determined. Higher total phenol concentration was observed in sample A in both the extract ME and AQ i.e. 15.6 mg GA/g and 14.3 respectively while the AQ extract in both the samples exhibited significant inhibitory activity towards lipid peroxidation by TBA method. Consequently, reducing power also increases with the concentration of extract. Moreover, AQ extract of both the sample were observed with appreciable amount of polysaccharides comparatively to ME extracts. Lichen samples also gave remarkable antioxidant capacity in AQ extract of sample A i.e.5.4 µg AA/g. In addition to this, in ME extracts of both samples showed comparable color change in TLC chromatogram. Therefore, our results clearly gave insight that lichen extract in water and organic solvent contain medicinally important bioactive compounds and which justifies their use in the traditional medicine. Key words: lichens, antimicrobial activity, MIC, phytochemical properties.

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EFFECTS OF BUTYRIC ACID ON THE BIOFILM FORMATION OF A. NAESLUNDII IN FLOW CELL SYSTEM

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Actinomyces naeslundii is an early colonizer and has important roles in the development of the oral biofilm. Short Chain Fatty Acids (SCFAs) is secreted extracellularly as a product of metabolism by Gram negative anaerobes e.g., Porphyromonas gingivalis and Fusobacterium nucleatum. Recently, we reported that one of SCFAs, butyric acid induced significant biofilm formation of A.naeslundii in 96 wells microtiter plate (Yoneda et al., Molecular Oral Microbiology, 2013 28:354-65.). As a next issue, to clear biofilm formation in flow condition and the mechanism of increased biofilm, we performed A.naeslundii's biofilm formation in flow cell system using Tryptic soy broth without dextrose and with 0.25% sucrose (TSB with sucrose) supplemented with butyric acid. Various concentrations (0, 6, 30, 40, 50 and 60 mM) of the butyric acid and other supplements {60 mM sodium butyrate (pH7.0) and 60 mM sodium butyrate (pH4.9) adjusted with hydrochloric acid} were applied in the flow cell culture. The biofilms were stained with BacLight LIVE/DEAD stain and examined using a confocal laser scanning microscope (CLSM). Z-stack images were collected via CLSM and analyzed by COMSTAT. The biofilm formation level was significantly higher in the condition containing 60 mM butyric acid than 30, 40, and 50 mM butyric acid. Furthermore, the biofilm formation failed in 0 and 6 mM butyric acid. The biofilm formation failed in 60 mM sodium butyrate (pH7.0), but 60 mM sodium butyrate (pH4.9) was similar

level to 60 mM butyric acid. The pH was 7.0, 6.7, 5.5, 5.0, 4.8 and 4.7 in TSB with sucrose containing 0, 6, 30, 40, 50 and 60 mM butyric acid respectively. Same pH conditions adjusted using hydrochloric acid were also tested in the assay but didn't induce significant biofilm formation. Therefore, 60 mM of butyric acid condition involving low pH are required for significant biofilm formation in the flow system. In the previous report, we found that the GroEL played as an adhesion of A.naeslundii in the biofilm formation assay condition without flow. As a next issue, we investigated how GroEL would contribute to the biofilm formation in the flow condition. The initial colonization of A.naeslundii was analyzed utilizing CLSM after incubation for 3 hours + 1hour after washing in TSB with sucrose. Initial colonization increased in 60 mM butyric acid condition. To observe contribution of GroEL to the initial colonization, anti-GroEL antibody was applied. As a result, anti-GroEL antibody inhibited clearly the initial colonization. In conclusion, the metabolic products such as butyric acid from Gram negative anaerobes stimulate the GroEL-dependent initial colonization of A.naeslundii resulting in significant biofilm formation under the low pH condition in the flow system.

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COMPARING THE RESPONSE OF PSEUDOMONAS AERUGINOSA TO NATURAL, SYNTHETIC, AND SEMI-SYNTHETIC ANTIMICROBIALS

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Opportunistic pathogens encounter antimicrobials during infection as well as outside the host. At sub-inhibitory levels, antimicrobials induce subtle changes in expression of stress-related and metabolic genes, which has led to the idea that antimicrobials not only function to inhibit bacterial growth but also act as cues. However, little is known about how bacteria respond to natural versus synthetic or semi-synthetic antimicrobials. The Gram-negative opportunistic pathogen Pseudomonas aeruginosa causes various acute and chronic infections in immunocompromised individuals and accounts for 15% of all nosocomial infections. P. aeruginosa is both intrinsically resistant and can acquire resistance to multiple antimicrobial classes. Here, we employed two genome-wide sequencing methods to: 1) elucidate the transcriptional response to sub-inhibitory levels of 17 natural, synthetic, and semi-synthetic antimicrobials; and 2) identify genes required for resistance to these antimicrobials. We hypothesized that P. aeruginosa has evolved an adaptive response to antibiotics commonly produced by soil microbes whereas synthetic or semi-synthetic antimicrobials will not elicit an adaptive response. Using RNA-sequencing, we found that the transcriptional response varied from having as little as 0 genes to as many as 500 genes differentially expressed upon exposure to sub-MIC levels of an antimicrobial. In general, P. aeruginosa had a more robust transcriptional response to natural antimicrobials than semi-synthetic antimicrobials. A closer look at the genes identified by RNA-seq indicated that oxidative stress-related genes are induced upon exposure to several different classes of antimicrobials. To identify genes required for resistance to these antimicrobials, we profiled the fitness of ~300,000 P. aeruginosa transposon mutants during growth in the presence of sub-inhibitory levels of antimicrobials using Transposon-sequencing (Tn-Seq). Tn-seq revealed that oxidative stress-related genes are also critical fitness determinants for multiple classes of antimicrobials. Our results reveal that P. aeruginosa possesses unique responses to natural, synthetic, and semi-synthetic antimicrobials and we anticipate this work will continue to yield important insights in the involvement of antimicrobials in growth inhibition as well as communication.

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SCANNING ELECTROCHEMICAL MICROSCOPY (SECM): A TOOLBOX TO STUDY MICROBIAL METABOLIC EXCHANGE

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Scanning electrochemical microcopy (SECM) has proved to be a powerful tool to study microbial metabolic exchanges at high spatial resolution (koley et. al). This technique has been used (koley et. al) to detect and quantify the reduced pyocyanin layer produced by pseudomonas aeruginosa biofilm. SECM is a technique that allows precise positioning of the electrochemical probe over the substrate of interest (e.g., biofilm) without touching or destroying it, thus making it a noninvasive method. The SECM probe or ultramicrosensor is positioned above the substrate at a known distance of 1- 10 µm with the aid of an approach curve (plot of probe current vs. z-direction distance). Later the probe can be moved in the x, y, and z direction over a range of 1-1,000 µm, a perfect working range for biofilm studies. The surface pattern of a single or multispecies biofilm fabricated by a new alginate-based electroaddressing technique is being used as a SECM substrate to study the polymicrobial metabolic exchanges. Time dependent 3D distribution of phenazine and its derivatives produced by the pseudomonas biofilm grown on these substrates will be addressed. Reference: 1. Koley, D., Ramsey, M. M., Bard, A. J. & Whiteley, M. Discovery of a biofilm electrocline using real-time 3D metabolite analysis. Proc. Natl. Acad. Sci. U. S. A. 108, 19996-20001 (2011).

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A FRET BASED SENSOR FOR QUANTITATIVE IN VIVO MEASUREMENTS OF PHRA-PEPTIDE SIGNALING IN BACILLUS SUBTILIS

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Extracellular signaling systems of the Rap-Phr type control a variety of cellular differentiation pathways in the Gram-positive model organism Bacillus subtilis. Rap-Phr systems are export-import systems where Phr-peptides are exported, processed, reimported and sensed intracellularly by Rap-proteins. Often several Rap-Phr systems act on the same pathway and are genetically interconnected. The resulting complexity makes it difficult to investigate these systems. Here we developed a FRET based PhrA-biosensor that consists of a pair of stable and functional fluorescent protein fusions. The sensor responds specifically and reproducibly to extracellular PhrA, as determined by acceptor photobleaching measurements and shows a high sensitivity in the range of low nanomolar concentrations. We show that PhrA can be detected in the supernatant of wild-type cells. Hence, the new biosensor might be used to measure extracellular concentration profiles and help to elucidate dynamical features of PhrA signaling during development. It might also facilitate the determination of biochemical rate constants by combining quantitative measurements with mathematical modeling. As a first step, we characterized the oligopeptide permease Opp, a shared network component of all Rap-Phr-systems. Overall, our data suggests that FRET-biosensors show a promising potential to serve in the quantitative analysis of complex signaling networks.

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BIOAFFINITY ANALYSIS OF QUORUM SENSING RECEPTOR SPNR WITH *SPN* BOX IN *SERRATIA MARCESCENS* USING A QUARTZ CRYSTAL MICROBALANCE TECHNIQUE

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Intracellular interaction of SpnR, a quorum sensing (QS) receptor, with the regulatory target DNA sequence in *Serratia marcescens* AS-1 was successfully analyzed by quartz crystal microbalance based on admittance analysis (QCM-A). *S. marcescens* is one of the opportunistic human pathogens and activation of the QS-related gene expression depends on a concentration of *N*-acyl-L-homoserine lactone (AHL). The QS-dependent production of an antibacterial red pigment prodigiosin is controlled by *N*-hexanoyl-L-homoserine lactone and *N*-(3-oxo-hexanoyl)-L-homoserine lactone. It is considered that SpnR stably binds to approximately 20 bp sequence of the QS-related DNA, *spn* box, possessing *lux* box-like sequence at low concentration of the AHL before the QS activation. To evaluate the bioaffinity using the QCM-A technique, the target promoter *spn* box was immobilized on a gold electrode of the 27 MHz QCM sensor. By using approximately 40 bp of double-stranded DNA fragment labelled at the 5'-end with biotin, the *spn* box sequence could be introduced to NeutrAvidin-immobilized self-assembly membrane formed on the Au-surface. The aqueous solution of maltose-binding protein (MBP) tagged SpnR produced by gene engineering technique was prepared as desired concentration. The aliquot of the MBP-SpnR solution was gently mixed to the *spn* box-modified electrode immersed in TE buffer solution (pH 7.8) after reaching the steady state output. A decrease of the resonant frequency observed just after adding MBP-SpnR clearly showed the effective interaction with the *spn* box. The apparent stability constant could be evaluated from a frequency change (ΔF_2), which was the index of the adsorbed mass on the electrode avoiding the effects on the viscosity change of the solution around the oscillating electrode.

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GENETIC DETERMINANTS OF FITNESS IN POLYMICROBIAL CHRONIC WOUND INFECTIONS

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Most naturally occurring infections are polymicrobial. Clinical and experimental observations suggest that when infections contain more than one bacterium, they are often worse. One particularly significant example of this is infections in chronic wounds, such as pressure ulcers (bedsores), surgical wounds, and diabetic ulcers. These wounds contribute to high healthcare costs, and are rapidly increasing in prevalence in the US. These chronic infections are often polymicrobial, and the opportunistic pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus* are among the most commonly isolated bacteria from infected chronic wounds. Infections containing both of these bacteria take longer to heal and are more resistant to antibiotic therapy. Yet specific molecular mechanisms potentiating this synergy are not well understood. To investigate the precise bases for synergy between these two organisms, we used genome-wide insertion mutant fitness profiling (Tn-seq) to characterize fitness determinants in monomicrobial and polymicrobial murine model chronic wound infections. Previous work has shown that *P. aeruginosa* must biosynthesize numerous metabolites in single-species infections, including riboflavin and lipoic acid, while it is able to harvest many others from the wound environment. Our polymicrobial Tn-seq analysis suggests that co-infection with *S. aureus* changes

the metabolic requirements of *P. aeruginosa in vivo*, requiring it to synthesize some metabolites previously available from wound environment and relieving biosynthetic requirements seen in monomicrobial infection. Our results also show that the MexEF-OprN multidrug efflux pump is required of *P. aeruginosa* specifically in co-infection, suggesting that efflux of small, possibly toxic molecules from the cytoplasm is a determinant of fitness in polymicrobial infections. Finally, we will detail advances made using genomic approaches to dissect the genetic determinants of *S. aureus* required for polymicrobial synergy in chronic wounds. Our results provide novel insight into the genetic requirements for *P. aeruginosa* and *S. aureus* polymicrobial chronic wound infections and demonstrate the power of using fitness profiling for probing bacterial virulence and persistence in infections.

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CELL-CELL COMMUNICATION AMONG THE STREPTOCOCCI: RGG PHEROMONES, INHIBITORS AND REGULATED BEHAVIORS

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Streptococcus pyogenes, a human pathogen, causes a variety of diseases including, but not limited to, pharyngitis, rheumatic fever and necrotizing fasciitis. It accounts for substantial mortality related to infections worldwide. Recent studies indicate that streptococci produce and respond to several secreted peptide signaling molecules (pheromones), including those known as SHPs (short hydrophobic peptides). Upon transport into the bacterial cell, the pheromones bind to and modulate activity of receptor proteins belonging to the Rgg family of transcription factors. In *S. pyogenes*, four Rgg paralogs exist, each serving as transcriptional regulators of genes associated with pathogenesis (RopB), biofilm development (Rgg2 and Rgg3), or a cryptic competence regulon (ComR). Our ongoing aims are to elucidate the mechanisms by which communication is propagated and regulated during the course of bacterial colonization and infection and to identify small molecules that disrupt signaling as a therapeutic strategy to treat or prevent disease. We have conducted genetic, biochemical and mass spectrometry analyses to identify several components of Rgg signaling pathways, that includes defining the mature pheromones. We have also begun identifying environmental signals and bacterial enzymes that control the expression and activity of signaling pathways. Finally, we have identified inhibitory compounds that specifically block individual Rgg pathways across several species.

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ACTIVATION OF VIBRIO CHOLERAE QUORUM SENSING VIA SMALL MOLECULES

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Quorum sensing is a form of bacterial cell-cell communication that uses extracellular signaling molecules called autoinducers to control individual vs. group behavior. In the pathogen Vibrio cholerae, the quorum-sensing response is initiated by the binding of the autoinducer CAI-1 to its cognate receptor CqsS. The histidine kinase CqsS controls the activity of the response regulator LuxO via phosphorylation. In the absence of autoinducer, the CqsS-LuxO cascade leads to virulence factor expression while binding and response to CAI-1 terminates the virulence program. Thus, compounds that activate the circuit (CqsS agonists or LuxO antagonists) could

lead to the development of medicinal molecules. A chemical screen was performed that yielded both types of compounds. Isolating overexpressed CqsS membranes revealed the synthetic CqsS agonists function by blocking His194 autophosphorylation, similar to CAI-1. Titration assays revealed that several agonists are more potent than the native autoinducer. While these agonists are structurally unrelated to the natural ligand, mutagenesis of CqsS suggests their binding sites overlap. Among the LuxO antagonists, both uncompetitive and competitive inhibitors of LuxO ATPase activity were identified. Characterizing these pro-QS molecules will provide tools for probing the quorum-sensing signaling mechanism and have the potential to be developed into therapeutics.

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IDENTIFICATION OF A SMALL MOLECULE SIGNALING FACTOR THAT REGULATES THE BIOSYNTHESIS OF THE ANTIFUNGAL POLYCYCLIC TETRAMATE MACROLACTAM HSAF IN LYSOBACTER ENZYMOGENES

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Lysobacter are emerging as a new source of antibiotics. The regulation of these antibiotics is not well understood. Here, we identified a small molecule metabolite (LeDSF3) that regulates the biosynthesis of the antifungal antibiotic HSAF, a polycyclic tetramate macrolactam with a structure and mode of action distinct from the existing antifungal drugs. LeDSF3 was isolated from the culture broth of L. enzymogenes, and its chemical structure was established by NMR and MS. The purified compound induced green fluorescence in a reporter strain of Xanthomonas campestris, which contained gfp gene under the control of a DSF (diffusible signaling factor)-inducible promoter. Exogenous addition of LeDSF3 in L. enzymogenes cultures significantly increased the HSAF yield, the transcription of HSAF biosynthetic gene, and the antifungal activity of the organism. The LeDSF3-regulated HSAF production is dependent on the two-component regulatory system RpfC/RpfG. Moreover, LeDSF3 up-regulated the expression of the global regulator Clp (cAMP receptor-like protein). The disruption of clp led to no HSAF production. Together, the results show that LeDSF3 is a fatty acid-derived, diffusible signaling factor positively regulating HSAF biosynthesis and that the signaling is mediated by the RfpC/RfpG-Clp pathway. These findings may facilitate the antibiotic production through applied genetics and molecular biotechnology in Lysobacter, a group of ubiquitous yet underexplored microorganisms.

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REGULATION OF VIOLACEIN PRODUCTION IN *CHROMOBACTERIUM VIOLACEUM*

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The biosynthesis of the violacein purple pigment by *Chromobacterium violaceum* is known to be positively regulated by an *N*-acyl homoserine lactone (AHL)-driven quorum sensing (QS) system. Wild type *C. violaceum* strain ATCC31532 produces low amounts of violacein whereas a transposon mutant of ATCC31532 has been reported to produce very large amounts of violacein. This suggests that violacein production in under negative regulation. We have studied this regulation showing that the violacein promoter is positively regulated by AHL-mediated QS and negatively regulated by a novel protein which we named VioS. VioS neither affected AHL levels nor the expression of *luxI/R* genes. However, it interfered with the positive regulation of the *vio* operon by the AHL QS system since in a *vioS*

mutants much higher levels of transcription of the *vio* operon could be detected. Interestingly the sequenced *C. violaceum* strain ATCC12472 naturally produces high amounts of violacein and introduction of *vioS* from strain ATCC31532 resulted in much lower levels of violacein production. We have also sequenced *C. violaceum* ATCC31532 and performed comparative genomics speculating possible DNA rearrangements in these strains. It is known that some AHL QS systems undergo negative regulation resulting in QS homeostasis; violacein production on the other hand is an example of strong competition between negative regulation and AHL QS at the target locus in order to carefully control the production levels of a QS regulated phenotype.

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