BLAST X PROGRAM

January 19, 2009 — Two-Component Systems

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SPEAKER ABSTRACTS

A STRUCTURAL INVESTIGATION OF RESPONSE REGULATOR AUTODEPHOSPHORYLATION

Yael Pazy[1], Amy C. Wollish[1], Stephanie A. Thomas[1], Peter J. Miller[2], Edward J. Collins[1,2], **Robert B. Bourret[1]**, and Ruth E. Silversmith[1]
Departments of Microbiology & Immunology[1] and Biochemistry & Biophysics[2], University of North Carolina, Chapel Hill, NC 27599

Assorted two-component regulatory systems are used to control a wide variety of biological processes, which occur over a broad range of time scales. To appropriately synchronize the adaptive responses implemented by response regulators with the environmental stimuli detected by sensor kinases, the kinetics of biochemical signaling reactions must be at least as fast as the biological process that they regulate. The fraction of response regulators in the phosphorylated state is determined by the net result of phosphorylation and dephosphorylation. Autodephosphorylation rates reported for various wildtype response regulators span a range of at least 40,000x, consistent with timescales ranging from about one second to one day. Furthermore, the range of rates observed suggests that autodephosphorylation rates are an important contributor to setting the overall timescales of two-component signaling systems.

We previously found that changing the amino acids at the variable active site positions corresponding to residues 59 and 89 of *Escherichia coli* CheY could alter response regulator autodephosphorylation rates about 100x (1). Thus, the particular amino acids found at positions '59' and '89' in response regulators can account for two orders of magnitude in autodephosphorylation rate, but other factors to account for an additional two to three orders of magnitude in reaction rate must also exist and remain to be identified. To begin to characterize the structural basis of response regulator autodephosphorylation rate, we determined high-resolution X-ray crystal structures for five CheY mutants that bear amino acid substitutions at positions 14, 59, and 89 and consequently exhibit autodephosphorylation rates six to 40 times slower than wildtype CheY. Each structure was determined in the presence of the phosphoryl group analog BeF_3^- and thus represents the starting point of the autodephosphorylation reaction. Comparison of mutant and wildtype CheY structures, which are matched at all but three residues and yet support different reaction rates, can potentially provide insight into the mechanistic basis by which positions '59' and '89' influence autodephosphorylation rate. Similarly, comparison of each mutant CheY structure with the structure of a wildtype response regulator that is matched at eight (three variable and five conserved) active site residues and yet catalyzes autodephosphorylation at rates 10-80x slower than the CheY mutants can potentially suggest candidates for additional factors that might contribute to autodephosphorylation rate.

Response regulator autodephosphorylation likely involves an inline attack on the phosphoryl group by a nucleophilic water molecule. The structural comparisons outlined above clearly suggest that autodephosphorylation rate is influenced by the extent to which amino acid sidechains at positions '59' or '89' sterically occlude access to the phosphoryl group. Additional factors potentially affecting autodephosphorylation rate will also be discussed.

Reference

1. Thomas, S.A., Brewster, J.A., & Bourret, R.B. (2008) Two nonconserved active site residues affect response regulator phosphoryl group stability. *Molecular Microbiology* **69,** 453-465.

INTRAMOLECULAR AUTOPHOSPHORYLATION OF THE *ESCHERICHIA COLI* ArcB SENSOR KINASE

Gabriela R. Peña-Sandoval, Luis A. Nuñez Oreza and Dimitris Georgellis
Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, 04510 México City, México.

The Arc two-component system is a complex signal transduction system that plays a key role in regulating energy metabolism at the level of transcription in bacteria. This system comprises the ArcB protein, a tripartite membrane-associated sensor kinase, and the ArcA protein, a typical response regulator. Under anoxic growth conditions, ArcB autophosphorylates and transphosphorylates ArcA, which in turn represses or activates the expression of its target operons. Under aerobic conditions, the kinase activity of ArcB is silenced by the oxidation of two cytosol-located redox-active cysteine residues that participate in intermolecular disulfide bond formation, a reaction in which the quinones provide the source of oxidative power.

Here we present results demonstrating that the putative leucine-zipper near the second transmembrane segment of ArcB is functional and necessary for proper ArcB signaling. Moreover, we provide data demonstrating that in contrast to the proposed model of intermolecular autophosphorylation, ArcB autophosphorylation is an intra-molecular reaction.

A BIFUNCTIONAL KINASE-PHOSPHATASE IN BACTERIAL CHEMOTAXIS

Steven L. Porter, Mark A.J. Roberts, Cerys S. Manning and Judith P. Armitage[1]
Oxford Centre for Integrative Systems Biology (OCISB), Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU

Phosphorylation based signaling pathways employ dephosphorylation mechanisms for signal termination. Histidine to aspartate phosphosignaling in the two-component system controlling bacterial chemotaxis has been studied extensively. *Rhodobacter sphaeroides* has a complex chemosensory pathway with multiple homologues of the *Escherichia coli* chemosensory proteins, although it lacks homologues of known signal terminating CheY-P phosphatases such as CheZ, CheC, FliY or CheX. Here we demonstrate that an unusual CheA homologue, $CheA_3$, is not only a phosphodonor for the principal CheY protein, $CheY_6$, but is also is a specific phosphatase for $CheY_6$-P. This phosphatase activity accelerates $CheY_6$-P dephosphorylation to a rate that is comparable with the measured stimulus response time of ~1 s. $CheA_3$ possesses only two of the five domains found in classical CheAs, the Hpt (P1) and regulatory (P5) domains, which are joined by a novel 794 amino acid sequence that is required for phosphatase activity. The P1 domain of $CheA_3$ is phosphorylated by $CheA_4$ and it subsequently acts as a phosphodonor for the response regulators. A $CheA_3$ mutant protein deleted for the 794 amino acid region lacked phosphatase activity, retained phosphotransfer function but did not support chemotaxis, suggesting that the phosphatase activity may be required for chemotaxis. Using a nested deletion approach we show that a 200 amino acid segment of $CheA_3$ is required for phosphatase activity. The phosphatase activity of previously identified non-hybrid histidine protein kinases depends upon the dimerization and histidine phosphorylation (DHp) domains. $CheA_3$, however, lacks a DHp domain, suggesting that $CheA_3$ is a novel phosphatase.

MESSAGE PASSING: PROTEIN STRUCTURE ASSEMBLY FROM SEQUENCE DATA FOR TWO-COMPONENT SIGNALING PROTEINS

Hendrik Szurmant[1], Martin Weigt[2,3], Robert White[1,1], Terry Hwa[3] and James A. Hoch[1]
[1] Division of Cellular Biology, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037
[2] Institute for Scientific Interchange, I-10133 Torino, Italy
[3] Center for Theoretical Biological Physics and Department of Physics, University of California at San Diego, La Jolla, CA 92093

The crystal structure of the *Bacillus subtilis* response regulator Spo0F in complex with the histidine kinase structural homologue Spo0B defined the active site of phosphotransfer and the spatial interactions of two-component systems of microbes and plants. The limited bioinformatic data available at the time was sufficient to deduce and understand the molecular basis for recognition specificity between histidine kinases and response regulators (J. A. Hoch and K. I. Varughese. 2001. J. Bacteriol. 183:4941-4949). Today, the availability of large protein databases generated from sequences of hundreds of bacterial genomes enables more sophisticated statistical approaches to extract interacting and specificity determining positions between proteins from protein databases. The goal of such studies is to identify protein interaction surfaces from sequencing data alone, without previous structural knowledge, i.e. co-crystal data. A number of co-variance based approaches producing nearly identical results have been applied to the highly amplified two-component systems as a means to verify mathematical data with structural knowledge of sensor kinase/response regulator interaction; including one presented by us at the BLAST IX in 2007 (R. A. White et al. 2007. Methods Enzymol. 422:75-101). While producing potential specificity determining position information, these methods have an important shortcoming in predicting spatial proximity. They cannot distinguish between directly correlated (interacting) and indirectly correlated (non-interacting) residue positions. To address this issue we developed a novel method that combines co-variance analysis with global inference analysis, adopted from use in statistical physics.

When applied to a set of over 2500 representatives of the bacterial two-component signal transduction system, the combination of covariance with global inference methods successfully and robustly identified residue pairs that are proximal in space without resorting to ad hoc tuning parameters, both for hetero-interactions between sensor kinase (SK) and response regulator (RR) proteins and for homo-interactions between RR proteins. The spectacular success of this approach illustrates the effectiveness of this combination approach in identifying direct interaction positions based on sequence information alone. We expect this method to be applicable for predicting interaction surfaces between proteins present in only one copy per genome as the number of sequenced genomes continues to expand, and for assembling multi-protein structures.

INTEGRATED CONTROL OF *CAULOBACTER* CELL ENVELOPE PHYSIOLOGY BY A HYBRID TWO-COMPONENT/ECF SIGMA FACTOR SIGNALING NETWORK

Robert Foreman, Erin Purcell, Aretha Fiebig, Dan Siegal-Gaskins & **Sean Crosson**
Department of Biochemistry and Molecular Biology, University of Chicago, 929 E. 57th St., Chicago, IL 60637

We present evidence that *Caulobacter crescentus* encodes a regulatory network that integrates information about two different signals, visible light and oxidative/osmotic stress, to regulate the cell envelope and cell adhesion. In this hybrid signaling system, light signals via the LovK histidine kinase and oxidative/osmotic stress signals via the ECF sigma factor σ^T are integrated to regulate cell envelope physiology. *Caulobacter* LovK, exhibits light-controlled autokinase activity and forms a two-component signaling system with the single-domain receiver protein, LovR. We have shown that the LovK/LovR system can function to modulate cell adhesion in response to blue light. LovK/LovR is a negative regulator of σ^T, an envelope stress sigma factor that is critical for cell survival under osmotic and oxidative stress. σ^T, in turn, is a positive transcriptional regulator of the *lovK/lovR* two-component system. This feedback-regulated signaling network can serve as a model to probe how bacterial cells integrate and coordinate their responses to multiple environmental queues.

THE ROLE OF SIGNAL TRANSDUCTION IN CELL WALL METABOLISM IN *BACILLUS SUBTILIS*

Paola Bisicchia, David, Noone, Efthimia Lioliou and Kevin M Devine.
Smurfit Institute of Genetics, Trinity College Dublin, Dublin 2. Ireland.

The cell wall of Gram positive bacteria is an extracellular structure, physically removed from the biosynthesis of the precursors used in its synthesis. Peptidoglycan and teichoic acid precursors are synthesized within the cytoplasm and transported across the cytoplasmic membrane where they are incorporated into the cell wall during growth and cell division. The spatial separation of these processes implies bidirectional signaling between the cell wall and cytoplasmic compartments, that recent work has begun to elucidate. We have shown that the essential YycFG two-component signal transduction system of *Bacillus subtilis* controls cell wall metabolism – during exponential growth, it activates expression of the YocH, YvcE and LytE autolysins and represses expression of YoeB (IseA), an inhibitor of autolysin activity, and YjeA a peptidoglycan deacetylase whose activity on peptidoglycan modulates its susceptibility to autolysin digestion (Howell *et al*., 2003; Bisicchia *et al*., 2007; Salzberg and Helmann, 2007; Yamomoto et al., 2008). Thus we propose that YycG senses some aspect(s) of the cell wall externally, perhaps the Lipid II intermediate, and transduces this information into the cytoplasm so that the cell wall synthetic activities in these two compartments are coordinated (Dubrac *et al*., 2008). We have also demonstrated a close connection between YycFG and the PhoPR two-component system that controls one of the phosphate limitation responses in *B. subtilis* (Hulett, 2002). YycFG and PhoPR are closely related phylogenetically - hybrid YycF'-'PhoP and PhoP'-'YycF response regulators are functional and there are similarities in the YycF and PhoP DNA binding sequences. We have also shown (i) that while YycG can phosphorylate only its cognate response regulator YycF, PhoR can phosphorylate both PhoP and YycF and (ii) that cells depleted for YycFG cannot mount a normal PhoPR-mediated phosphate limitation response. From these observations, we postulated that the roles of YycFG and PhoPR might be linked during cell wall metabolism and phosphate limitation.

In this talk we will present the results of further analysis on the relationships between the YycFG and PhoPR two-component systems and their roles in cell wall metabolism during growth and phosphate limitation.

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THE WalK/WalR ESSENTIAL SIGNAL TRANSDUCTION PATHWAY AND CELL WALL HOMEOSTASIS IN *STAPHYLOCOCCUS AUREUS*

Sarah Dubrac [1], Aurélia Delauné [1], Olivier Poupel [1], Adeline Mallet [2], Tarek Msadek [1]
Biology of Gram-positive Pathogens [1], Department of Microbiology, Plateforme de Microscopie Ultrastructurale [2], Imagopole, Institut Pasteur, 25 rue du Dr. Roux, 75015 Paris, France

The highly conserved WalK/WalR (aka YycG/YycF) two-component system is specific to low G+C % Gram-positive bacteria. While this system is essential for cell viability, both the nature of its regulon and its physiological role remained mostly uncharacterized. We have recently shown that the *S. aureus* WalKR system positively controls autolytic activity, in particular that of the major autolysins, AtlA, LytM and Sle1, and identified at least ten genes belonging to the WalKR regulon that are known or thought to be involved in *S. aureus* cell wall degradation. While none of these genes appears to be essential, we have shown that their global regulation by the WalKR system results in a drastic down regulation of cell wall dynamics, with a complete arrest of both cell wall biosynthesis and turn over under WalKR depletion. As a consequence of these molecular disorders TEM observations revealed that the cell wall of WalKR depleted cells was significantly thicker and division septa were abnormally distributed. Recent advances presented here have shown that this global regulation is directly linked to WalKR essentiality. While the *walRK* genes are essential, the WalKR system is inducible since it is assumed that the WalR response regulator is only active when phosphorylated. While the activation signal is still unknown, several recent results suggest that it could be related to cell wall homeostasis.

As cell wall metabolism is a major parameter influencing virulence and particularly the innate immune response, we are now interested in characterizing the impact of WalKR on *S. aureus* virulence. Beyond the regulation of genes involved in cell wall metabolism, we have also shown that the WalKR system activates expression of at least two genes involved in interactions with the extracellular host matrix and influences the capacity of *S. aureus* to adhere to the host matrix.

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DYNAMIC ASSEMBLY AND DISASSEMBLY OF THE TYPE IV MOLECULAR MACHINE

Iryna Bulyha and Lotte Søgaard-Andersen
Max Planck Institute for Terrestrial Microbiology, D-35043 Marburg, Germany

Myxococcus xanthus harbors two gliding motility systems, A and S. The S(ocial)-system depends on type IV pili (T4P) and is generally active only when the cells are within contact distance of each other. T4P undergo cycles of assembly and retraction. The force for S-motility is generated by retraction of T4P. T4P are localized in a unipolar pattern and are present only at the leading pole of the rod-shaped cells. *M. xanthus* cells periodically undergo cellular reversals in which the old leading pole (which harbors T4P) becomes the new lagging pole (which does not harbor T4P). These observations suggest that in parallel with a cellular reversal, the pole at which T4P assemble switches. The molecular mechanisms regulating the T4P extension/retraction cycle and underlying T4P pole switching remain unknown.

To investigate these mechanisms, we focused on the cellular localization of five highly conserved T4P biogenesis proteins (PilB, PilT, PilM, PilC and PilQ), which are present in all known T4P systems. PilB and PilT are cytoplasmic proteins and members of the secretion ATPase superfamily of proteins; PilB is required for assembly of T4P, while PilT is necessary for T4P retraction. PilM shows similarity to MreB/FtsA and is indispensable for T4P assembly. PilC is an inner membrane protein and is necessary for T4P assembly. The PilQ secretin forms a gated oligomeric channel for the pilus in the outer membrane. Using immuno-fluorescence microscopy, and time-lapse fluorescence microscopy with functional YFP-fusions, we show that PilQ, PilC and PilM are localized in clusters at both cell poles. These clusters have equal intensities and they *do not oscillate* between the two poles during reversals. The analysis of PilB and PilT localization revealed that both proteins are localized in polar clusters. PilB is predominantly localized in a cluster at the leading pole and PilT is predominantly localized in a cluster at the lagging cell pole. This localization is dynamic and the two proteins *oscillate* between the poles during reversals. These observations show that T4P function depends on two sets of proteins: one set is statically localized at both cell poles, and the other set is dynamically localized. Based on these findings we suggest that during cellular reversals, the T4P machinery is disassembled at the old leading pole and reassembled at the new leading pole.

Moreover, we will present the data which suggest that the T4P assembly/retraction cycle relies on a PilB/PilT competition-based mechanism. According to this model, PilB at the leading cell pole stimulates T4P assembly, and the occasional accumulation of PilT at the leading cell pole results in retraction. Therefore, the two dynamically localized ATPases determine whether assembly or disassembly of pilus takes place.

A "FOUR COMPONENT" SIGNAL TRANSDUCTION SYSTEM REGULATES DEVELOPMENTAL PROGRESSION IN *MYXOCOCCUS XANTHUS*

Sakthimala Jagadeesan, Bongsoo Lee, and **Penelope I. Higgs**
Department of Ecophysiology, Max Planck Institute for Terrestrial Microbiology, D35043 Marburg, Germany

Myxococcus xanthus responds to starvation by entering a multicellular developmental program in which 10^5 cells first aggregate into mounds and then within these mounds, differentiate into environmentally resistant spores. Under standard laboratory conditions, formation of spores within the mounds (fruiting bodies) takes approximately 72 hours. We have previously demonstrated that progression through the developmental program appears to be regulated by an atypical two component signal (TCS) transduction system consisting of four TCS homologs (RedC, RedD, RedE, and RedF). While RedC appears to be a typical membrane bound histidine kinase, RedD consists solely of two receiver domains. RedE is a soluble histidine kinase-like protein, and RedF is a single receiver domain response regulator. Based on a combination of genetic and biochemical analyses, we propose a model for how these four Red proteins function together to regulate progression through the developmental program. Our data suggests that development is repressed when the RedC histidine kinase phosphorylates RedF, a single domain response regulator. Developmental repression is relieved when, in response to an unknown signal(s), RedC is instead induced to phosphorylate the response regulator RedD. Surprisingly, the phosphoryl group is then transferred from RedD to the histidine kinase-like protein, RedE. RedE is then likely made accessible to RedF-P, whereupon it removes RedF's phosphoryl group. We present the data that supports this model. Furthermore, we will address how progression through the developmental program is modulated by the Red system.

INDEPENDENCE AND INTERDEPENDENCE OF Dif AND Frz CHEMOSENSORY PATHWAYS IN *MYXOCOCCUS XANTHUS* CHEMOTAXIS

Qian Xu, Wesley P. Black, Christena L. Cadieux and **Zhaomin Yang**
Biological Sciences, Virginia Tech, Blacksburg, Virginia 24061-0910, USA

Dif and Frz, two *Myxococcus xanthus* chemosensory pathways, are required in phosphatidylethanolamine (PE) chemotaxis for excitation and adaptation, respectively. DifA and FrzCD, the homologs of methyl-accepting chemoreceptors in the two pathways, were examined for methylation in the context of chemotaxis and inter-pathway interactions. Evidence indicates that DifA may not undergo methylation but signals transmitting through DifA do modulate FrzCD methylation. Results also revealed that *M. xanthus* possesses Dif-dependent and Dif-independent PE sensing mechanisms. Previous studies showed that FrzCD methylation is decreased by negative chemostimuli but increased by attractants such as PE. Results here demonstrate that the Dif-dependent sensory mechanism suppresses the increase in FrzCD methylation in attractant response and elevates FrzCD methylation upon negative stimulation. In other words, FrzCD methylation is governed by opposing forces from Dif-dependent and Dif-independent sensing mechanisms. We propose that the Dif-independent but Frz-dependent PE sensing leads to increases in FrzCD methylation and subsequent adaptation, while the Dif-dependent PE signaling suppresses or diminishes the increase in FrzCD methylation to decelerate or delay adaptation. We contend that these antagonistic interactions are crucial for effective chemotaxis in this gliding bacterium to ensure that adaptation does not occur too quickly relative to the slow speed of *M. xanthus* movement.

PREDATAXIS BEHAVIOR IN *MYXOCOCCUS XANTHUS*

Jeb Berleman, Jodie Scott, Tatiana Chumley, and John R. Kirby*
University of Iowa, Department of Microbiology, Iowa City, IA 52246, USA

Spatial organization of cells is important for both multicellular development and tactic responses to a changing environment. We find that the slow-moving, gliding bacterium, *Myxococcus xanthus,* utilizes a Che-like pathway to regulate multicellular rippling during predation of other microbial species. Tracking of GFP-labeled cells indicates directed movement of *M. xanthus* cells during the formation of rippling wave structures. Quantitative analysis of rippling indicates that ripple wavelength is adaptable and dependent on prey cell availability. Methylation of the receptor, FrzCD, is required for this adaptation: a *frzF* methyltransferase mutant is unable to construct ripples, whereas a *frzG* methylesterase mutant forms numerous, tightly packed ripples. Both the *frzF* and *frzG* mutant strains are defective in directing cell movement through prey colonies. These data indicate that the transition to an organized multicellular state during predation in *M. xanthus* relies on the tactic behavior of individual cells, mediated by a Che-like signal transduction pathway. Predataxis behavior differs from chemotaxis behavior in that it seems to depend heavily on tactile-stimulation, as opposed to chemical-stimulation.

DYNAMIC LOCALIZATION OF FrzCD IN *MYXOCOCCUS XANTHUS*

Emilia M.F. Mauriello[1], David P. Astling[1], Oleksii Sliusarenko[2] and David R. Zusman[1]
University of California, Department of Molecular and Cell Biology, Berkeley, CA 94720, USA[1]; Yale University, Department of Molecular, Cell and Developmental Biology New Haven, CT 06520, USA[2]

Directional motility in the gliding bacterium *Myxococcus xanthus* requires controlled cell reversals mediated by the Frz chemosensory system. FrzCD, a cytoplasmic chemoreceptor, does not form membrane bound polar clusters typical for most bacteria, but rather cytoplasmic clusters that are helically arranged and span the cell length. This unusual localization is maintained in the absence of the CheA homologs FrzE or CheA4, and the CheW homologs FrzA or FrzB. In contrast, MCPs lose their respective polar or cytoplasmic localization in *Escherichia coli* and *Rhodobacter spheroides* strains lacking CheA and/or CheW (1, 2). The distribution of FrzCD in living cells was found to be dynamic: FrzCD was localized in clusters that continuously changed their size, number, and position. The number of FrzCD clusters was correlated with cellular reversal frequency: fewer clusters were observed in hypo-reversing mutants and additional clusters observed in hyper-reversing mutants. When moving cells made side-to-side contacts, FrzCD clusters in adjacent cells showed transient alignments. These events were frequently followed by one of the interacting cells reversing. These observations suggest that FrzCD detects signals from a cell-contact sensitive signaling system and then re-localizes as it directs reversals to distributed motility engines.

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IDENTIFYING NOVEL BACTERIAL CYTOSKELETAL ELEMENTS AND CYTOSKELETAL INTERACTORS THROUGH HIGH-THROUGHPUT IMAGING

John Werner, Michael Ingerson-Mahar, Jonathan Guberman, and **Zemer Gitai**
Princeton University, Department of Molecular Biology, LTL-355 Washington Rd., Princeton, NJ 08540

Bacterial cytoskeletal proteins polymerize into filamentous structures that represent key regulators of a wide array of cellular processes, including cell shape determination, cell division, and cell polarity. While bacterial homologs of all three major eukaryotic cytoskeletal families have already been described, the upstream regulators of bacterial cytoskeletal assembly and downstream effectors of cytoskeletal function remain poorly understood. In addition, recent studies have suggested that additional filament-forming proteins remain uncharacterized.

All known cytoskeletal elements in both bacteria and eukaryotes have distinct non-uniform subcellular distributions. Focusing on the asymmetric bacterium, *Caulobacter crescentus*, we thus employed a directed high-throughput imaging approach to identify both novel bacterial cytoskeletal proteins and proteins that act upstream or downstream of the previously-characterized cytoskeletons. We developed a pipeline of high-throughput methods for generating fluorescent protein fusions, expressing them in *Caulobacter*, imaging their subcellular distribution at high resolution, and quantitating the resulting imaging data. By using this pipeline to analyze over 2,800 *Caulobacter* proteins as both N-and C-terminal mCherry fusions, we identified a set of ~300 localized *Caulobacter* proteins. This set included the three known *Caulobacter* cytoskeletons (the MreB actin homolog, FtsZ tubulin homolog, and Crescentin intermediate-filament). We also identified a novel protein that localizes to a tight line that hugs a short region of the inner curvature of *Caulobacter* cells. This protein may polymerize on its own, as it is capable of forming linear structures when expressed in heterologous systems such as E. coli or S. pombe. Preliminary studies suggests that this previously-uncharacterized cytoskeletal element plays a role in cell shape determination and may exhibit crosstalk with other cytoskeletal proteins.

To identify upstream regulators of cytoskeletal assembly, we modified our pipeline to allow us to assay the effects of overexpressing genes of interest on the localization patterns of MreB, FtsZ, and Crescentin. A pilot screen of ~200 conserved proteins with no known function identified four proteins that perturbed FtsZ, one that perturbed MreB, one that perturbed both MreB and FtsZ, and one that perturbed Crescentin. The functions and mechanisms of action of these candidate cytoskeletal regulators are currently being examined. Finally, to identify downstream cytoskeletal effectors, we imaged our library of localized *Caulobacter* proteins in the presence of the MreB-delocalizing compound, A22. We found a large number of proteins that are either delocalized or mislocalized by A22 and are currently determining the cellular functions of these proteins as well as the nature of their direct or indirect associations with MreB.

THE ROLE OF POSITIVE FEEDBACK IN CONTROLLING FLAGELLA ASSEMBLY DYNAMICS

Supreet Saini[1], Christy Aldridge[2], Jonathan Brown[2], Philip Aldridge[2], **Christopher Rao**[1]
[1]Department of Chemical and Biomolecular Engineering, University of Illinois, Urbana, IL 61801, United States
[2]Institute for Cell and Molecular Biosciences, Newcastle University, Framlington Place, Newcastle upon Tyne NE2 4HH, United Kingdom

Flagellar assembly in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) proceeds in a sequential manner, starting from the base of the flagella and concluding at the filament tip. A key regulatory step in the assembly process is the σ^{28}-FlgM checkpoint, which prevents the activation of σ^{28}-dependent P_{class3} promoters prior to completion of the hook basal body. This regulatory checkpoint is typically assumed to involve a binary decision process: either proceed with P_{class3} gene expression or not, depending on the state of assembly. However, mathematical modeling suggests that this binary checkpoint may in fact result in more subtle, rheostat-like control. σ^{28} is involved in a positive feedback loop, as $\iota\tau$ positively regulates its own expression along with the expression of FliZ, an activator of P_{class2} gene expression. In addition, the ability of σ^{28} to regulate gene expression depends on the concentration of FlgM in the cell. This suggests that the response of the σ^{28} positive feedback loop is tuned by late protein secretion. In fact, our modeling and experimental results suggests that σ^{28} and FlgM are not only involved in establishing the binary checkpoint between P_{class2} and P_{class3} gene expression but are also involved in fine tuning the relative timing of expression. In particular, the positive feedback loop involving σ^{28} and FliZ establishes the delay between P_{class2} and P_{class3} gene expression.

In this talk, we will discuss our recent work investigating the positive feedback loops involving σ^{28} and FliZ. We have recently shown that FliZ is an $FlhD_4C_2$-dependent activator of P_{class2} gene expression. In addition, our results indicate the FliZ speeds up the induction of P_{class2} genes in a secretion-dependent manner. With regards to σ^{28}, we have found that autoregulation controls the relative timing of gene expression. In cells lacking FlgM, both P_{class2} and P_{class3} genes are induced at the same times. Conversely, when the rate of FlgM secretion is reduced, the delay between P_{class2} and P_{class3} gene expression is exaggerated. These results suggest that timing is responsive to the rate of late protein secretion. Moreover, mathematical modeling predicts that this control is due to autoregulation by σ^{28}. To test this model, we have rewired the flagellar gene circuit by replacing the native P_{fliA} promoter with $P_{class1}/P_{class2}/P_{class3}$ promoters. Consistent with the model predictions, these promoter replacement experiments show that autoregulation plays a key role in enforcing the timing of flagellar gene expression. Last, we also investigated gene expression dynamics at single-cell resolution, and our preliminary results suggest that the dynamics may exhibit bistability, consistent with control involving feedback. Collectively, our results suggest that the regulation of flagellar gene expression is complex and involves multiple layers of control.

CRYSTAL STRUCTURE OF FLIT, A BACTERIAL FLAGELLAR EXPORT CHAPERONE FOR THE FILAMENT CAP PROTREN HAP2 (FliD)

Katsumi Imada, Tohru Minamino, Miki Kinoshita and Keiichi Namba
Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan

The bacterial flagellum is a filamentous organelle responsible for motility. Since the flagellum extends from the cytoplasm to the cell exterior, the external component proteins have to be exported from the cytoplasm. The protein subunits are exported by the flagellar specific export apparatus, which is a member of the type III secretion system. The export apparatus is believed to be located within the C-ring of the flagellar basal body and consists of at least six integral membrane proteins (FlhA, FlhB, FliO, FliP, FliQ, FliR) and three soluble proteins (FliH, FliI, FliJ). In addition to these proteins, other cytoplasmic proteins (FlgN, FliA, FliS, FliT) act as substrate-specific chaperons that facilitate the export of their substrates.

FliT is a flagellar export chaperone for FliD (HAP2), which forms a capping complex at the distal end of the flagellar filament and promotes incorporation of flagellin subunits into the growing filament, and prevents FliD from premature aggregation in the cytoplasm. FliT is not only involved in protein export but also in regulation of flagellar gene expression. FliT negatively regulates transcription of the flagellar class 2 operons by binding to $FlhD_4C_2$ complex, which is a transcriptional activator.

We have determined a crystal structure of FliT at 3.2 Å resolution. The structure and following genetic and biochemical studies have revealed that the C-terminal region of FliT regulates its interactions with other flagellar proteins. We will discuss the molecular mechanisms of protein export and gene expression based on the FliT structure.

STRUCTURAL INSIGHT INTO ACTIVE FLAGELLAR MOTOR FORMATION THROUGH THE PERIPLASMIC REGION OF MOTB

Seiji Kojima[1], Mayuko Sakuma[1], Yuki Sudo[1], Chojiro Kojima[2], Tohru Minamino[3], Keiichi Namba[3], Michio Homma[1] and Katsumi Imada[3*]
[1]Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-Ku, Nagoya 464-8602, Japan; [2]Laboratory of Biophysics, Graduate School of Biological Sciences, Nara Institute of Science and Technology (NAIST), 8916-5 Takayama, Ikoma, Nara, 630-0192 Japan; [3]Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan

Bacterial flagellar motor is a supramolecular nano-machine powered by the transmembrane gradient of protons or sodium ions, and spins flagellar filaments to drive cell motility. Motor torque is generated by the rotor-stator interaction that is coupled with ion flow through the ion-channel in the stator unit, which is composed of four MotA and two MotB subunits. About ten stators are assembled around the perimeter of the rotor and anchored to peptidoglycan layer by the peptidoglycan-binding (PGB) domain of MotB. The rotor-stator assembly is not a rigid complex, so each stator unit can dynamically be exchanged in the functional motor, and is activated only when assembled around the rotor. However, the mechanisms of the stator assembly and the activation of the proton flow remain unclear. Here, we report the crystal structure of a C-terminal fragment of MotB ($MotB_C$), which includes the PGB domain and covers the whole periplasmic region essential for cell motility (PEM), at 1.75 Å resolution. The structure, and subsequent mutational and biochemical analyses indicate that dimer formation by the PGB domains is required for motility through the regulation of the arrangement of the transmembrane segment. Moreover, we show that large structural changes in the N-terminal helices should be coupled with both peptidoglycan binding and activation of the stator. This work provides novel structural insight into the dynamic behavior of the ion-channel complex regulated by the periplasmic domain, and the activation mechanism of the complex coupled with completion of the assembly where it works.

STATOR SELECTION IN *SHEWANELLA ONEIDENSIS* MR-1

Anja Paulick, Andrea Koerdt, **Kai M. Thormann**
Department of Ecophysiology, MPI für terrestrische Mikrobiologie, Karl-von-Frisch-Strasse, D-35043 Marburg, Germany

The Gram-negative metal-ion reducing bacterium *Shewanella oneidensis* MR-1 is motile by means of a single polar flagellum. We identified two potential stator systems, PomAB and MotAB, each sufficient as a force generator to drive flagellar rotation. Physiological studies demonstrate that PomAB is sodium-dependent while MotAB is powered by proton motive force. Homology comparisons strongly indicate that the MotAB system has been acquired by horizontal gene transfer, probably as a consequence of long-term adaptation from a marine to a low-sodium freshwater environment. As in *S. oneidensis* MR-1, a number of bacterial species possess more than one stator system to power a single flagellar system but it is yet unclear, how selection of the stators is achieved.

Expression analysis at the single cell level showed that both stator systems of *S. oneidensis* MR-1 are expressed simultaneously, and functional fusions of PomB and MotB to mCherry revealed that both stator systems are present in the cell at the same time. While the Pom system is efficiently localizing to the flagellated cell pole under all conditions, the Mot stator is located in the cell membrane and only found at the cell pole at high abundance in media with low sodium content. At low sodium, both stator systems are localizing to the flagellated cell pole in the majority of the cell population, thus indicating that under such conditions a hybrid motor may be formed. We conclude that stator selection occurs at the level of protein localization by alterations in the localization efficiency in response to sodium levels.

In *Vibrio* species, two additional proteins, MotX and MotY, are involved in stator recruitment and sodium-dependent swimming. We therefore analyzed whether *S. oneidensis* MR-1 orthologs to MotX and MotY play a role in stator selection. Mutant and localization analyses demonstrated that both proteins are required for function of the Pom as well as the Mot stator system. As opposed to the *Vibrio* system, in *S. oneidensis* MR-1, MotX and MotY are not required for stator recruitment and also do not play a role in stator selection in response to sodium conditions.

TAKING CONTROL OF THE BACTERIAL FLAGELLAR MOTOR

Mathieu Gauthier, Dany Truchon and Simon Rainville
Department of Physics, Engineering Physics and Optics and Centre d'optique photonique et laser, Laval University, Québec, Québec, CANADA

The bacterial flagellar motor is a fairly complex machine, requiring 40-50 genes for its expression, assembly and control. Furthermore, it is embedded in the multiple layers of the bacterial membrane. That explains why, unlike many other molecular motors, it has not yet been studied *in vitro*. As spectacular studies of linear motors (like kinesin, myosin and dynein) have clearly demonstrated, an *in vitro* system provides the essential control over experimental parameters to achieve the precise study of the motor's physical and chemical characteristics. Here, we report significant progress towards the development of a unique *in vitro* system to study quantitatively the bacterial flagellar motor.

Our system consists of a filamentous *Escherichia coli* bacterium partly introduced inside a micropipette. Femtosecond laser pulses (60 fs and ~ 15 nJ/pulse) are then tightly-focused on the part of the bacterium that is located inside the micropipette. This vaporizes a submicrometer-sized hole in the wall of the bacterium, thereby granting us access to the inside of the cell and the control over the proton-motive force that powers the motor. Using a patch-clamp amplifier, we applied an external voltage between the inside and the outside of the micropipette. If the hole in the bacterium is open, that voltage should translate into a membrane potential powering the motors outside of the micropipette. As we varied the applied potential, variations in the motor's rotation speed were observed. For these preliminary results, the rotation speed was observed directly using video microscopy of fluorescently labeled filaments. That system opens numerous possibilities to study the flagellar motor and other membrane components.

EXPERIMENTAL EVIDENCE FOR CONFORMATIONAL SPREAD IN THE BACTERIAL SWITCH COMPLEX

Richard W. Branch[1], Fan Bai[1], Dan V. Nicolau[2], Teuta Pilizota[1], Bradley Steel[1], Philip K. Maini[2], Richard M. Berry[1]
[1]Clarendon Laboratory, Department of Physics, University of Oxford, Parks Road, Oxford OX1 3PU, UK. [2]Centre for Mathematical Biology, Mathematical Institute, University of Oxford, St.Giles Road, Oxford OX1 3LB, UK.

The bacterial switch complex in *E. coli* controls the direction of rotation of the bacterial flagellar motor between clockwise and counterclockwise modes. The complex takes the form of a ring composed of about 110 FliN, 34 FliM and 26 FliG protein subunits. Regulation is through binding of the signaling molecule CheY-P to FliM. FliG interfaces with the torque-generating stator units of the motor. The precise mechanism by which the complex executes a switch is unclear.

The complex displays the ultrasensitive nature typical of allosteric proteins, with a steep sigmoidal relationship existing between [CheY-P] and motor rotational bias. Allosteric regulation of proteins has classically been understood in terms of the Monod-Wyman-Changeux (MWC) or Koshland-Nemethy-Filmer (KNF) models. However, it is unrealistic that MWC-type concerted transitions could be responsible for quaternary conformational changes of such a large complex, and cooperative binding studies *in vitro* and *in vivo* have precluded a KNF-type induced-fit mechanism.

The MWC and KNF models are recognized as limiting cases of a general allosteric scheme that has recently been described in a model of conformational spread. The model has been shown to be capable of reproducing motor switching kinetics. A directly observable consequence of conformational spread in the switch complex would be the variation of motor speed associated with the conformational spread of ring subunit state. In particular, the duration of switch events should be finite and broadly distributed due to the diffusive random walk of conformational spread, and incomplete switches should be observed due to incomplete growth and shrinkage of subunit state domains.

We have used high-resolution back-focal-plane interferometry of polystyrene beads attached to truncated WT *E. coli* flagella to resolve intermediate states of the motor predicted by conformational spread, and demonstrate detailed quantitative agreement between our measurements and conformational spread simulations. Individual switch events are not instantaneous, but follow a broad distribution of switch times with mean ~ 20 ms. The shortest switch events are observed to last less than 1ms, while the longest require over 100ms and take several revolutions to complete. Intervals between switches are exponentially distributed at all values of bias. Incomplete switches reaching a range of intermediate speeds are observed. The events are Poisson distributed in time with a bias-dependent frequency.

DO INDIVIDUAL BACTERIAL FLAGELLAR MOTORS USE HYSTERESIS TO MAINTAIN A ROBUST OUTPUT IN A NOISY ENVIRONMENT? – AN EXPERIMENTAL STUDY

Peter Reuven[1], Oleg Krichevsky[2], Michael Eisenbach[1]
[1] Department of Biological Chemistry, Weizmann Institute of Science, 76 100, Israel
[2] Department of Physics, Ben-Gurion University, Beer Sheva, 84 105, Israel

It is known that despite imperfections of intracellular environment, flagellar motor outputs are robust against stochastic fluctuations of CheY signal. Indirect evidence from our lab suggests that, to maintain stability, the motor complex might damp out fluctuations in the intracellular level of CheY by having a hysteresis feature - two different thresholds for switching. In this case, hysteresis means that the default-state motor switches at a higher threshold from counterclockwise to clockwise state compared to a lower threshold when switching back. Such behavior will produce hysteretic loop in input/output characteristics of flagellar motor.

Our aim is to pin-point the level at which the noise-filtering (via hysteresis) occurs in the chemotactic network. We are studying flagellar rotation of single cells as a function of their intracellular CheY-P concentration, changing the concentration up and down in order to cover both - counterclockwise to clockwise and clockwise to counterclockwise - switching routes.
Since it is impossible to distinguish CheY from CheY-P in vivo, one has to work under conditions that maintain CheY constantly fully phosphorylated. The challenge was how to effectively decrease the concentration of the phosphorylated signal.

Knowing that we cannot play with the level of phosphorylation we opt to play with the level of the CheY-P protein instead. While to increase protein concentration is a routine task, to decrease it is less obvious. We cloned a bi-modal, inducible plasmid expressing a CheY fused to yellow fluorescent protein (YFP) and ssrA degradation tag. YFP is used for quantifying the signal whereas the degradation tag makes it possible to shorten the lifetime of CheY-YFP. The core assumption of our approach is that heat-shock-induced proteases accelerate the degradation of the ssrA-targeted CheY-YFP protein. We have verified this assumption experimentally.

We monitor the degradation of CheY-YFP by a decrease in fluorescence intensity and this decrease is correlated with the change of the direction of motor rotation. Input/output characteristics of individual flagellar motors is build from these correlations.

Advancing this study will, hopefully, enable us to deeper understand how the mechanisms of intracellular interactions affect the logic of cell's behavior.

DYNAMICS OF THE BACTERIAL FLAGELLAR MOTOR WITH MULTIPLE STATORS

Giovanni Meacci and **Yuhai Tu**
IBM T. J. Watson Research Center

The bacterial flagellar motor drives the rotation of flagellar filaments and enables many species of bacteria to swim. Torque is generated by interaction of stator units, anchored to the peptidoglycan cell wall, with the rotor. Recent experiments [Yuan, J. & Berg, H. C. (2008) PNAS 105, 1182-1185] show that near zero load the speed of the motor is independent of the number of stators. Here, we introduce a mathematical model of the motor dynamics that explains this behavior based on a general assumption that the stepping rate of a stator depends on the torque exerted by the stator on the rotor. We find that the motor dynamics can be characterized by two time scales: the moving-time interval for the mechanical rotation of the rotor and the waiting-time interval determined by the chemical transitions of the stators. We show that these two time scales depend differently on the load, and that their crossover provides the microscopic explanation for the existence of two regimes in the torque-speed curves observed experimentally. We also analyze the speed fluctuation for a single motor using our model. We show that the motion is smoothed by having more stator units. However, the mechanism for such fluctuation reduction is different depending on the load. We predict that the speed fluctuation is determined by the number of steps per revolution only at low load and is controlled by external noise for high load. Our model can be generalized to study other molecular motor systems with multiple power-generating units.

EFFECT OF OSMOLYTES ON REGULATING THE ACTIVITIES OF THE SSK1 RESPONSE REGULATOR FROM *SACCHAROMYCES CEREVISIAE*

Alla O. Kaserer, Paul F. Cook and Ann H. West
Department of Chemistry and Biochemistry, The University of Oklahoma, 620 Parrington Oval, Norman, OK 73019

The multi-step His-Asp phosphorelay system in *Saccharomyces cerevisiae* allows cells to adapt to osmotic, oxidative and other environmental stresses. The pathway consists of a hybrid histidine kinase SLN1, a histidine-containing phosphotransfer (HPt) protein YPD1 and two response regulator proteins, SSK1 and SKN7. Under non-osmotic stress conditions, the SLN1 kinase is active and phosphoryl groups are shuttled to SSK1 via YPD1. We have previously demonstrated that YPD1 stabilizes the phosphorylated form of SSK1. The cellular response to hyperosmotic stress involves rapid efflux of water and change in intracellular ion and osmolyte concentration. It is our hypothesis that these changes may affect rates of phosphotransfer within the SLN1-YPD1-SSK1 phosphorelay system and the phosphorylated lifetime of response regulators. Therefore, we examined the effect of different solute concentrations on dephosphorylation of SSK1 and phosphotransfer rates within the phosphorelay system using half-life studies and rapid quench kinetics, respectively. These studies provide new insight and offer a better understanding of how this His-Asp multi-step phosphorelay is environmentally regulated.

REGULATION OF *Escherichia coli* MOTILITY BY THE NITRIC OXIDE SENSITIVE TRANSCRIPTIONAL REPRESSOR NsrR

Jonathan D. Partridge and Stephen Spiro
Department of Molecular and Cell Biology, The University of Texas at Dallas, 800 W. Campbell Road, Richardson, Texas 75080, USA

There is circumstantial evidence implicating the water-soluble free radical nitric oxide (NO) as a regulator of motility, chemotaxis and biofilm development. Heme-containing NO-binding domains of methyl accepting chemotaxis proteins from *Clostridium botulinum* and *Thermoanaerobacter tengcongensis* have been characterized, though the prediction that these proteins mediate taxis towards or away from NO has not been tested. In transcriptomics experiments, the expression of some motility genes has been observed to be perturbed by exposure of cultures to sources of NO or nitrosative stress (imposed by *S*-nitrosothiols), although both positive and negative responses have been reported, and the regulators involved were not identified. In the non-pathogenic organism *Nitrosomonas europaea*, NO stimulates biofilm formation. In *Pseudomonas aeruginosa* and *Staphylococcus aureus*, there is evidence that NO inhibits biofilm formation, or stimulates dispersal, and NO stimulates swimming and swarming motility in *P. aeruginosa*. In no case has a molecular mechanism been described which accounts for the effects of NO on biofilm development or motility.

NO is made in bacteria either as a by-product of nitrite reduction to ammonia, or as an intermediate of denitrification, and is made by the inducible NO synthase of host phagocytes. Thus pathogenic bacteria can be exposed both to endogenously-generated NO, and to the NO made by host cells. In *Escerichia coli*, the regulatory proteins NorR and NsrR mediate adaptive responses to NO, by controlling the expression of genes encoding enzymes that reduce or oxidize NO to less toxic species. The key NO detoxifying activities are the flavohemoglobin (encoded by the *hmp* gene) and the flavorubredoxin (encoded by *norVW*), the expression of which is regulated by NsrR and NorR, respectively. As far as is known, the *norVW* promoter is the sole target for regulation by NorR, while NsrR appears to control a large regulon of genes and operons.

The extent of the NsrR regulon of *E. coli* has been assessed computationally, and by a transcriptomics analysis of a strain in which NsrR was titrated by the presence of multiple copies of a cloned NsrR binding site. We believe that neither approach has provided a comprehensive inventory of all of the genes regulated by NsrR. Therefore, we used chromatin immunoprecipitation and microarray analysis (ChIP-chip) to identify NsrR binding sites in the *E. coli* genome. Surprisingly, we found NsrR binding sites associated with the promoter regions of three transcription units (*mqsR-ygiT, fliAZY* and *fliLMNOPQR*) containing genes with well-established or suspected roles in motility and/or biofilm development. We have confirmed that the *fliA* and *fliL* promoters are subject to regulation by NsrR and NO, and have identified an NsrR binding site in the *fliA* promoter. We have shown that NsrR is a negative regulator of motility in both K12 and UPEC strains of *E. coli*. These results provide, for the first time, a molecular mechanism by which NO might control bacterial motility.

SYNTHETIC LETHALITY UNCOVERS A NOVEL LINK BETWEEN THE MalT AND OmpR REGULONS

Sylvia A. Reimann and Alan J. Wolfe
Loyola University Chicago, Maywood, Il

Synthetic lethality (SL) is a genetic term for the inviability of a double mutant combination of two fully viable single mutants. SL is commonly interpreted as redundancy at an essential metabolic step. However, a second, less well-known, class of SL exists: the so-called "defect-damage-repair" (DDR) cycles that link apparently unrelated metabolic pathways (Ting *et al*., 2008).

We have isolated an SL mutant of the form *ompR* SL(*ompR*), where SL(*ompR*) refers to a mutation that causes death when present in a cell that lacks the two-component response regulator OmpR. This global regulator is required for proper assembly of the cell envelope and we reasoned that the nature of the SL(*ompR*) mutation would provide further insight into the role of OmpR and its regulon.

Using a combined genetic/genomic approach, we mapped the SL(*ompR*) mutation to *malT*, which encodes the transcription factor MalT. Because overexpression of the MalT inhibitor MalK did not rescue growth, we proposed that the *malT* mutation leads to a constitutively active form (MalT^{c}). To test this hypothesis, we deleted the MalT-dependent *lamB*, which encodes an outer membrane porin, and found that this deletion suppressed the SL of the *ompR malT*^{c} mutant.

Since LamB and OmpR do not perform redundant functions, we propose that the observed SL is of the DDR variety, as follows: the defect (MalT^{c} activity) leads to damage (constitutive expression of LamB) that is repaired by one or more members of the OmpR regulon. To further understand the role of OmpR, we are currently seeking OmpR regulon members that can suppress the SL of the *ompR malT*^{c} mutant.

A CHEMOTAXIS-LIKE SIGNALING PATHWAY REGULATES THE EXPRESSION OF EXTRACELLULAR MATERIALS IN *GEOBACTER SULFURREDUCENS*

Hoa T. Tran[1], Derek R. Lovley[2], and Robert M. Weis[1]
Departments of Chemistry[1] and Microbiology[2], University of Massachusetts, Amherst, MA 01003, USA.

The chemotaxis pathway that regulates cell motility toward chemical attractants is well-studied in *Escherichia coli*. By contrast, multiple chemotaxis clusters have been found in many other bacteria, and evidence is accumulating that these cells use chemotaxis-like pathways to regulate diverse cellular functions. The genome of *Geobacter sulfurreducens*, a δ-*Proteobacterium* found predominantly in the Fe(III) reducing zone of sedimentary environment contains ~70 chemotaxis gene homologs arranged in 6 major clusters. Cluster 5 (Che5) has a complete set of chemotaxis homologs, including the kinase *cheA* (1 copy), *cheW* (2 copies), *cheR* (1), *cheB* (1), *cheY* (3) and four other non-*che* genes. There are 34 chemoreceptor homologs in the genome, but none is found in the Che5 cluster. Che5-type clusters have been identified in the genomes of several δ-*Proteobacteria*, yet their functions are not known. Here, we report that *G. sulfurreducens* Che5 cluster regulates gene expression, and in particular the synthesis of extracellular material that is abundant in OmcS and OmcZ, two *c*-type cytochromes bound to the outer membrane. OmcS and OmcZ are essential for cell growth in insoluble electron acceptors and for effective electricity production on electrodes. Deletion mutants of the homologs of *cheW* (*gsu2218*, *gsu2220*), *cheA* (*gsu2222*) and *cheR* (*gsu2215*) increased OmcS production and decreased OmcZ. In contrast, deletion mutants of *cheB* (*gsu2214*), one of the three *cheY*s (*gsu2223*) and a non-*che* gene (*gsu2216*) decreased OmcS and increased OmcZ production. The chemoreceptors that signal through these Che5 proteins are hypothesized to belong to a single MA class. Evidence that supports this idea is based on the phenotypes of deletion mutants in two *mcp* genes (*gsu1704,* and *gsu2372*), which are similar the *cheA* mutant. Wherever the functional parallels can be drawn, the function of the homologues in *Geobacter* Che5 signaling pathway is similar to their counterpart in the *E. coli* chemotaxis pathway. In addition to changes in OmcS and OmcZ expression, the *cheA*, *cheR* and *cheW* (*gsu2220*) mutants promote cell aggregation and overproduce non-PilA filamentous material. Moreover, microarray data of the *cheR* mutant, and quantitative RT-PCR data from the other *che* mutants indicate that the Che5 cluster alter the expression of ~175 genes. A substantial fraction of these are predicted to contain export signals that will result in an to extracellular location. Taken together, these data demonstrate that *G. sulfurreducens* Che5 cluster, with one class of chemoreceptors, regulates the extracellular matrix material biosynthesis.

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THE TWO-COMPONENT REGULATORY SYSTEM BarA/SirA IS AT THE TOP OF A MULTI-FACTORIAL REGULATORY CASCADE CONTROLLING THE EXPRESSION OF THE SPI-1 AND SPI-2 VIRULENCE REGULONS IN *SALMONELLA*

Luary C. Martínez, José L. Puente and Víctor H. Bustamante
Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México. Cuernavaca, Mor., México.

Horizontal gene transfer of pathogenicity islands has been a major event in the evolution of pathogenic bacteria. Integration of regulatory networks to control the expression of the gained genes has represented another essential step in this process. *Salmonella* Pathogenicity Islands 1 and 2 (SPI-1 and SPI-2) are required at different phases during *Salmonella* infection in humans and animals. A positive regulatory cascade comprising the SPI-1-encoded regulators HilD, HilA and InvF induces expression of the SPI-1 regulon in response to conditions resembling the intestinal environment, such as growth in Luria-Bertani (LB) rich medium. Two global two-component regulatory systems, OmpR/EnvZ and PhoP/PhoQ, control the expression of the SsrA/B two-component system encoded within SPI-2, which specifically induces the expression of the SPI-2 regulon genes in response to conditions resembling the intracellular environment, mimicked *in vitro* by growth at low concentrations of phosphate and magnesium. Interestingly, we have recently shown that HilD also induces expression of the SsrA/B system, and thus of the SPI-2 regulon, at late stationary phase in LB cultures, indicating that SPI-2 expression is also controlled by a SPI-1/SPI-2 cross-talk mechanism.

The results presented here, together with previous reports, better define the complex and multi-factorial regulatory cascade that controls SPI-1 and SPI-2 expression through HilD. We show that the global two-component system BarA/SirA activates the transcription of two small RNA molecules, *csrB* and *csrC*. These molecules counteract the negative effect exerted by the CsrA RNA binding protein on *hilD* mRNA stability, ensuring the synthesis of the appropriate concentration of HilD required for the expression of HilA and SsrA/B, the central positive regulators of the SPI-1 and SPI-2 regulons, respectively. Furthermore, we demonstrated that growth conditions affecting SPI-1 expression in LB (*e.g.* low salt, acidic pH or temperatures below 37°C), similarly repress the expression of the SPI-2 regulon. However, while acidic pH seems to negatively regulate the regulatory cascade by affecting the activity of the BarA sensor kinase, growth at low salt concentration or at low temperature seems to directly repress *hilD* expression through an as yet unidentified mechanism.

INTERACTION OF THE TRANSCRIPTIONAL REGULATORY COMPLEX, FlhDC, WITH ITS TARGET DNA

Yi-Ying Lee, and Philip Matsumura
Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, 835 S. Wolcott Ave., M/ C 790, Chicago, Illinois 60612-7344

The bacterial flagellum is the structure that allows bacteria to move and respond to nutritional and chemical signals in their environment. It is a complex suborganelle and the transcriptional regulation of the 40 plus structural genes is organized in a highly regulated cascade. At the top of the hierarchy is the master operon which codes for FlhD and FlhC. These two positive transcriptional regulators form a unique heteroheximeric complex which binds upstream of the -35 region and requires sigma 70 for transcription. This complex has an unusually large 'footprint' of 48 base pair and bends the DNA 110 degrees. We have proposed that the DNA bind on the circumference of this toroid shaped FlhDC complex. Although we have determined the sequence 3 footprints on FlhDC regulated promoters, it is not possible to determine a consensus binding site in these 3 sequences. In this study, we have determined which bases are important for DNA binding and activity for FlhDC regulated promoter activity. First, we have divided the FlhDC footprint in the *fliA* promoter into five segments and found that two of the segments or 40% of the footprint were not required for binding. The remaining 30 base pairs were divided into 3-5 base segments and randomly mutagenized and screened for the ability to bind and activate the *fliA* promoter. Analysis of these data suggests a consensus of ^{12}A, ^{15}A, ^{34}T, ^{36}A, ^{37}T, ^{44}A, ^{45}T in $FlhD_4C_2$ footprint fragment were important for activity. Five of these bases demonstrated high specificity. Finally, this consensus was tested and found to be important in other FlhDC regulated promoter regions.

A NOVEL AMINO ACID BINDING STRUCTURE IN BACTERIAL CHEMOTAXIS

George D. Glekas and George W. Ordal
Department of Biochemistry, University of Illinois at Urbana-Champaign
409 MSB, Urbana, IL 61801

Simple flagellated bacteria, such as *Bacillus subtilis*, possess the ability to sense their environment and move to more favorable conditions, where there are, for instance, more nutrients like amino acids, by the process of chemotaxis. The initial stage is binding of an amino acid by receptors on the outside of the cell. This binding causes conformational changes that affects the activity of enzymes on the inside of the cell and alters the movement of the bacteria. The main paradigm for understanding these events is the bacterium *Escherichia col*i. However, we have discovered that, in fact, the mechanism used by *E. coli* is not used by most bacteria and that the mechanism used by the distantly related bacterium *B. subtilis* is more likely to be the general mechanism. Unlike in *E. coli*, where binding of attractant causes a shift of the receptor polypeptide that goes from the outside of the cell to the cell interior toward the cell interior, attractant causes rotational movement of the receptors without any comparable interior shifting

We seek to understand how this rotational movement occurs in the asparagine receptor McpB. To do this, we have discovered that the most likely conformation of the exterior part of the receptor is far different from that in the *E. coli* receptor. Molecular and homology modeling of the McpB sensing domain has led to a structural model that reveals a dual PAS domain structure similar to the crystal structure of the LuxQ sensor. PAS domains are known to be conserved structures capable of binding a great many “small” molecules. Further mutagenetic analyses of putative asparagine-binding residues have not only confirmed the validity of the structural model, they have revealed certain residues that greatly effect chemo-attractant binding. Using both *in vivo* chemotactic assays and *in vitro* isothermal titration calorimetry performed on purified mutant receptor exterior regions, three residues, all in the upper PAS domain, have been shown to lower the affinity of the McpB receptor for asparagine. Mutations in the lower PAS domain show no such effect. Further structural and mutagenetic studies have shown a similar dual PAS architecture in the *B. subtilis* proline receptor McpC, with similar residues responsible for amino acid binding. Extensive homology modeling shows that eight of the ten *B. subtilis* chemoreceptors have PAS domains in their sensing domains.
We are now in the process of modeling the consequences of binding attractant at these residues on the expected structure of the receptor.

STRUCTURE AND FUNCTION OF THE *HELICOBACTER PYLORI* CHEMORECEPTOR TlpB

John Goers, Nathan Henderson, **S. James Remington**, Karen Ottemann* and Karen Guillemin
Institute of Molecular Biology, University of Oregon, Eugene OR, 97403
*Environmental Toxicology, UC Santa Cruz, Santa Cruz, CA 95064

We have shown that the *Helicobacter pylori* chemoreceptor TlpB is required for chemotaxis away from acid and from the quorum-sensing molecule autoinducer-2. Here we report the determination of an atomic resolution crystal structure for the periplasmic domain of TlpB. The structure reveals a PAS domain inserted into a pair of antiparallel helices and shows unexpected structural homology to the LuxQ receptor from *Vibrio harveyi*. Furthermore, the PAS domain tightly binds urea, suggesting that the TlpB receptor may be associated with detection of urea. We present mutational and physical evidence for interaction of TlpB with urea and its role in chemotaxis.

THE TM2-HAMP CONNECTION

Gus A. Wright, Rachel L. Crowder and Michael D. Manson
Department of Biology, Texas A&M University, College Station, TX 77843

The HAMP domain—a conserved protein motif in most Histidine kinases, Adenylate cyclases, Methyl-accepting chemotaxis proteins, and Phosphatases—typically conforms to a helix–connector-helix domain architecture. Recently, an NMR solution structure was determined for the Af1503 HAMP from the archaebacteria *Archeoglobus fulgidis* (Hulko, et al, Cell 126: 929-940, 2006). This structure revealed that the HAMP domain is a parallel four-helix bundle with a knob-on-knob packing. Similar structures of HAMP domains are proposed to exist in all 5 chemoreceptors of *Escherichia coli*, including the aspartate chemoreceptor Tar. The HAMP domain of Tar receives information from TM2 and regulates the transmission of the signal to the kinase signaling domain. Mutations were introduced into the TM2-HAMP connector region of Tar to determine if manipulating the input signal into the HAMP affects the output signal to the signaling domain. MLLT residues between R214 and P219 were deleted (-4 through -1), and additional LLT tandem repeats were added up to 8 residues after P219 (+1 through +8). Aspartate sensitivity, rotational bias, mean reversal frequency, and *in vivo* methylation were measured for these mutants. The results suggest that adding helical turns in this region destabilizes, or “relaxes”, the HAMP/signaling domains; while, removing helical turns from this region stabilizes, or “tightens”, the HAMP/signaling domains. In order to determine if increasing flexibility of the TM2/HAMP connector affects the output signal, a second set of mutants were constructed to replace the MLLT region with four tandem glycine residues (4G). Glycine residues were then subtracted (-4G through -1G) and added (+1G through +5G). Data collected from this experiment suggests that increasing the flexibility of this region dampens the input signal from TM2, in addition to destabilizing the HAMP/signaling domains.

STRUCTURE, ASSEMBLY AND CONFORMATIONAL CHANGES IN CHEMORECEPTORS STUDIED IN INTACT BACTERIAL CELLS USING CRYO-ELECTRON TOMOGRAPHY

Cezar M. Khursigara, Xiongwu Wu*, Peijun Zhang, Jon Lefman, Mario J. Borgnia, Yuhai Tu‡, Jacqueline Milne and Sriram Subramaniam
National Cancer Institute and *National Heart, Lung and Blood Institute, NIH, Bethesda, MD 20892.
‡T. J. Watson Research Center, IBM, Yorktown Heights, NY 10598

Bacteria respond to changes in their chemical environment by activating an assembly of proteins that collectively represent the bacterial chemotaxis apparatus. In Gram-negative bacteria the core-signaling unit of the chemotaxis machinery is a ternary complex composed of chemoreceptors, CheA and CheW that localize primarily to the poles of the cell and form extended arrays. Using cryo-electron tomography, we describe and compare the architecture, localization and spatial relationship between macromolecular complexes involved in chemotaxis signaling and cellular motility in three different Gram-negative bacteria. In addition, by combining the tomographic analysis with 3D averaging methods we demonstrate that trimeric chemoreceptors in *E. coli* display two distinct conformations that differ principally in arrangement of the HAMP domains within each trimer.

DISCRETE SIGNAL-ON AND -OFF CONFORMATIONS IN THE AER HAMP DOMAIN

Kylie J. Watts, Mark S. Johnson and Barry L. Taylor
Dept. Microbiology and Mol. Genetics, Loma Linda University, Loma Linda, CA, USA

The PAS-FAD sensor of the aerotaxis receptor, Aer, signals through HAMP and signaling domains that are similar to these domains in other chemoreceptors. Our previous crosslinking studies showed that the AS-1 and AS-2 helices of the Aer-HAMP domain might form a four-helix bundle similar to the Af1503 and Tar HAMP domains. In this study, AS-1 residues were crosslinked to AS-2′ residues in di-cys Aer mutants (using 13 proximal and 4 distal di-cys pairs). The results confirmed a parallel four-helix HAMP bundle for Aer, but one in which AS-2 is rotated compared to the orientation of AS-2 in Af1503 or Tar.

We extended our HAMP crosslinking studies to probe for structural differences between the signal-on (CW) and signal-off (CCW) states. In our previous crosslinking studies, we used the oxidant copper phenanthroline, which maintains Aer in the signal-off state. In order to generate snapshots of the signal-on state, CW lesions such as PAS-N85S were engineered into the Aer di-cys and single-cys mutants. When the AS-1 to AS-2′ di-cys crosslinking experiments were repeated in mutants containing N85S, several di-cys pairs showed significant increases in dimer formation rates. These di-cys pairs were located at the distal end of the HAMP four-helix bundle. In contrast, no significant crosslinking changes were observed at the proximal end of the four-helix bundle. The data supports a model in which the distal ends of the HAMP helices move closer together during signal transduction. This could be due to an inward lateral movement of the helices, and may include some element of rotation. However, the entire Aer-HAMP domain does not appear to rotate as has been proposed for Af1503.

In Aer, HAMP lesions that lock the receptor in the signal-on (CW) state cluster at the distal end of a HAMP four-helix bundle, indicating a possible site for PAS-HAMP interactions during signal transduction. We used PEG-maleimide to determine in vivo the solvent-accessible surface of the HAMP and proximal signaling domains (residues 206-275). Solvent accessibility was restricted for most AS-2, but not AS-1 or connector, residues in Aer. This indicates that AS-2 residues that are exposed to solvent in Af1503 and Tar, and are predicted to be exposed in an Aer-HAMP model, are buried in vivo in Aer. We are currently investigating whether these residues are buried in a PAS-HAMP contact domain. We are also probing the surface of the HAMP domain in the signal-on state (with N85S) to determine whether there are differences in accessibility between the two signaling states.

INVESTIGATING THE STRUCTURE OF TERNARY COMPLEX OF HISTIDINE KINASE CheA, COUPLING PROTEIN CheW, AND CHEMORECEPTOR BY PULSED DIPOLAR ESR SPECTROSCOPY

Jaya Bhatnagar, Peter P. Borbat, Jack H. Freed, Brian R. Crane
Cornell University, B 150 Caldwell Hall, Ithaca, NY 14853

A central question in understanding the mechanism of chemotaxis involves the nature of interactions between histidine kinase CheA, adaptor protein CheW and receptors. Pulsed dipolar ESR spectroscopy (PDS) has developed as a valuable technique for structural characterization of protein complexes. PDS provides long-range distance information between spin-labeled residues in the proteins. A set of distance measurements can be subsequently used to model the assembly structure of the whole complex. In our previous work, we demonstrated the success of this approach in predicting the structure of complex of CheW with CheA. We have now applied PDS to the ternary complex formed by CheA, CheW and soluble chemoreceptor fragments. Our results indicate changes in distance distributions from spin-labeled sites on P4, P5 domains and CheW in the presence of unlabeled receptor. Dipolar signals between spin-labeled receptor and CheAΔ289 (domains P3, P4 and P5 together) or CheW provide important insights about the relative position and orientation of the three components with respect to each other. Based on this data we have developed a structural model of the ternary complex and the conformational changes CheA undergoes upon binding to receptor.

THE CHEMOTACTIC CORE SIGNALLING COMPLEX IS ULTRASTABLE

Annette H. Erbse and Joseph J. Falke
Department of Chemistry and Biochemistry, University of Colorado, Boulder, Campus Box 215, Boulder, CO 80309

The chemotatic core complex, composed of the transmembrane receptor, the histidine-kinase CheA and the coupling protein CheW, is the central building block of the extensive, highly cooperative polar signaling clusters in bacteria involved in sensing chemical gradients. Recent studies have shown that the receptors are organized hexagonal arrays of trimers-of-dimers. But it is still unclear how these arrays are formed and stabilized, how CheA and CheW are incorporated into single core complexes and how the complexes are interconnected to build the cooperative signaling network. Here we focus on the stability of the core complex. We show that the isolated, membrane-bound core complex is stable for at least 24 hours, both when it is assembled *in vivo* and *in vitro.* All three components are needed to achieve this ultra-stability, which is dependent on electrostatic interactions. By contrast, the stability is independent of ligand binding, receptor methylation or kinase activity. We propose that the assembly of the signaling clusters is cooperative, such that interactions between CheA , CheW and the receptor trimers-of-dimers are needed not only for receptor regulated kinase activity of individual core complexes, but also to position neighbouring complexes during the formation of a multi-linked network. The resulting ultra-stable network is the foundation for the ordered organization and the hypersensitivity of the signaling patches.

ELECTRON CRYOTOMOGRAPHY OF BACTERIAL CHEMOTAXIS ARRAYS

Ariane Briegel[1], H. Jane Ding[1], Zhuo Li[1], John Werner[2], Zemer Gitai[2], D. Prabha Dias[1], Rasmus B. Jensen[3], Elitza Tocheva[1] and Grant Jensen[1]

[1] California Institute of Technology, CA
[2] Princeton University, NJ
[3] University of Roskilde, Denmark

Motile prokaryotes are able to sense and to respond to ambient conditions through a process known as chemotaxis. Attractants and repellents bind to the sensing domain of methyl-accepting chemotaxis proteins (MCPs), thereby regulating the activity of the histidine kinase CheA. Together with the linking protein CheW, CheA is located at the distal tip of the cytoplasmic signaling domain of the MCPs. If activated, CheA phophorylates CheY (CheY-P), which in turn controls the direction of flagellar rotation. Together with CheA and a linking protein CheW, the MCPs form extended chemotaxis arrays at the cell poles.

Electron cryotomograhy (ECT) makes it possible to visualize chemoreceptor clusters in prokaryotes *in vivo* at macromolecular resolution (4-8 nm). While high-resolution structures of the individual chemotaxis proteins are available, their arrangement and position in the arrays remain unclear. Understanding this "mesoscale" architecture of the clusters is critical, however, since it is vital to the arrays' cooperative signal amplification and regulation. In order to unambiguously identify the chemotaxis arrays inside cells, we have correlated ECT with fluorescent light microscopy (FLM), using slightly fixed and immobilized *Caulobacter crescentus* cells with a fusion of the red-fluorescent protein, mCherry, to the C-terminus of the chemoreceptor (McpA). After plunge freezing, we imaged the same cells by ECT. In combination with ECT of near-native wild-type and mutant cells, we used the correlated FLM and ECT approach to identify the chemotactic array, its location and its *in-vivo* structure. We demonstrate that in wild-type *Caulobacter crescentus* cells preserved in a near-native state, the chemoreceptors are hexagonally packed with a lattice spacing of 12 nm, just a few tens of nanometers away from the flagellar motor that they control. The arrays were always found on the concave side of the cell, further demonstrating that *Caulobacter* cells maintain dorsal/ventral as well as anterior/posterior asymmetry. Placing the known crystal structure of a trimer of receptor dimers at each vertex of the lattice accounts well for the density, supporting an array composition unlike the published models for *Escherichia coli* [1] or *Thermotoga maritima* [2].
We are now in the process of comparing the chemotaxis arrays of a wide range of bacteria to determine the similarities and differences of these macromolecular assemblies at the ‘mesoscale’ level.

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TWO REGULATORY PROTEINS CONTROL THE SWIM-OR-STICK SWITCH IN ROSEOBACTERS

Robert Belas
University of Maryland Biotechnology Institute, Center of Marine Biotechnology, 701 East Pratt St., Baltimore, MD 21202

Members of the *Roseobacter* clade of α-Proteobacteria are among the most abundant and ecologically relevant marine bacteria. One of the most salient features of the roseobacters from aspects of marine ecology is their ability to enter into close physical and physiological relationships with “red tide” phytoplankton such as dinoflagellates. For example, *Silicibacter* sp. TM1040, our model roseobacter, forms a symbiosis with the dinoflagellate *Pfiesteria piscicida*, such that the dinoflagellate cannot live without TM1040. Aiding TM1040 in development of the symbiosis is a biphasic swim-or-stick lifestyle wherein a genetic regulatory circuit controls whether the bacteria are motile and chemotactic or sessile and develop a biofilm. Bacterial swimming and chemotaxis behavior are initial, essential steps in establishment of the symbiosis. Once near the host surface, motility and flagellar synthesis are downregulated, while biofilm formation and synthesis of an antibiotic are upregulated. The abilities to swim using flagella and to form a biofilm via adhesins have been demonstrated to be important traits for both pathogenic and symbiotic bacteria. While it is generally agreed that motility and biofilm development are mutually exclusive, the molecular mechanisms that underlie the lifestyle switch remain virtually unknown for most bacterial species. We have used genetic screens to search for mutants defective in either the motile or the sessile phenotype, and have discovered many new genes including two previously unknown and novel regulatory proteins, FlaC and FlaD that are envisaged to act together with cyclic dimeric GMP to play important roles in the swim-or-stick switch. FlaC is predicted to function as a response regulator protein, with homology to a protein of *Caulobacter crescentus* known to be important for cell envelope function. FlaC$^-$ cells are skewed towards the motile phase, e.g., their populations have a greater percentage of motile cells and fewer rosettes, and have defects in antibiotic synthesis and biofilm formation. Thus, FlaC determines whether the switch is in the swim or stick position. FlaD is predicted to be a MarR-type DNA-binding protein. Mutations in *flaD* result in nonmotile cells that synthesize but cannot rotate their flagella, i.e., they produce paralyzed flagella. We hypothesize that FlaD is involved in the function of the flagellar motor, either by (1) acting directly to control transcription of the class IV *fliL* operon or (2) acting indirectly to control transcription or activity of a protein that acts as a ‘clutch’ to engage or disengage the flagellar motor. The implications of FlaC and FlaD activities in the swim-or-stick strategy and their impact on the symbiosis will be discussed.

DELETION ANALYSIS OF RcsC REVEALS A NOVEL SIGNALING-PATHWAY CONTROLLING BIOFILM FORMATION IN *ESCHERICHIA COLI*

Ricardo Oropeza, Rosalva Salgado, Ismael Hernandez-Lucas and Edmundo Calva
Departmento de Microbiologia Molecular, Instituto de Biotecnologia, Universidad Nacional Autonoma de Mexico. Av. Universidad 2001. Col. Chamilpa. Cuernavaca, Morelos. Mexico, CP 62210

RcsC is a hybrid histidine kinase that forms part of a phosphorelay signal transduction pathway with RcsD and RcsB. Besides the typical domains of a sensor kinase, i.e. the periplasmic (P), linker (L), dimerization and H-containing (A), and ATP- binding (B), RcsC possesses a receiver domain (D) at the carboxy-terminal domain.

In order to study the role played by each of the RcsC domains, four plasmids containing several of these domains were constructed (i.e. PLAB, LAB, AB and ABD) and transformed in *Escherichia coli* wild type. Different amounts of biofilm were produced, assessed by crystal violet staining, depending on the RcsC domains expressed by the plasmid.

E. coli transformed with the plasmid expressing the ABD subdomains produced the highest amount of biofilm, while the lowest amount of biofilm was produced under the control of the PLAB expressing plasmid. This phenotype was observed in the same ratio when the plasmids were transformed in a *ΔrcsCDB* strain.

Several mutants on genes involved in biofilm formation were transformed with this set of plasmids. Biofilm formation was abolished in the *pgaABCD* and *nhaR* backgrounds but not in the *csrB* and *uvrY* backgrounds. Our results suggest the existence of a signaling pathway depending of RcsC but independent of RcsD and RcsB, activating biofilm formation by the *pgaABCD* operon.

REGULATION OF CELL FATE IN BACILLUS SUBTILIS BIOFILMS

H. C. Vlamakis[1*], C. Aguilar[1*], R. Losick[2], and R. Kolter[1]
[1]Harvard Medical School, Boston, MA, [2]Harvard University, Cambridge, MA.
*These authors contributed equally to this work.

Many microbial populations differentiate from free-living planktonic cells into surface-associated multicellular communities known as biofilms. Within a biofilm, motile *Bacillus subtilis* cells differentiate into non-motile chains of cells that form parallel bundles held together by an extracellular matrix. These bundles eventually produce aerial structures that serve as preferential sites for sporulation. By analyzing strains harboring multiple cell-type specific promoter fusions we can visualize the spatial anatomy of at least three physiologically distinct cell populations within mature biofilms. Motile, matrix-producing, and sporulating cells localize to distinct regions within the biofilm and the localization and percentage of each cell type is dynamic. Mutants unable to produce extracellular matrix form unstructured biofilms that are deficient in sporulation. This suggests that in architecturally complex biofilms, spore formation is coupled to the production of extracellular matrix. The coupling of matrix production and sporulation could be explained by the phosphorylation state of the master transcriptional regulator Spo0A. Spo0A is phosphorylated both directly and through a phosphorelay by at least five different histidine kinase proteins. When cells have low levels of Spo0A-P, matrix genes are expressed; however, at higher levels of Spo0A-P, sporulation commences. We have found that a deletion of *kinD*, a gene encoding one of the kinases that feed into the Spo0A phosphorelay, is sufficient to restore sporulation to matrix-deficient mutants. We hypothesize that KinD is not acting as a kinase under these conditions, but rather functions as a phosphatase to delay sporulation until matrix (or a matrix-encased signal) is sensed.

PROTEIN MISFOLDING DONE RIGHT: THE BIOGENESIS OF BACTERIAL AMYLOID FIBERS

Xuan Wang, Neal Hammer and **Matt Chapman**
University of Michigan, Department of Molecular, Cellular and Developmental Biology, Ann Arbor, MI, 48109

Many *Enterobacteriaceae spp.,* including *E. coli,* produce surface-localized amyloid fibers called curli. Curli fibers are associated with biofilm formation, host cell adhesion and invasion, and immune system activation. Unlike disease-associated amyloid formation, curli biogenesis is a directed and highly regulated process. The major curli subunit protein, CsgA, polymerizes into amyloid after interacting the CsgB nucleator protein. CsgB presents an amyloid-like template to CsgA on the cell surface that initiates fiber formation. CsgA has five imperfect repeating units (R1-R5) that are each predicted to form strand-loop-strand structures. Asn and Gln residues in R1 and R5 were found to be required for efficient amyloid formation and for interaction with the CsgB nucleator protein. Furthermore, the polymerization of CsgA was tempered by the presence of conserved aspartic residues in R2, R3 and R4. When these aspartic acid residues were changed to alanine (CsgA*), polymerization was significantly faster *in vitro*. Even more remarkable was the observation that CsgA* assembled into an amyloid fiber *in vivo* in the absence of CsgB. The ability of CsgA* to polymerize into amyloid more efficiently, and in the absence of CsgB, was not without consequences. Cells expressing CsgA* grew more slowly when compared to cells expressing wild type CsgA. This analysis suggests that aspartic acid residues can potently inhibit functional amyloid formation. CsgA has apparently evolved to efficiently assemble into an amyloid *in vivo* only in the presence of CsgB. This suggests an elegant mechanism to control amyloid formation by regulating the temporal and spatial interactions between CsgA and CsgB.

RHODOBACTER SPHAEROIDES, A BACTERIUM WITH TWO FLAGELLAR SYSTEMS AND MULTIPLE CHEMOTAXIS GENE HOMOLOGS

Ana Martínez del Campo[1], Sebastian Poggio[2], Teresa Ballado[1], Aurora Osorio[2], Javier de la Mora[1], Laura Camarena[2] and Georges Dreyfus[1]
Instituto de Fisiología Celular[1], Instituto de Investigaciones Biomédicas[2], Universidad Nacional Autónoma de México, 04510 México DF, México.

Rhodobacter sphaeroides has two flagellar systems (fla1 and fla2). One of these systems has been shown to be functional and is required for the synthesis of the well-characterized single subpolar flagellum (fla1), while the other was found only after the genome sequence of this bacterium was completed (fla2). In this work we found that the second flagellar system of *R. sphaeroides* can be expressed and encodes a functional flagellum. This second flagellar system produces polar flagella that are required for swimming. Phylogenic analysis suggests that the flagellar system that was initially characterized, was in fact, acquired by horizontal transfer from a γ-proteobacterium, while the second flagellar system contains the native genes.

In addition to having two flagellar systems, this photosynthetic bacterium posses several reiterated chemotactic genes (2 *cheB*, 3 *cheR*, 4 *cheA* and *cheW* and 6 *cheY*), which are encoded in three operons (cheOp1, cheOp2 and cheOp3). In spite of this, only some of the gene copies are required when the cell is swimming with the fla1 flagellum. The presence of a second functional flagellum (fla2) suggests that some of these genes could be involved in its tactic control. To test this hypothesis we proceeded to individually mutate each *cheY* gene. We show evidence that CheY1, CheY2 and CheY5 control de chemotactic behavior mediated by fla2 flagella. Additionally, we identified that open reading frame *RSP6099* encodes the fla2 FliM protein. Furthermore CheY1, CheY2 and CheY5 are located within cheOp1, which is not essential for chemotaxis mediated by the fla1 system. This raises the question: What is the role of cheOp1?

MOTILITY, CHEMOTAXIS AND VIRULENCE OF *BORRELIA BURGDORFERI*, THE LYME DISEASE SPIROCHETE

M. A. Motaleb[1], P. Stewart[2]. A. Bestor[2], P. Rosa[2] and N. Charon[3]
[1]Dept of Microbiology & Immunology, East Carolina University, Greenville, NC
[2]Human Bacterial Pathogenesis, NIH, RML, Hamilton, MT
[3]Dept of Microbiology, Immunology & cell Biology, West Virginia University, Morgantown, WV

Borrelia burgdorferi is the causative agent of Lyme disease. It is the most prevalent arthropod borne infection in the United States with 27,444 reported cases on 2007. The disease is a multiple-systemic disorder with various clinical manifestations including erythema migrans rash, arthritis, cardiac, musculoskeletal and neurological manifestations.

B. burgdorferi exists in nature in an enzootic cycle. *Ixodes scapularis* ticks (commonly known as deer ticks) acquire the infection when they feed on an infected host, mainly rodents. During subsequent tick feeding, which lasts for several days, *B. burgdorferi* migrate from the tick midgut, pass through the salivary glands, and are then transmitted to the mammal through the saliva. *B. burgdorferi* is highly invasive. After being deposited in the skin following a tick bite, the spirochetes can invade many tissues including the joints, heart, and nervous system.

Motility and chemotaxis are critical for bacterial survival and adaptation in diverse environmental conditions. In several species of bacteria, motility and chemotaxis have been shown to be associated with the disease process. Results obtained using *B. burgdorferi* with mutations in key motility and chemotaxis genes also indicate that these activities are required for the pathogenesis of Lyme disease. These studies could lead to the development of a novel pharmacological agent to treat/prevent Lyme disease.

PLEIOTROPIC PHENOTYPES OF A *YERSINIA ENTEROCOLICIA FLHD* MUTANT INCLUDE REDUCED LETHALITY IN A CHICKEN EMBRYO MODEL

Birgit M. Prüß[1], Megan K.T. Ramsett[1], Nathan J. Carr[1], Jyoti G. Iyer[1], Penelope S. Gibbs[1], Philip Matsumura[2], and Shelley M. Horne[1]
[1]Veterinary & Microbiological Sciences Department, North Dakota State University, Fargo ND 58108
[2]Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago IL 60612

The goal of this study was to correlate phenotypes with gene regulation by several flagellar regulators in *Yersinia enterocolitica*. FlhD/FlhC was initially described as a flagella transcriptional activator and later recognized as a global regulator in several enteric bacteria (*1, 2*). In *Y. enterocolitica*, FlhD/FlhC positively affected the expression levels of genes of histidine degradation and pyrimidine biosynthesis, while repressing the urease genes (*1*). A second protein that is involved in the regulation of flagellar genes, the sigma factor FliA, exhibited a negative effect upon the expression levels of seven plasmid-encoded virulence genes (*3*). In addition, eight flagellar operons were regulated by FliA. Among the differences to *Escherichia coli* were a 10 fold regulation of *fliZ* expression by FliA and a lack of FliA regulation of the *flgM* operon.

Phenotypes relating to FlhD/FlhC and FliA gene regulation were investigated. These phenotypes included growth on carbon and nitrogen sources, and virulence (*4*). Growth was determined with Phenotype MicroArrays (Biolog). Compared to the wild-type strain, *flhD* and *fliA* mutants exhibited increased growth on purines as carbon sources and decreased growth on pyrimidines and histidine as nitrogen sources. Several dipeptides provided differential growth conditions between the wild-type strain and both mutants. Gene regulation was determined for the *dpp* (dipeptide transport) and *opp* (oligopeptide transport) genes and was found to correlate with the observed phenotypes. Phenotypes relating to virulence were determined with the chicken embryo lethality assay that was previously established and used for *E. coli* strains (*5*). Relative to the wild-type strain, the *flhD* mutant caused a reduced lethality in this assay, while the *fliA* mutant caused lethality similar to the wild-type. Mutants were able to colonize infected embryo organs at levels that were comparable to the wild-type. In addition, a mutant in *flhB*, encoding one component of the flagellar type III secretion system also caused a reduced embryo lethality. Since genes of the type III secretion system are regulated by FlhD/FlhC and not by FliA, we believe that the lethality phenotype of the *flhD* mutant is due to regulation of the type III secretion genes.

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REGULATION OF MOTILITY BY QUORUM SENSING IN *SINORHIZOBIUM MELILOTI* AND ITS ROLE IN SYMBIOSIS ESTABLISHMENT

Juan E. González*, Nataliya Gurich, Jennifer L. Morris, Konrad Mueller, and Arati V. Patankar
Department of Molecular and Cell Biology, University of Texas at Dallas, Richardson, Texas, USA

Quorum sensing is a mechanism widely used by bacteria to coordinate their behavior in response to a particular cell population density. Signal molecules, termed autoinducers, are produced by bacteria, and at a high population density, accumulate in the environment. Once a threshold level of autoinducer is reached, they bind to their cognate transcriptional regulators and activate or repress expression of target genes, thereby preparing the bacteria for behaviors associated with high cell density, such as interacting with eukaryotic hosts.

In *Sinorhizobium meliloti*, this mechanism is utilized to appropriately modulate gene expression and permit the establishment of a nitrogen-fixing symbiosis with its host plant *Medicago sativa*. *S. meliloti* possesses a quorum-sensing system composed of two transcriptional regulators, SinR and ExpR, and the SinR-controlled autoinducer synthase SinI, which is responsible for the biosynthesis of the signal molecule in the form of an *N*-acyl homoserine lactone (AHL). These AHLs, in conjunction with the ExpR regulator, control a variety of downstream genes. The concentration of AHLs varies with changes in population density. As a result, expression of quorum-sensing-dependent genes may exhibit different patterns during various stages of bacterial growth. Work in our laboratory has shown that the *S. meliloti* ExpR/Sin quorum-sensing system regulates over 200 genes, including those involved in exopolysaccharide synthesis, motility and chemotaxis, metal transport, and other metabolic functions, thereby playing an important role during plant-bacteria interactions.

Inoculation of plants with a *sinI*-deficient strain results in a delay in invasion as well as a significant reduction in the total number of nodules per plant when compared to the wild type, resulting in plant development deficiencies. Concurrently, expression of most of the motility and chemotaxis genes in the *sinI* mutant fail to be down-regulated by quorum sensing at high cell population density. Microarray and real-time PCR analyses revealed that the ExpR/Sin system adjusts the expression of the transcriptional regulators VisN/VisR and Rem, which in turn modulate downstream motility genes in a population-density-dependent manner to decrease motility. Recently we have shown that mutating flagellar production in a *sinI* mutant restores bacterial competency for symbiosis establishment to wild type levels, suggesting that the elimination of flagella during the invasion process is crucial. Therefore, down-regulation of motility and chemotaxis by the ExpR/Sin quorum-sensing system plays an essential role in successful plant invasion by *S. meliloti*.

ENGINEERED SINGLE- AND MULTI-CELL CHEMOTAXIS IN *E. COLI*

Shalom D. Goldberg[1], Paige Derr[2], William F. DeGrado[1], and Mark Goulian[2,3]
[1] Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA, 19104
[2] Department of Physics, University of Pennsylvania, 209 South 33rd Street, Philadelphia, PA 19104
[3] Department of Biology, University of Pennsylvania, 433 S University Avenue, Philadelphia, PA 19104

We have engineered the chemotaxis system of *E. coli* to enable responses to molecules that are not attractants for wild-type cells. The system depends on an artificially introduced enzymatic activity that converts the target molecule into a ligand for an *E. coli* chemoreceptor, thereby allowing the cells to respond to the new attractant. Two systems, designed to respond to asparagine and to phenylacetyl glycine respectively, showed robust chemotactic responses. In addition, their behavior in a mixed population was suggestive of a “hitchhiker” effect in which cells producing the ligand can induce chemotaxis of neighboring cells lacking the enzymatic activity. This behavior was exploited to design a complex system of two strains that are mutually interdependent for their activity, which functions as a simple microbial consortium.

PHOTO-ENERGY CONVERSION AND SENSORY TRANSDUCTION OF MICROBIAL RHODOPSINS IN PHOTOSYNTHETIC MICROBES

So Young Kim, Keon Ah Lee, Ah Reum Choi, Song-I Han, and **Kwang-Hwan Jung**
Department of Life Science and Interdisciplinary Program of Integrated Biotechnology, Sogang Univeristy, Seoul 121-742, Korea (kjung@sogang.ac.kr)

Microbial rhodopsins, seven transmembrane proteins which contain *all-trans/13 cis* retinal as a chromophore, have been known for three decades and extensively studies in extreme halophiles. Photosynthetic microbes possess lots of photoactive proteins including chlorophyll-based pigments, phytochromes, phototropin-related blue light receptors, and cryptochromes. Surprisingly, recent genome sequencing projects discovered additional photoactive receptors, retinal-based rhodopsins, in cyanobacterial and algal genera. Analysis of the *Anabaena* and *Chlamyrhodopsin* revealed that they have sensory functions, which based on our work with haloarchaeal rhodopsins, may use a variety of signaling mechanisms. *Anabaena* rhodopsin is interacted with a tetramer of 14kDa soluble transducer (ASRT) and one of their putative functions is a global regulation of phycobilin protein. The *Anabaena* rhodopsin shows a visible light-absorbing pigment (540-550nm) and it has mixed photochemical reaction of all trans and 13 cis form of retinal in ground state. Two *Chlamydomonas* rhodopsins are involved in phototaxis and photophobic responses based on electrical measurements by RNA*i* experiment. The rhodopsins from *Gloeobacter violaceus* and *Acetabularia acetabulum* is light-driven proton pump coexisted with photosynthetic machinery. The genes were functionally expressed in *Escherichia coli* and bound all-*trans* retinal to form a pigment in the presence of N- and C-terminal MISTIC sequences. *Gloeobacter* and *Acetabularia* rhodopsin I showed a light-driven proton pumping activity similar to proteorhodopsin.

FUNCTION OF MULTIPLE CHEMOTAXIS-LIKE PATHWAYS IN MEDIATING CHANGES IN MOTILITY PATTERNS AND CELLULAR MORPHOLOGY IN *AZOSPIRILLUM BRASILENSE*

Amber N. Bible[1], Zhihong Xie[1], Matthew Russell[1] and Gladys Alexandre[1,2]
[1]Department of Biochemistry, Cellular and Molecular Biology and [2]Department of Microbiology, The University of Tennessee, Knoxville, TN 37996

Molecular details on bacterial chemotaxis have been derived from studies of model organisms such as *Escherichia coli* and *Bacillus subtilis* which genome encode for a single chemotaxis pathway that functions to modulate changes in motility patterns. Comparative genomics analysis indicates that the genome of many bacteria possess multiple chemotaxis-like (Che) pathways. *A. brasilense* is a plant-associated bacterium that can differentiate in at least four different cell types (swimmer, swarmer, aggregated and cyst cells). Transition from one cell type to the other depends on the environmental (especially nutritional) conditions. One of the 4 Che-like *pathways (Che1)* encoded within the genome of the alphaproteobacterium *A. brasilense* was recently shown to regulate changes in motility patterns, cell-to-cell aggregation concomitant with changes in cell length (Bible et al., 2008). We will present evidence for the role of two Che pathways and several chemoreceptors in controlling the ability of cells to modulate multiple cellular responses, including cell length, that suggest that cross-regulation between parallel chemotaxis pathways may function to coordinate and integrate a set of cellular functions. Experimental evidence suggests that proteins that function in the methylation/demethylation of chemoreceptors may have a critical role in this cross-regulation. The implications in the lifestyle of this bacterium will also be discussed in lights of recent experimental evidence obtained.

Bible, A. N., Stephens, B. B., Ortega, D. R., Xie, Z. and G. Alexandre (2008) Function of a chemotaxis-like signal transduction pathway in modulating motility, cell clumping, and cell length in the alphaproteobacterium *Azospirillum brasilense*. *J Bacteriol* 190: 6365-6375.

PROBING ADAPTATION KINETICS *IN VIVO* BY FLUORESCENCE RESONANCE ENERGY TRANSFER

Thomas S. Shimizu[1], Yuhai Tu[2] and Howard C. Berg[1]
[1]Department of Molecular & Cellular Biology, Harvard University, Cambridge, MA 02138.
[2]T. J. Watson Research Center, IBM, Yorktown Heights, NY 10598.

Bacteria sense spatial gradients by taking time derivatives of ligand concentrations measured during runs of a random walk[1]. The remarkable sensitivity to shallow gradients in *Escherichia coli* has been explained mainly by cooperativity between receptors and ultrasensitivity of the flagellar motor. We have revisited the experimental findings of Block, Segall and Berg[2], where the chemotactic response of tethered cells to time-varying stimuli were characterized quantitatively. A simple theoretical model[3] that combines robust adaptation[4] with an allosteric model of receptor cooperativity[5-7] can explain the general features of responses to temporal ramps and oscillatory stimuli.

A notable feature of this model is that the steady-state amplitude of responses to exponential ramps do not depend on the degree of receptor cooperativity (the parameter *N* of an MWC-type allosteric model[5]). The time required to reach this steady state, however, depends inversely on *N*, so cooperativity speeds up computation of the derivative signal, but does not determine its amplitude. The latter is instead determined by the adaptation kinetics, and this relation allows us to infer quantitative characteristics of adaptation *in vivo* from measured ramp-response data.

Here we present novel experiments in which the chemotactic responses of *E. coli* populations during time-varying stimuli are monitored by fluorescence resonance energy transfer[8] (FRET). This approach is far more efficient than the earlier experiments of Block et al.[2], in which the chemotactic responses of individual cells were characterized through the stochastic output of the motor. We find that the sensitivity of *E. coli* to gradients depends strongly on temperature, and using our model framework, we analyze how ultrasensitvity in the adaptation system[9] contributes to gradient sensitivity *in vivo*.

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MINOR RECEPTOR SIGNALLING IN *E. COLI*

Silke Neumann, Ned Wingreen* and Victor Sourjik
Ruprecht-Karls-Universität Heidelberg, Zentrum für molekulare Biologie Heidelberg (ZMBH), Im Neuenheimer Feld 282, 69120 Heidelberg, Germany
* Department of Molecular Biology - Princeton University

Ligand recognition in the chemotaxis pathway of *E. coli* proceeds through binding of ligands to transmembrane receptors, either directly or indirectly through periplasmic binding proteins. *E. coli* has five types of receptors, with two high-abundance (or major) receptors – Tsr for serine and Tar for aspartate and maltose – and three low-abundance (or minor) receptors – Tap for dipeptides, Trg for ribose, galactose and glucose, and Aer for redox potential. Together with the histidine kinase CheA, receptors form chemosensory complexes which in turn are organized in tight clusters where receptors of different ligand specificities are intermixed. Signal processing is thought to occur within these receptor clusters through allosteric interactions between receptor dimers. To compare signal processing by minor and major receptors, we systematically investigated responses mediated by Trg and Tap, and by Tar and Tsr in respect to response sensitivity, relation between receptor occupancy and kinase inactivation, dynamic range of the response, adaptation time to a range of stimuli, as well as integration of signals that are sensed by different receptors using an *in vivo* FRET-based kinase assay. Our experimental analysis shows that signals are amplified and integrated differently by the two receptor populations, but in both cases signal processing can be quantitatively explained by the same allosteric model.

A SYSTEMS BIOLOGY APPROACH TO UNDERSTANDING HOW *BACILLUS* MAKES UP ITS MIND

Arnaud Chastanet[1], Guocheng Yuan[2], Thomas M. Norman[1], Jun Liu[3] and Richard Losick[1]
[1]Molecular and Cellular Biology Department, Harvard University,
[2]Department of Biostatistics and Computational Biology, Harvard School of Public Health,
[3]Department of Statistics, Harvard University.

Understanding how cells make decisions and differentiate are key biological questions. Mechanisms underlying such behaviors integrate multiple environmental signals in intricate networks in order to appropriately respond to the situations. The sporulation process that takes place in *Bacillus subtilis* under adverse conditions perfectly exemplifies this kind of question. For this, numerous signals and control systems are integrated at the level of a ”decider” protein called Spo0A, a transcriptional regulator belonging to the two-component systems family. The decision to sporulate is taken during the first two hours after optimal sporulation conditions have been reached. During this time, Spo0A accumulates slowly reaching a high level at hour two. It has been previously shown that while some of its targets are activated at low concentration, thus early on, others are switched on later, when the maximal quantity of the regulator has been achieved. Interestingly, even in optimal conditions, only a fraction of the population will finally decide to sporulate, a phenomenon described as bistability.

We are attempting to understand how this two-stage activation of Spo0A is achieved through an interdisciplinary approach combining the methods of genetics and mathematics. The time resolved picture of the regulatory process we have obtained has revealed a multiple step process involving successive switches. First, Spo0A activity is rising during log phase, activating some switches. Then under conditions of nutrient limitation, Spo0A is further activated to a mid and variable extent throughout the population. During this period, “low-threshold” genes are turned ON. In an ultimate step, a bistable switch allows Spo0A to be activated to a high level but only in a portion of the population. These cells express high threshold genes and proceed to sporulate.