Temperature affects c-di-GMP signaling and biofilm formation in

*Vibrio cholerae*

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Biofilm formation is crucial to the environmental survival and transmission of *Vibrio cholerae*, the facultative human pathogen responsible for the disease cholera. During its infectious cycle *V. cholerae* experiences fluctuations in temperature within the aquatic environment and during the transition between human host and aquatic reservoirs. In this study, we report that biofilm formation is induced at low temperatures through increased levels of the signaling molecule, cyclic diguanylate (c-di-GMP). Strains harboring in-frame deletions of all *V. cholerae* genes that are predicted to encode diguanylate cyclases (DGCs) or phosphodiesterases (PDEs) were screened for their involvement in low-temperature-induced biofilm formation and *Vibrio* polysaccharide (VPS) gene expression. Of the 52 mutants tested, deletions of six DGCs and three PDEs were found to affect these phenotypes at low temperatures. Unlike wild type, a strain lacking all six DGCs did not exhibit a low-temperature-dependent increase in c-di-GMP, indicating that these DGCs are required for temperature modulation of c-di-GMP levels. We also show that temperature modulates c-di-GMP levels in a similar fashion in the Gram-negative pathogen *Pseudomonas aeruginosa* but not in the Gram-positive pathogen *Listeria monocytogenes*. This study uncovers the role of temperature in environmental regulation of biofilm formation and c-di-GMP signaling.
Translational Control of the Caulobacter Cell Cycle

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Cellular differentiation is an essential process by which cells carrying identical genes develop into specialized cell types with distinct functions. An important goal in understanding cellular differentiation is to determine how the genetic information encoded in the genome is expressed properly in time and space to ensure the correct cell fate. The bacterium Caulobacter crescentus has proven to be an excellent model organism for studying cellular differentiation processes that occur as a function of the cell cycle. In Caulobacter each cell division is asymmetric, yielding a daughter with a different cell fate. This process requires rapid and specific changes in gene expression during the cell cycle that are controlled at many levels, including transcriptional regulation, transient DNA methylation, differential proteolysis, and protein phospho-signaling. However, relatively little is known about the cell cycle control of mRNA translation.

To understand the role of translational control in Caulobacter cell cycle progression and cell differentiation, we employed a recently developed method, ribosome profiling, to monitor genome-wide changes in translation throughout the cell cycle. In agreement with data in E. coli and B. subtilis, ribosomes were observed to pause at internal Shine-Dalgarno sites within the coding DNA sequence. Despite an identical anti-Shine-Dalgarno sequence in the ribosomal RNA across virtually all bacteria, only half of bacterial genes contain Shine-Dalgarno sites preceding the start codon, including some genomes such as Caulobacter (23.5%) with very poor usage of this sequence. Therefore, the conserved function of the Shine-Dalgarno sequence may be ribosome pausing, not translation initiation.

While the transcription of 20% of the mRNAs in the Caulobacter genome is cell cycle-regulated, approximately 55% of cell cycle-regulated mRNAs are additionally regulated at the level of translation, including 70 mRNAs whose cell cycle-regulated expression is exclusively controlled at the level of translation. Genes undergoing cell cycle-regulated translational control include many critical genes involved in polar morphogenesis, DNA replication, and cell division. Overall, our data show that translational control provides an additional layer of regulation of the Caulobacter cell cycle and that this control is largely used to optimally tune the timing of gene expression.
Using optically reversible spatial mutations to dissect the asymmetric developmental program of a bacterium

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Understanding the role of the spatiotemporal organization of molecules within a cell in determining its fate is a critical challenge in biology. The stem cell like Caulobacter bacterium provides the simplest model system for studying the role of protein localization in establishing asymmetric cell division. Caulobacter’s cell cycle regulatory circuit is controlled by a dynamically localized signaling network and targeted proteolysis. This network has been well characterized both genetically and biochemically. However, little is known about the underlying spatiotemporal mechanisms controlling this circuit. Optogenetics techniques offer an unprecedented way to reversibly alter protein localization in vivo in seconds time resolution. We have adapted a light-inducible dimerization system to Caulobacter and introduced light controlled "spatial-mutations" for driving a diffuse protein to specific cellular addresses as well as light controlled “degradation-mutations” for fast protein degradation. Using these assays, in combination with mathematical modeling, we are dissecting Caulobacter’s regulation pathways by altering the localization and availability of its key regulatory proteins.

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How cells control their size and maintain size homeostasis is a fundamental open question. Cell-size homeostasis has been discussed in the context of two major paradigms: sizer, in which the cell actively monitors its size and triggers the cell cycle once it reaches a critical size, and timer, in which the cell attempts to grow for a specific amount of time before division. These paradigms, in conjunction with the “growth law” [1] and the quantitative bacterial cell cycle model [2], inspired numerous theoretical models [3-9] and experimental investigations from growth [10,11] to cell cycle and size control [12–15]. However, experimental evidence involved difficult-to-verify assumptions or population-averaged data, which allowed different interpretations [1–5,16–20] or limited conclusions [4–9]. In particular, population-averaged data and correlations are inconclusive as the averaging process masks causal effects at the cellular level. In this work, we extended a microfluidic “mother machine” [21] and monitored hundreds of thousands of Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis* cells under a wide range of steady-state growth conditions. Our combined experimental results and quantitative analysis demonstrate that cells add a constant volume each generation irrespective of their newborn sizes, conclusively supporting the so-called constant Δ model. This model was introduced for *E. coli* [6,7] and recently revisited [9], but experimental evidence was limited to correlations. This “adder” principle quantitatively explains experimental data at both the population and single-cell levels, including the origin and the hierarchy of variability in the size-control mechanisms, and how cells maintain size homeostasis.
Identification and characterization of VpsR and VpsT recognition sites in *Vibrio cholerae*

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The ability to form biofilms is critical for environmental survival and transmission of *Vibrio cholerae*, a facultative human pathogen responsible for the disease cholera. Biofilm formation is controlled by several transcriptional regulators and alternative sigma factors.

In this study, we report that the two main positive regulators of biofilm formation, VpsR and VpsT bind to non-overlapping target sequences in the regulatory region of *vpsL in vitro*. VpsR binds to a proximal (R1-box) as well as a distal (R2-box) site with respect to the transcriptional start site identified upstream of *vpsL*. The VpsT binding site (T-box) is located between the R1 and R2-boxes. The T- and R-boxes are necessary to achieve wild-type expression levels of *vpsL*. However the T and R2-boxes are dispensable in a genetic background lacking H-NS, suggesting that their primary role is to prevent H-NS dependent repression. *In silico* analysis of the regulatory regions of VpsR and VpsT targets resulted in the identification of conserved recognition motifs for VpsR and VpsT, and revealed that operons involved in biofilm formation and *vpsT* are co-regulated by VpsR and VpsT.

Furthermore, a comparative genomics analysis revealed substantial variability in the promoter region of *vpsT* and *vpsL* genes among extant *V. cholerae* isolates, suggesting that regulation of biofilm formation is under active selection.
Sinorhizobium meliloti DksA affects efficient root nodule symbiosis

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The soil-dwelling α-proteobacterium Sinorhizobium meliloti symbiotically interacts with legume plants by invading the root and fixing molecular nitrogen in special compartments, the root nodules. To establish an effective interaction, gene regulation must be continuously controlled in both host and symbiont. In bacteria, the stringent response (SR) is one regulatory mechanism employed to adapt to changing environmental conditions; This occurs due to the synergistic action of DksA and the small alarmone ppGpp that bind to RNA polymerase and thus alter the kinetic properties of the promoter/RNAP complex. We are taking a genetic approach to investigate the relevance of the SR for the symbiosis between S. meliloti and its host Medicago sativa.

The S. meliloti genome encodes two DksA paralogs, DksA and DksA2, which differ in the amino acid sequence of two major protein domains, and one RelA protein, which synthesizes and hydrolyzes ppGpp. Complete single deletion mutants as well as the corresponding double and triple mutants for the three genes were constructed and tested for their behavior in free-living or symbiotic conditions. We found that \( \Delta dksA \) is impaired in growth on minimal medium lacking amino acids, induces a lower number of nodules on host plants, and has lower nitrogenase activity in early nodules. However, \( \Delta dksA2 \) behaves like the wild type strain in all conditions tested. \( \Delta relA \) shows severe growth and symbiosis defects, which is consistent with previous reports on another relA mutant strain. The double and triple mutants behave like the corresponding single mutant with the strongest phenotype. Furthermore, dksA, but not dksA2 can rescue the metabolic defects of an E. coli dksA mutant strain, suggesting distinct functions of the paralogs.

These findings show that DksA contributes as a transcriptional regulator to free-living and symbiotic performance in S. meliloti, whereas the function of DksA2 remains unknown at this time.
**S. meliloti** ECF σ factors are not required for Nod or Fix phenotypes on alfalfa


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We constructed marked insertional mutations with a derivative of pVO155 (1) in each of the ECF-type sigma factor (rpoE) genes in *Sinorhizobium meliloti*. All of the single insertions showed normal behavior on *Medicago sativa* (alfalfa) and *M. truncatula*. We constructed double mutants by transducing one marked mutation into a second mutant background. Among the 45 double mutants we assayed, we found four Nod-minus (no formation of nodules), one non-infective (small nodules, limited infection, no release of bacteroids) and eight Fix-minus (no dinitrogen reduction) strains.

Complementation of Nod-minus and non-infective strains with corresponding wild-type sigma factor genes failed to restore nodulation or defective symbiosis, respectively. We suspected that background mutations might be responsible for the symbiosis phenotypes.

We determined the complete genome sequence for each of the double-mutants that display a severe symbiosis phenotype to look for background mutations. All four Nod-minus mutants carried an insertion element in nodC, and the non-infective mutant carried one in nodF. Clones carrying nodD1ABC and nodFE, respectively, restored normal symbiosis to the Nod-minus and non-infective mutants. Genome sequence combined with complementation tests thus show that the non-nodulating and infection defects are not due to the sigma factor gene mutations. We are presently sequencing the genomes of all of the Fix-minus strains to see if these carry background mutations in known *nif* and *fix* genes.

In a second approach, we carried out a systematic deletion of every alternative sigma factor open reading frame. We used sacB selection (2) to create unmarked precise deletions. To make multiple deletions, we transduced marked single-recombination constructs into already deleted strains, followed by resolution of the recombination with sacB counterselection. Strain RFF625 is deleted for all 11 ECF sigma factors. We determined the sequence of this strain and discovered a point mutation in the gene encoding malate dehydrogenase (*mdh*). When we restored the *mdh* mutation to wild type by site-directed mutagenesis and tested the resulting strain on alfalfa, it elicited pink nodules that we scored as Fix-plus.

In sum, our data point strongly to the conclusion that the ECF sigma factors are not required for basic nodulation and nitrogen fixation phenotypes on alfalfa. Other phenotypic studies are ongoing, to examine competitiveness, symbiosis with other plant hosts, and diverse stress responses.


(2) J. Quandt and M. Hynes (1993) Gene 127:15-21
Genome-wide identification of transcriptional target genes of ExoS/ChvI in the symbiotic bacterium *Sinorhizobium meliloti*

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The ExoS/ChvI signaling pathway in *Sinorhizobium meliloti* is important for establishing a nitrogen-fixing symbiosis between the bacteria and its legume plant host. ExoS/ChvI signaling also regulates multiple free-living phenotypes of the bacteria. This signaling pathway is conserved in other alpha-proteobacteria, including the plant pathogen *Agrobacterium tumefaciens* and the mammalian pathogens *Brucella spp.*, and is required for successful interactions of these bacteria with their respective eukaryotic hosts. To better understand why this signaling pathway is so critical for the biology of the bacteria, we performed a genome-wide study to identify genes that are directly regulated transcriptionally by ExoS/ChvI in *S. meliloti*. From these results, we also identified a motif that is important for binding of ChvI to DNA.
Isolation and characterization of bacteria from the plant rhizosphere

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Plant Growth Promoting Rhizobacteria (PGPR) are bacteria that reside in the rhizosphere of plants and have a beneficial effect on plant growth. The bacteria do this either by directly promoting plant growth by making nutrients available, producing phytohormones, etc., or indirectly by controlling plant pathogens. There has been much interest in the use of PGPR as a biofertilizer, as it would reduce the use of chemical fertilizers and promote more sustainable agricultural practices.

The isolation and characterization of potential PGPR has become the focus of the second semester of the General Biology Laboratory at LMU. During the course, students learn and use basic tools and techniques of traditional and molecular microbiology, record and analyze their data for meaning and importance, prepare data in effective figures and tables, and practice various forms of scientific communication. Furthermore, there are opportunities for free inquiry. Overall, the goal is to provide students an “authentic” research experience with the opportunity to think, work, and present their data as biologists and to cultivate in students an engagement in the process of science.

Students isolate bacteria from the rhizosphere of plants, screen selected isolates biochemically to determine whether they have properties associated with PGPR, and inoculate plants to determine whether their isolates promote plant growth. Along with the phenotypic characterization of their bacterial isolates, students do molecular analysis on their isolates of the 16S rRNA gene. Students determine the identity of their isolates by comparison of this sequence to the database and derive phylogenetic relationships between their organisms and those of their classmates. Over 300 isolates have been characterized in the course, with species of Bacillus, Streptomyces, Flavobacterium, and Microbacterium appearing as the most numerous. Over the three years this project has been in place, consistent trends have been seen in the biochemical makeup of the organisms that students have characterized, with about 39% producing auxin, 59% showing cellulase production, 34% solubilizing phosphate, 35% showing plant growth promotion, and 10% showing plant growth inhibition.
Analysis of the Mutualistic Interaction between Alfalfa—Rhizobium—
Micromonospora

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One of the major challenges for the 21st century will be the attainment of a sustainable crop production. Agriculture derived from the Green Revolution, which helped bring about a greater use of pesticides, fertilizers, and herbicides of chemical origin, as well as the genetic improvement of plant germplasm, resulted in an increase in agricultural productivity. Decades ago, the cost and risks derived of this kind of agriculture were described \textsuperscript{(1,4,5)} and in response, a new agricultural revolution is beginning, using probiotic microorganisms as an alternative to chemicals. Gram-positive, filamentous actinobacteria, well-known soil bacteria, are well adapted to the soil environment. Certain Streptomyces species were recently shown to enter root tissues and establish an endophytic lifestyle with plants \textsuperscript{(3)}. Micromonospora species have also been found inside nodules in several legume genera \textsuperscript{(2,9)} However, the beneficial plant-microbe interactions of Gram-positive microbes were ignored in breeding strategies, even after their importance in soil ecosystems was proven \textsuperscript{(8)}. The study, comprehension, and definition of their PGPR traits are of major importance for the success of this new agriculture. Our study focuses on these traits in endophytic Micromonospora strains previously isolated from alfalfa nodules.

We showed that many Micromonospora isolates significantly increased the number of nodules and promoted growth in alfalfa. Multivariate data analysis allowed us to select two strains (ALFPr18c and ALFB5), which were responsible for the largest increase in several important agricultural parameters, namely dry weight, shoot length and root length, when coinoculated with a wild type strain of Ensifer meliloti. The probiotic effect was also evident in the increase of N, P and K \textsuperscript{(6)}. Given that the N content in most treatments with the largest increase in several important parameters, namely dry weight, shoot length and root length, when coinoculated with a wild type strain of Ensifer meliloti. The probiotic effect was also evident in the increase of N, P and K \textsuperscript{(6)}. Given that the N content in most treatments with Micromonospora was significantly higher than the control, a study on nitrogen fixation was performed. We could not find any evidence of nitrogen fixation in any of the strains tested, with any of the different methodologies used \textsuperscript{(7)}.

The results of this study strongly support that Micromonospora is a common inhabitant of the nitrogen fixing nodules of alfalfa, that this plant-microbe interaction has a beneficial effect on nodulation by \textit{E. meliloti} acting as RHB and promotes the growth and nutrition of the plant, including nitrogen nutrition, by means other than biological fixation.

The novel Spindle-shaped Structure and Infection Kinetics of the Archaeal Virus SSV1


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The *Sulfolobus* spindle-shaped viruses (SSVs) of the family Fuselloviridae were the first discovered and probably the best studied archaeal viruses. SSVs are found throughout the world in high temperature (>70°C) and low pH (pH<4) environments, where their hosts *Sulfolobus solfataricus* and its close relatives thrive. The type virus, SSV1, encodes a positively supercoiled, 15.5 kbp circular dsDNA genome that is packaged within a spindle-shaped capsid. The spindle morphology is unique to Archaea but widespread therein. We have solved the structure of SSV1 to 32 ångstroms resolution, the first structure of a spindle-shaped virus to be solved to this resolution. The structure provides unprecedented detail of the tail structure which presumably interacts with the host cell. The overall architecture of the structure appears to be two modified fullerene cones, similar to the nucleocapsid of HIV. In order to obtain sufficient virus particles for a higher resolution structure we have developed techniques to obtain microgram quantities of virus. Since we can now reproducibly prepare large quantities of virus, we have also been able to perform, for the first time, synchronized infections of Sulfolobus with SSV. SSV1 infection seems to have a long eclipse phase, nearly 24 hours. However, once past this eclipse phase very large quantities of virus are produced, matching levels observed with induction with ultraviolet irradiation. Despite high genetic similarity, the SSV from Kamchatka, Russia, SSV-K (aka SSV-9) has very different infection kinetics and influence on host growth. On the other hand mutant SSV viruses appear to be unchanged in their growth kinetics.
Identification of inner-membrane proteins required for contact-dependent growth inhibition (CDI)

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Contact-dependent growth inhibition (CDI) is a mechanism of inter-bacterial competition mediated by the CdiB/CdiA family of two-partner secretion proteins. CdiB is an outer-membrane β-barrel protein required for the secretion and presentation of CdiA effector proteins on CDI+ inhibitor cells. CdiA proteins are very large and extend several hundred angstroms from the inhibitor-cell surface to interact with specific receptors on neighboring target bacteria. Upon binding its receptor, CdiA delivers a toxin derived from its C-terminus (CdiA-CT) into the target cell. Remarkably, the CdiA-CT region is highly variable between CDI+ bacteria, indicating that these systems can deliver a variety of toxins with distinct activities. Many CdiA-CTs are nucleases and must presumably be translocated into the target-cell cytoplasm to inhibit growth, but the mechanism of CDI toxin translocation remains poorly understood. To identify target-cell factors required for toxin translocation, we performed a series of selections for *E. coli* mutants that are resistant to different CDI toxins. These selections isolated a collection of mutants with disruptions in the genes for a variety of inner-membrane proteins. In general, each mutation provides resistance to a specific toxin, however some mutants are resistant to multiple CdiA-CTs that share related N-terminal domains. Because the N-terminal domain is not required for CdiA-CT nuclease activities *in vitro*, we propose that this region functions in toxin translocation. Together, these findings suggest that CDI exploits multiple parallel import pathways, with each CdiA-CT toxin recognizing a different membrane protein to enter target bacteria.
Characterization of type VI secretion system-1 (T6SS-1) in Enterobacter cloacae

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Enterobacter cloacae ATCC 13047 effectively outcompetes many enterobacterial species during co-culture on solid media. This competitive fitness advantage depends on the type VI secretion system-1 (T6SS-1) locus, which is constitutively expressed under laboratory conditions. Hemolysin co-regulated protein (Hcp), of which there are 5 in E. cloacae, has been shown to be required for T6S activity. Here, we find that deletion of hcp3 (the only hcp gene encoded by the T6SS-1 locus) abrogates all T6S activity, suggesting that it is the only hcp paralog expressed under laboratory conditions. Thus far, we have identified 4 T6SS effectors deployed under laboratory conditions in E. cloacae, the most potent of which are Rhs1 and Rhs2 proteins. Somewhat surprisingly, we find that deletion of both rhs1 and rhs2 phenocopies the ∆hcp3 mutation, indicating that at least one functional Rhs protein is required for T6SS in E. cloacae. Rhs1 and Rhs2 each contain an N-terminal PAAR domain, which has recently been shown to be required for T6S activity in Vibrio cholerae and Acinetobacter baylyi. However, expression of the Rhs PAAR domains alone is not sufficient to restore T6S activity in ∆rhs1 ∆rhs2 cells. Together, these data suggest that Rhs plays an essential, yet uncharacterized, structural role in the E. cloacae T6S apparatus.
Competition experiment and community sequencing identifies fast growing mutants

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We are interested in engineering cyanobacteria to serve as high value biomass for biofuel and bio-chemical production. In general, increasing the absolute amount or the specific nature of its lipid, carbohydrate and protein components raises biomass value. One barrier to optimized biomass production is the presence of unwanted or unnecessary biosynthetic pathways that funnel carbon, energy (reductant) and other resources away from the product(s) of interest. However, the elimination of the genes encoding these pathways can often cause unforeseen growth defects. Here, we describe a batch growth competition assay targeted at identifying genes that are unnecessary for sustained growth. We inoculated a turbidostat with approximately 2500 different strains of *Synechococcus elongatus* PCC 7942. Each strain had a unique transposon insertion mutation. We sampled over seventeen days of culturing and analyzed the sample populations using Next Gen sequencing, which allowed us to quantify the relative abundance of each mutant. We found three hundred mutants that eventually dominated the population, and verified the improved growth rate of eight isolates in mono-culture. This competition assay was useful for selecting mutant strains with improved growth.
A novel class of methyl-accepting chemotaxis proteins found in

*Vibrio fischeri*

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Chemotaxis is an important and widely conserved mechanism by which microbes navigate their environment. The bioluminescent bacterium *Vibrio fischeri* is the monospecific symbiont of the Hawaiian bobtail squid, *Euprymna scolopes*. The bacteria are present as a planktonic population in the waters surrounding Hawaii, and each generation of squid isolate their symbionts after hatching. During colonization, the bacteria must navigate several complex environments to reach epithelium-lined crypts in the interior of the host’s light organ, where they multiply and provide light for the host. In chemotaxis, attraction/repulsion signaling is initiated by the binding of ligands to the periplasmic domain of methyl-accepting chemotaxis proteins (MCPs). These proteins are found in many different families of microbes, and the number of MCPs each species of bacteria carries is highly variable. *V. fischeri* encodes 42 MCPs in its genome, and many of the ligand-binding domains of these MCPs appear to be redundant. Previous work has shown that MCPs are able to sense a wide range of ligands, including metabolic intermediates, quorum-signaling compounds, amino acids, and sugars. I will present the first initial characterization of VfcB and its ortholog, VfcB2, which have been found to represent a novel class of receptors capable of mediating attraction to fatty acids. Fatty-acid sensing is likely to be important in *Vibrio* species, as *V. fischeri* has previously been shown to directly incorporate fatty acids shed by the host within the light organ, and *V. cholerae* has been shown to similarly incorporate fatty acids encountered in silt deposits. As research groups begin to focus on chemotaxis characterization in bacteria with larger numbers of MCPs, we will begin to gain a better understanding of what, and how many, environmental cues microbes use to navigate.
How Motility and Chemotaxis promote *Helicobacter pylori* pathogenesis

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*Helicobacter pylori* chronically infects more than 50% of the human population worldwide, living in the gastrointestinal tract. Motility is essential for normal stomach colonization. While a non-motile mutant shows a severe defect in colonizing mouse stomachs, a chemotaxis mutant shows only slightly reduced numbers compared to wildtype. Interestingly, this reduction is predominantly due to a defect of colonization in a part of the stomach called the antrum. However, after one month of infection, both wildtype and non-chemotactic strains show similar colonization. In this situation, despite reaching wild-type numbers, the non-chemotactic mutant causes less inflammation. To better understand what *H. pylori* is sensing in the stomach that drives both disease and colonization, we started to study chemotaxis in a novel model system, gastric organoids. Gastric organoids are established from adult stem cells in gastric glands isolated from either the antrum or the other section, the corpus, and are highly proliferative containing differentiated gastric cell types. This model system allows us to have the advantages of high throughput numbers and direct imaging of motility and chemotaxis in conditions similar to the ones within the stomach. To understand how chemotaxis is connected to pathogenesis we focus on the pro-apoptotic toxin VacA. We hypothesized that chemotaxis plays a role in positioning the bacterium for VacA delivery. To address this question, we created a non-chemotactic *cheY* mutant, a *vacA* mutant, and a *cheY vacA* double mutant in *H. pylori* to study differences in colonization, attachment, and host cell apoptosis. We first focused on two well established systems, AGS cell tissue culture and the *in vivo* system with C57BL/6 mice. We found that in tissue culture chemotaxis does not play a significant role in apoptosis, while VacA is required for this outcome. In this system the double mutant shows no additive effects. In contrast, the *in vivo* experiments showed that both chemotaxis and VacA play a role for apoptosis in mice. In the gastric organoids model, we employed the same mutants with the addition of GFP expression. In this model, we found that chemotaxis is required for normal attachment in gastric organoids. These results suggest that gastric organoids maintain key facets of intact tissue and will allow dissection of chemotaxis-dependent processes.
Integration of aromatic acid chemotaxis, transport, and catabolism in *Pseudomonas putida*

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The ability of bacteria to utilize chemotaxis to travel towards or away from specific chemicals allows cells to locate a niche that is optimal for growth and survival. The catabolically versatile soil bacterium *Pseudomonas putida* is able to recognize aromatic and hydroaromatic compounds that are metabolized through the β-ketoadipate catabolic pathway as chemoattractants. A screen of *P. putida* F1 mutants each of which was lacking one of the genes encoding the 18 putative canonical methyl-accepting chemotaxis proteins (MCPs) revealed that the gene *pcaY* encodes the MCP required for metabolism–independent chemotaxis to vanillate, vanillin, 4-hydroxybenzoate, benzoate, protocatechuate, quinate, shikimate, and ten substituted benzoates that do not serve as growth substrates for *P. putida* F1. Chemotaxis was induced during growth on the aromatic compounds, and a *pcaY-lacZ* fusion revealed that *pcaY* is expressed in the presence of aromatic growth substrates. Specifically, β-ketoadipate, the common intermediate in the degradation pathway, was responsible for induction of *pcaY*, and a mutant strain lacking the transcriptional activator PcaR, which controls expression of genes for aromatic acid degradation (*pca regulon*), lost the ability to induce *pcaY* expression. These results suggest that chemotaxis and degradation are coordinately regulated and that *pcaY* is a member of the *pca* regulon. The *pca* regulon includes three distantly located gene clusters encoding five enzymes required for the conversion of 4-hydroxybenzoate to TCA cycle intermediates, as well as the major facilitator superfamily transport protein PcaK, which had previously been implicated to play a role in 4-hydroxybenzoate chemotaxis. Our results indicate that PcaK modulates the chemotactic response by facilitating uptake of 4-hydroxybenzoate, which leads to the accumulation of β-ketoadipate, thereby increasing *pcaY* expression. Taken together, the results show that chemotaxis, transport, and metabolism of aromatic compounds are intricately linked in *P. putida*. 
The role of energy taxis in sensing phenylpropanoid compounds by

*Pseudomonas putida*

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Chemotaxis has been extensively studied in the gut microbe *E. coli*, which is attracted to a variety of amino acids and sugars. However, chemotaxis is less well-studied in bacteria that reside in the soil, such as *Pseudomonas putida* F1, which are exposed to a considerably wider range of compounds than is typically found in the mammalian gut. We characterized the chemotactic response of *P. putida* F1 to the phenylpropanoid p-coumarate, a ubiquitous compound that is a primary component of lignin and a growth substrate for *P. putida* F1. The chemotactic response of *P. putida* F1 to p-coumarate is induced following growth on p-coumarate, and requires metabolism of p-coumarate. A mutant lacking the MarR-type repressor CouR, encoded in the p-coumarate degradation cluster, was constitutively attracted to p-coumarate. We showed that the energy taxis receptor in *P. putida* F1, Aer2, is required for chemotaxis to p-coumarate. Together these findings indicate that, rather than being sensed directly by a chemoreceptor protein, p-coumarate is detected indirectly, via the energy generated as a result of its metabolism. Two similar phenylpropanoid compounds, ferulate, and caffeate, which are metabolized via the same pathway as p-coumarate, are also sensed via energy taxis in *P. putida* F1. It is possible that energy taxis is an adaptation that allows bacteria to respond to a large number of structurally distinct attractants without the need for numerous specific chemoreceptors, while at the same time prioritizing responses to optimal growth substrates.
Denitrifying bacteria are a favorite topic for culture-independent study. Important players in the nitrogen cycle, and host to several well-understood functional genes for which numerous PCR assays exist, denitrifiers have been quantified, identified, and scrutinized in countless environments around the globe. A topic of perpetual interest is whether measurable environmental factors can predict the distribution of these diverse and ubiquitous nitrate-respiring bacteria. Unfortunately, few studies have attempted to separate the trends in denitrifier communities from those of the microbial community as a whole. (As an example, salinity has been found to be the single most reliable factor structuring denitrifier communities globally; yet salinity is known to be one of the most reliable factors structuring the communities of all organisms). So the question remains: do denitrifiers occupy a specialized niche that separates them from the majority of the microbial community? This question is especially interesting in light of the fact that denitrifier marker genes have undergone some horizontal gene transfer, so the environmental patterns observed in phylogenetic diversity of the genes may not correlate with patterns in phylogenetic diversity of the organisms.

We addressed this question in the San Francisco Bay-Estuary, where denitrifiers form a sizeable percentage of the sediment microbial community. We sampled sediment along the major salinity gradient of the estuary, regularly for one year, and examined microbial abundance and diversity using qPCR and high-throughput sequencing of both the nirS functional gene (cytochrome cd1 dissimilatory nitrite reductase) and the 16S ribosomal subunit. This approach allowed us not only to reveal striking geographic patterns in the denitrifier populations that would not have been apparent using sparser sampling methods (e.g., clone libraries with Sanger sequencing), but also to show that those geographic patterns, with few exceptions, strongly resemble patterns in the broader benthic community.
Nitrate perturbation stimulates chemolithoautotrophic activity in an aquifer at 
Rifle, CO

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Global climate change is leading to altered precipitation events in many parts of the world. This in turn impacts the seasonal fluctuations of water tables, resulting in perennially suboxic and anoxic regions of aquifers being subjected to changing redox conditions. Characterizing the phylogenetic diversity and active metabolic pathways of aquifer microbial communities as they respond to environmental perturbations is key to predicting the response of large-scale ecosystems to climate-induced changes. In an experiment designed to mimic these changes at an accelerated pace, we injected up to 1.5 mM nitrate into an anoxic aquifer at Rifle, CO, over a two-month period. The aquifer has naturally fluctuating levels of nitrate and contains a large reservoir of reduced Fe- and S-containing compounds. The injected nitrate was rapidly consumed and remained below detection in downgradient wells. Planktonic biomass was collected throughout the time course on 0.2-\(\mu\)m filters and extracted for gDNA and RNA. We employed a metatranscriptomic analysis on Illumina sequence data from rRNA-subtracted cDNA libraries that were assembled and mapped to phylogenetically binned Rifle metagenome data. The phylogenetic diversity was ascertained through 16S rRNA Illumina iTag analysis.

Phylogenetic and metatranscriptomic analyses revealed an emergence of chemolithoautotrophic microorganisms relevant to C, S, N, and Fe cycling. Sulfide oxidation was evident through highly expressed subunits of APS reductase with high sequence identity (98-99%) and synteny to the mapped scaffold of the obligately chemolithoautotrophic S- and Fe(II)-oxidizing \textit{Thiobacillus denitrificans}. While genes for autotrophic carbon assimilation were represented by an array of Proteobacterial species, the most highly expressed RuBisCo gene had 89% identity to a Betaproteobacterium. All of the major pathways for N cycling were represented in the metatranscriptome, excepting aerobic ammonia oxidation. However, genes from anaerobic ammonia oxidizing (anammox) bacteria closely related to the planctomycete KSU-1, including the key enzyme in the anammox reaction, hydrazine oxidoreductase (\textit{hzoB}), were highly expressed at the initiation of the experiment. Genes belonging to the Fe(II)-oxidizing Betaproteobacteria Gallionellaceae family composed 80% of the top 30 most highly expressed transcripts at the time of maximum nitrate injection. The increase in Gallionellaceae expression correlating with increasing nitrate concentration suggests the presence of nitrate-dependent Fe(II)-oxidizing species. Based on this, we returned to the Rifle site well, re-injected nitrate, and collected groundwater from which we isolated a Betaproteobacterium that performs nitrate-dependent Fe(II) oxidation.
Extracellular Enzymes Mediate Electron Uptake in Biocorrosion and Bioelectrosynthesis

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Microorganisms can drastically accelerate anaerobic iron corrosion, causing significant economic loss. In the last 10 years, novel anaerobic and highly corroding sulfate-reducing and methanogenic microorganisms have been isolated. These microorganisms effectively corrode iron apparently by taking up electrons directly from the iron surface rather than by consuming hydrogen formed chemically by proton-reduction during Fe corrosion. A direct uptake mechanism of cathodic electrons was also implied for various electrosynthetic microorganisms. However, the underlying molecular mechanisms of these electron uptake reactions are unknown. Here we show that cell-free, surface-associated enzymes, such as hydrogenases and formate dehydrogenase, are sufficient to mediate an apparent ‘direct’ electron uptake. These redox enzymes catalyze the formation of compounds such as H₂ or formate that are rapidly consumed by the microorganism. Cell-free spent culture medium of the iron-corroding and electro-methanogenic archaeon Methanococcus maripaludis greatly enhanced H₂ and formate formation from Fe(0) and cathodic electrode surfaces compared to medium-only controls. Thus, surface-sorbed extracellular redox enzymes provide a mechanistic explanation for a ‘direct’ electron uptake reaction suggested for iron corrosion and microbial electrosynthesis.
Catabolism of *Pseudomonas aeruginosa*-derived phenazines by rapidly growing mycobacteria

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Phenazines are small molecules, produced by a variety of bacteria, which have profound impacts on the structure of microbial communities. For example, the secreted phenazines produced by the opportunistic human pathogen *Pseudomonas aeruginosa* likely benefit the producers by functioning to maintain redox homeostasis while simultaneously inhibiting other organisms in polymicrobial infections. Despite the importance of these compounds to shaping ecosystem diversity, little is know about the fate of phenazines in situ. Using a culture dependent approach, strain CT6, an organism related to *Mycobacterium fortuitum*, was isolated using the *Pseudomonas*-derived compound phenazine-1-carboxylic acid (PCA) as the sole source of carbon and energy. Additionally, strain CT6 was found to degrade the clinically important phenazine pyocyanin (PYO). Genome sequencing of strain CT6 and comparative genomics, revealed a cluster of four dioxygenase genes predicted to be involved in PCA degradation. qPCR revealed that these genes are induced >1,000 fold in the presence of both PCA and PYO. Finally, an allele replacement knockout of one of these genes (MFORT_16334::GmR) resulted in an inability to grow with PCA as the sole carbon source. These data suggest the involvement of a dedicated set of dioxygenases in the degradation of phenazines and that microbially mediated phenazine degradation may modulate phenazine concentrations in natural and clinical ecosystems.
Tetrahymanol production in bacteria requires an uncharacterized protein not found in tetrahymanol producing ciliates.

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Tetrahymanol is a pentacyclic isoprenoid lipid first discovered in the ciliated protozoan, *Tetrahymena thermophila*. *Tetrahymena*, a sterol auxotroph, has been shown to produce tetrahymanol under conditions of sterol starvation in low oxygen environments. As a result, gammacerane, the diagenetic product of tetrahymanol, which can be preserved in the rock record, is often interpreted to represent both the occurrence of eukaryotic ciliates deep in time and ancient anoxic environments. However, tetrahymanol biosynthesis has also been shown to occur in two alphaproteobacterial species, *Rhodopseudomonas* and *Bradyrhizobium*, and nothing is known about the biosynthesis and physiological function of this lipid in bacteria. The biosynthesis of tetrahymanol in *Tetrahymena* requires only a squalene-tetrahymanol cyclase to cyclize squalene directly to tetrahymanol. Most bacteria that produce tetrahymanol also produce hopanoids and thus are thought to utilize a squalene-hopanoid cyclase (Shc) to produce both hopanoids and tetrahymanol. However, studies have shown that bacterial Shcs can only convert squalene to the hopanoid structure and do not produce tetrahymanol in vitro. We therefore hypothesized that there was an alternate mechanism for producing tetrahymanol in bacteria.

In this study, we demonstrate the production of tetrahymanol by a third bacterial species, *Methylomicrobium alcaliphilum*, an alkaliphilic halotolerant gammaproteobacterial aerobic methanotroph originally isolated from a soda lake in Siberia. Utilizing comparative genomics and gene deletion analysis, we identify a protein of unknown function (MEALZ_1626) that is required for the production of tetrahymanol in *M. alcaliphilum*. Phylogenetic analysis revealed that every sequenced *Rhodopseudomonas* and *Bradyrhizobium* strain contains a copy of this protein in their genome, in agreement with the occurrence of tetrahymanol in these species. In addition, this protein is found in the genomes of other alphaproteobacteria, *Methylomicrobium* species, and some *Desulfovibrio* species. We have begun studies to determine the biochemical mechanism of MEALZ_1626 and hypothesize that this protein oxidizes squalene to a hydrosqualene, which can then be cyclized by the bacterial Shc to tetrahymanol. In addition, we are characterizing an MEALZ_1626 mutant for any growth phenotypes associated with a low oxygen environment. Through these studies we should be able to get a better understanding of tetrahymanol biology in the bacterial domain, which could lead to a better interpretation of the occurrence of tetrahymanol in the rock record.
Hopanoids are steroid-like lipids found in some bacteria. The carbon backbone of hopanoids can be preserved as ancient molecular fossils (hopanes), dating back to at least 1.6 billion years ago. The variance in 2-methylhopane abundance at discrete intervals in Earth history suggests that these compounds may serve as biomarkers to understand changing environmental conditions. But to use 2-methylhopanes for such a purpose, we need to know what their progenitors do in cells today. To better understand the biological function of hopanoid methylation, we determined the concentrations of short (diplopterol (Dip)) and long (bacteriohopanetetrol (BHT)) chain hopanoids and their 2-methylated species in the inner and outer membranes of *Rhodopseudomonas palustris* TIE-1 by GC-MS. Fluorescence polarization was used to measure membrane rigidity in small unilamellar vesicles made of model or native *R. palustris* lipids with hopanoid concentrations that are physiologically relevant. Our results show that hopanoids, especially the long chain ones, are enriched in the outer membrane of *R. palustris* TIE-1. Furthermore, 2-methylation of hopanoids increases their capacity to rigidify membranes. However, the magnitude of this membrane rigidifying effect is greatly dependent on the lipid milieu. These quantitative biophysical analyses are helping to constrain our interpretation of ancient 2-methylhopanes.
Discovery of polyphosphate and iron-sulfide bodies in the archaea:  

*Methanospirillum huntgatei* JF1 and *Archaeoglobus fulgidus* V16

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Storage granules are an important component of metabolism in many organisms spanning the bacterial, eukaryal, and archaeal domains, but systematic analysis of their organization inside cells is lacking. In this study, we identify and characterize granule-like inclusion bodies in a methanogenic archaeon, *Methanospirillum huntgatei*, an anaerobic microorganism that plays an important role in nutrient recycling in the ecosystem. We also identify bodies in the thermophilic Euryarchaeaean, *Archaeoglobus fulgidus* strain V16. Using cryo-electron microscopy, we show that granules in mature *M. huntgatei* are amorphous in structure with a uniform size. Energy dispersive X-ray spectroscopy analysis establishes that each granule is a polyphosphate body (PPB) that consists of high concentrations of phosphorous and oxygen, and increased levels of iron and magnesium. By scanning transmission electron tomography, we further estimate that the mass density within a PPB is a little less than metal titanium at room temperature and is about 4 times higher than that of the surrounding cytoplasm. Finally, three-dimensional cryo-electron tomography reveals that PPBs are positioned off-center in their radial locations relative to the cylindrical axis of the cell, and almost uniformly placed near cell ends. This positioning ability points to a genetic program that spatially and temporally directs the accumulation of polyphosphate into a storage granule, perhaps for energy-consuming activities, such as cell maintenance, division or motility.

Dense granules were also very apparent in *Archaeoglobus fulgidus* strain V16 cells imaged by cryo electron microscopy (cryoEM), but were either poorly or not seen in negatively stained cells or *A. fulgidus* cell ghost preparations. By cryo electron tomography (cryoET), we observe that each cell may contain one or more dense granules, which were located near the cell periphery adjacent to the cell membrane. Surprisingly, elemental analysis by energy dispersive X-ray (EDX) spectroscopy and scanning transmission electron microscopy (STEM) revealed that each cell contained not just one, but often two types of granules with different elemental compositions. One type, named the iron-sulfide body (ISB), was composed mainly of the elements iron and sulfur, plus copper. The other type, called the polyphosphate body (PPB), was composed mainly of phosphorous and oxygen, plus magnesium, calcium, and aluminum. PPBs have been found in other archaeal and bacterial cells and are polyphosphate-containing granules likely used for energy storage and/or during for metal ion sequestration/detoxification. The newly discovered *A. fulgidus* ISBs may result from the reduction of sulfate to sulfide via anaerobic energy harvesting pathways and may be also associated with energy and/or metal storage in support of cell survival in harsh habitats.
Metagenomic approach reveals patterns of bacterial and archaeal assembly and functions after long-term land-use change in Southern Brazil

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Knowledge about microbial assembly and functions, due conversion of forests into agricultural systems help us to understand ecological consequences of land-use change and allow to develop monitoring and soil management strategies. The aim of this work was to evaluate the possible shifts in microbial assembly and functions, after a long-term (15-year at least) conversion of forest into grassland or no-till cropping. We collected samples from soils at 0-10 cm profile, in a geogrid system, in two municipalities and two seasons, at the Atlantic Rainforest Biome, Southern Brazil. High throughput sequencing of shotgun metagenomic libraries were accomplished to reach soil archaeal and bacterial communities. Taxonomic alpha-diversity did not vary after long-term conversion. Although, taxonomic beta-diversity was lower in grassland, compared to forest, which represents a simplification of microbial communities in that system. Functional alpha-diversity has shown no differences among land-uses. Despite of that, functional beta-diversity was lower in grassland, compared to forest, similarly as found for taxonomic results. Since general taxonomic and functional patterns were similar, as visualized by non-multidimensional scaling, we have looked into major groups and functions that have responded to land-use change, through Welch’s t-test with Benjamini-Hochberg correction. We found that the phylum Acidobacteria has decreased its relative abundance in no-till cropping, probably due long-term liming effect. Instead of that, Bacteroidetes, Chloroflexi, Gemmatimonadetes and Nitrospirae have increased in this system. The phyla Gemmatimonadetes, Nitrospirae and Thaumarchaeota have shown lower abundance in grassland, in detriment of forest. Differently, Planctomycetes and Verrucomicrobia have relatively enlarged their populations in grassland. Several biological functions have shifted after conversion of forest in grassland. Functions like carbohydrates metabolism, regulation and cell signaling, respiration and defense metabolism were significantly increased, while amino acids and derivatives metabolism and fatty acids, lipids and isoprenoids metabolism were repressed. Comparing no-till to forest, only amino acids and derivatives metabolism and nucleosides/nucleotides metabolism were significantly increased after long-term land-use change, while photosynthesis and defense metabolism have slightly decreased. The number of taxonomic correlations into microbial communities, as revealed by Spearman’s, was more dependent on site and season than the land-use. Functional correlations have slightly increased in grassland, compared to forest. Correlations between taxonomy and functions have increased after conversion, with exception to no-till in one of the municipalities, in the summer. Both taxonomic and functional correlations with environmental factors have significantly increased in grassland. The patterns of assembly and functional resilience in grassland are linked to physicochemical and other environmental factors, which is unlikely to occur to forest and no-till. The shifts in abundance on major groups and correlation matrices, revealed that long-term no-till can recover functional patterns found in the forest, even with differences in taxonomic structure, and apart from soil environmental conditions. [Funding: CNPq; FAPESC; FAPESP]