POSTER ABSTRACTS
CHARACTERIZATION OF TWO SETS OF SUB-POLAR FLAGELLA IN *BRADYRHIZOBIUM JAPONICUM*

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*Bradyrhizobium japonicum* is one of the soil bacteria that form nodules on soybean roots. The cell has two sets of flagellar systems, one thick flagellum and a few thin flagella, that are uniquely growing at sub-polar positions. The thick flagella appear semi-coiled in morphology and the thin flagella were observed to be in a tight-curly form by dark-field microscopy. Flagellin genes were identified from the amino acid sequence of each flagellin. Flagellar genes for the thick flagellum are scattered within several clusters on the genome, while those genes for the thin flagellum are compactly organized in a single cluster. Both types of flagella are apparently powered by proton-driven motors. The swimming propulsion is mainly supplied by the thick flagellum. *B. japonicum* flagellar systems resemble the polar-lateral flagellar systems of *Vibrio* species but differ in several aspects.
THE REAL ROLE OF FliK IN THE HOOK LENGTH CONTROL

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The role of FliK in the hook length control has recently centered around two theories. One theory claims that FliK is directly involved in hook length measurement as a ruler, while the other theory claims that the C ring is the measuring cup and that FliK plays a subsidiary role in this event, turning on the substrate-specificity switching at a proper time. In this study, we systematically created fliK mutants with deletions and insertions at various sites of FliK and analyzed their effects on the hook length. Only when certain specific sites of FliK were deleted or inserted did the cell produced hooks of a regulated length, in which case the hook length was proportional to the molecular size of chimeric FliK. Deletions or insertions in other sites of FliK gave rise to the FliK null phenotype, or polyhooks; hooks of uncontrolled length. In many of the deletion mutants, FliK was secreted into medium but the hook length was not controlled. On the other hand, in some deletion mutants, FliK was not secreted but the hook length was controlled, indicating that FliK secretion is not essential for the hook length control. Thus, a correlation between FliK length and hook length was observed, but this was not dependent on FliK secretion. We, as a claimer of the latter theory, argue against a direct ruler model and discuss the true role of FliK in hook length control.
REDEFINING THE REGULATORY NETWORK THAT CONTROLS FLAGELLAR ASSEMBLY IN SALMONELLA.

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Efficient protein secretion in bacteria requires temporal control of secretion-substrate production and delivery to the secretion-apparatus. The motility organelle, the flagellum, is structurally comprised of a basal body, a hook and a long filament that acts as the propeller. Flagellar assembly requires an associated Type III secretion (T3S) apparatus to secrete flagellar subunits through the growing structure. Assembly is temporally regulated by a checkpoint dictated by the length of the intermediate hook-basal body structure. Three promoter classes organised into a transcriptional hierarchy control flagellar gene expression coupled to flagellar assembly.

A substrate specificity switch of the flagellar T3S-apparatus defines the assembly checkpoint and is coupled to post-switch subunit production by the flagellar specific transcription factor $\sigma^{28}$. Proteins destined for post-switch secretion by the T3S-apparatus require T3S-chaperones. The T3S-chaperones are molecular timing devices: acting as secretion chaperones for flagellar subunits during one stage of flagellar assembly and regulators of gene expression at later assembly stages.

Using a bioluminescence microplate-based reporter assay of flagellar promoter activity we have begun to define the complete regulatory network that couples flagellar gene expression to assembly in Salmonella enterica. Using multiple combinations of null mutations in genes coding T3S-chaperones and their substrates we have shown: 1) the T3S-chaperone FliT regulates two flagellar promoter classes antagonistically 2) the FliT substrate, FliD, acts as an anti-regulator of FliT 3) there are two further regulatory circuits responsive to basal body assembly and filament polymerisation and 4) Secretion substrate competition influences $\sigma^{28}$-dependent post-switch gene expression via changes in secretion of its anti-$\sigma^{28}$ factor, FlgM.
A DEDICATED CHEMORECEPTOR FOR AEROTAXIS UNDER NITROGEN FIXING CONDITIONS IN AZOSPIRILLUM BRASILENSE

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Azospirillum brasilense fix nitrogen under microaerophilic conditions in the free-living state. Nitrogen fixation is energetically expensive and the enzyme nitrogenase is inhibited by oxygen. It has been previously proposed that A. brasilense locate low oxygen concentrations where intracellular energy levels are optimum and allow nitrogen fixation by aerotaxis. Here, we describe a chemoreceptor, named Tlp3, which serves as energy taxis transducer and mediates aerotaxis under nitrogen fixation conditions in A. brasilense. The tlp3 gene is located in a genomic region that comprises structural and regulatory genes for nitrogen fixation and encode for a protein predicted to be a PAS domain-containing soluble receptor. A tlp3 mutant was constructed, and its chemotaxis and aerotaxis abilities were compared to that of the wild type. Interestingly, although tlp3 is expressed constitutively (and the protein is present under all growth conditions), it contributes to aerotaxis and mediates energy taxis responses only under nitrogen fixing conditions. This suggests that Tlp3 senses as energy parameter that is related to nitrogen fixation. We show that a mutant lacking tlp3 does not locate the optimum oxygen concentration in an energy gradient that affects the induction of nitrogen fixation abilities. We also determined the role of Tlp3 in colonization of the wheat rhizosphere.
STIMULUS PERCEPTION BY THE SENSOR KINASE KdpD OF *ESCHERICHIA COLI*

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Signal transduction by the histidine kinase/response regulator system KdpD/KdpE regulates the expression of the *kdpFABC* operon coding for the high affinity potassium uptake system KdpFABC in *Escherichia coli*. Upon stimulus perception, i.e. K⁺-limitation or high osmolality, the membrane-bound KdpD becomes autophosphorylated at a conserved histidine residue in the C-terminal transmitter domain. The phosphoryl group is then transferred to the cytoplasmic response regulator KdpE, which binds in its phosphorylated and dimerized form with high affinity upstream of the *kdpFABC* promoter region, thereby triggering *kdpFABC* expression.

The large sensing domain of the histidine kinase KdpD, which is responsible for stimulus perception encompasses about two third of the protein. It includes the cytoplasmic N-terminal domain, four transmembrane helices, an “arginine-cluster”, about 110 P/Q enriched amino acids a coiled-coil sequence connecting the sensing domain to the transmitter domain of KdpD.

*In vivo* and *in vitro* analysis of KdpD derivatives (point mutations, deletions, domain swapping) revealed the importance of the C-terminal part of the sensing domain for a proper signal perception mechanism of the histidine kinase.
THE INTERACTIONS OF THE MULTIPLE CheY RESPONSE REGULATORS OF RHODOBACTER SPHAEROIDES WITH THE FLAGELLAR SWITCH PROTEIN FliM

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The roles of the six CheY homologues of \textit{R. sphaeroides} have been investigated. The mutations that activate \textit{E. coli} CheY (D13K, Y106W, and I95V) do not cause activation of the \textit{R. sphaeroides} CheYs. Thus the \textit{R. sphaeroides} CheYs may differ in both the nature of the phosphorylation-induced conformational change and their subsequent interactions with the flagellar motor. A number of techniques have been employed to investigate the CheYs of \textit{R. sphaeroides}:

1. \textit{R. sphaeroides} CheY\textsubscript{1}, CheY\textsubscript{3}, CheY\textsubscript{4} and CheY\textsubscript{5} have a tryptophan residue at the equivalent position of Y106 in \textit{E. coli} CheY. Phosphorylation induced changes in fluorescence intensity of this tryptophan residue in purified CheY\textsubscript{1}, CheY\textsubscript{3}, CheY\textsubscript{4} and CheY\textsubscript{5} proteins were monitored.

2. The fluorescence anisotropy of a fluoresceinated peptide corresponding to the first 18 residues of FliM was used to quantify CheY binding \textit{in vitro}. However, despite varying a range of conditions no binding was detected. Purification of full length fluorescently tagged FliM is now in progress; this will be used in the CheY binding assays.

3. Finally, we are currently identifying residues in FliM that are necessary for CheY binding. This is facilitated by the use of the phosphorylation site mutant of CheY\textsubscript{6}, CheY\textsubscript{6} (D56N), which produces a stopped motor.

These techniques in combination are yielding results that are increasing our understanding of how the multiple CheY homologues of \textit{R. sphaeroides} control the rotation of the flagellar motor.
STUDIES OF THE *E. coli* FLAGELLAR MOTOR PROTEIN FliM

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*Escherichia coli* swims by rotating 4-6 long helical filaments to propel the cell through its environment. The flagellar motor that rotates the filament is bidirectional and composed of two parts: the rotor and the stator, the stator being the fixed component against which the rotor spins. The stator is composed of two integral membrane proteins, MotA and MotB, the stoichiometry of each complex being \((\text{MotA}_4:\text{MotB}_2)\). The rotor is composed of multiple rings, among which the C-ring which is localized at the base of the motor and is the switch complex that gives the bidirectionality to the *E. coli* motor. The C-ring comprises FliG (~25 copies), FliM (~34 copies) and FliN (>100 copies).

Recent studies using GFP-MotB expressed from the genome of *E. coli* in place of the wild-type gene have shown that there are on average 11 stator per motor. These stator units are not fixed in the flagellar motor but diffuse rapidly in and out of the whole motor (Leake et al.). This result was surprising, as it had been assumed that the stator was the fixed part of the motor. In light of this result, it becomes interesting to wonder whether the stator is the only part of the motor to be labile, or if other protein(s) undergo similar diffusion and dynamic exchange.

In particular, electron microscopy images seem to indicate that the attachment of the C-ring to the rest of the rotor might to be labile, even though most workers assume that both the MS- and the C-rings rotate as a single unit. The N-terminus of FliM, a protein of the C-ring, has therefore been tagged with eYFP and expressed from the genome. Data will be presented on the construction of the eYFP-FliM mutant as well as preliminary data on the dynamics of FliM. The consequences of these results and future work will be discussed.
SITE-SPECIFIC MUTAGENESIS OF CheB PROTEINS FROM RHODOBACTER SPHAEROIDES

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Chemotaxis is employed by many bacteria to allow directed movement towards more favorable environments. The chemotaxis pathway in *Escherichia coli* has been well characterized, but in other bacterial chemotaxis systems, such as that found in *Rhodobacter sphaeroides*, there is a much higher degree of complexity. An illustration of this is its three complete chemotaxis operons, compared to the single operon in *E. coli*.

The ability to adapt to the environment and adjust the sensitivity to stimulant concentration gradients is required for the bacterium to remain responsive. In *E. coli* the methylesterase, CheB, and the methyltransferase, CheR, have been shown to be critical for adaptation.

Work on the CheB protein found in *E. coli* has identified a number of key residues including aspartates at (D56) or near to (D11) the site of phosphorylation and the histidine residue (H190) in the methylesterase catalytic site. These residues appear to be conserved in the two *R. sphaeroides* CheB proteins, according to sequence comparisons. Directed mutagenesis of these homologues has been used to determine whether their roles are equivalent. The phenotypes of the *R. sphaeroides* mutants will be discussed.
A NOVEL CHEMOTAXIS PHOSPHATASE FROM *RHODOBACTER SPHAEROIDES*

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*Rhodobacter sphaeroides* has a complex chemosensory pathway with multiple homologues of the *Escherichia coli* chemosensory proteins, except for CheZ. Many bacteria lack CheZ homologues and have alternative CheY-P phosphatases such as CheC, FliY and CheX. *R. sphaeroides* does not have homologues of any of these alternative phosphatases.

In this study, we demonstrate that one of the CheA homologues of *R. sphaeroides*, CheA₃, possesses specific phosphatase activity for the principal CheY protein, CheY₆ but not for any of the other CheY-Ps. CheA₃ possesses only two of the five domains found in classical CheAs; the Hpt (P1) and regulatory (P5) domains, which are joined by a novel 751 amino acid sequence that contains the CheY₆-P phosphatase activity. CheA₃ is phosphorylated by CheA₄, and CheA₃-P phosphotransfers to CheY₆. CheA₃ is therefore both a phosphodonor and a specific phosphatase for its cognate response regulator, CheY₆.
THE ROLES OF HAMP DOMAINS IN TlpT IN RHODOBACTER SPHAEROIDES

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Methyl accepting chemotaxis proteins are dimeric receptors whose periplasmic ligand binding domains signal across the cell membrane to the cytoplasmic signalling domain. This link is mediated by HAMP domains which are found after the cytoplasmic end of the transmembrane region of the receptor. This domain is essential for chemotaxis and it is thought to interact with the membrane during its signal transduction mechanism.

Rhodobacter sphaeroides is a purple non-sulphur bacterium that is tactic to a wide range of stimuli. Previous work has identified two clusters of chemotaxis proteins: one found at the cell poles akin to E. coli and one located in the cytoplasm. The cytoplasmic chemotaxis cluster contains soluble receptors (TLPs) which share homology to MCPs.

We have identified two putative HAMP domains in the soluble chemoreceptor, TlpT. As TlpT lacks transmembrane domains the role of the HAMP domains in signalling is of interest. Previous studies showed that TlpT is important for cluster formation. We have therefore examined (a) the role of the HAMP domains in signalling by a cytoplasmic receptor and (b) using TlpT-YFP fusions, the role of the HAMP in the clustering of cytoplasmic chemotaxis.
LIGAND-INDUCED ASYMMETRY IN HISTIDINE SENSOR KINASE COMPLEX REGULATES QUORUM SENSING

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Bacteria sense their environment using receptors of the histidine sensor kinase family, but how kinase activity is regulated by ligand binding is not well understood. Autoinducer-2 (AI-2), a secreted signaling molecule originally identified in studies of the marine bacterium Vibrio harveyi, regulates quorum-sensing responses and allows communication between different bacterial species. AI-2 signal transduction in V. harveyi requires the integral membrane receptor LuxPQ, comprised of periplasmic binding protein (LuxP) and histidine sensor kinase (LuxQ) subunits. Combined X-ray crystallographic and functional studies show that AI-2 binding causes a major conformational change within LuxP, which in turn stabilizes a quaternary arrangement in which two LuxPQ monomers are asymmetrically associated. We propose that formation of this asymmetric quaternary structure is responsible for repressing the kinase activity of both LuxQ subunits and triggering the transition of V. harveyi into quorum-sensing mode.
GENETIC ANALYSIS OF ANTIBIOTIC SYNTHESIS IN *SILICIBACTER* SP.TM1040

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*Silicibacter* sp. TM1040 forms an obligate symbiosis with the dinoflagellate *Pfiesteria piscicida*. We have shown that *Silicibacter* sp. TM1040 both degrades the algal osmolyte dimethylsulfoniopropionate (DMSP) and is chemoresponsive to DMSP, its degradation products, and other dinoflagellate compounds. We recently discovered that this bacterium produces a sulfur-containing antibiotic, tropodithietic acid (TDA). The synthesis of TDA is up-regulated when the bacteria are grown in static broth conditions, co-occurs with the production of a melanin-like pigment, the formation of biofilms and rosettes of attached cells, loss of flagella and motility, and requires the presence of a sulfur substrate, notably DMSP, for its production. Therefore, the production of TDA, whose synthesis is a significant output of the cells, is likely to have profound effects on the sulfur cycle, the structure and composition of bacterial communities, and the interaction of roseobacters with their phytoplankton hosts. Using random transposon mutagenesis and knowledge of the annotated TM1040 genome, a genetic study was conducted to identify the genes responsible for the biosynthesis and regulation of TDA activity. A total of 135 loss-of-function mutants were obtained from a bank of over 12,000 mutants, and of these, 62 unique genes were found to be required for TDA synthesis and expression. A genomic analysis of six of these genes encoding either sensory or regulatory proteins suggests that TDA expression involves a response to environmental stress derived from chemical signals received by two methyl-accepting chemotaxis proteins (MCPs), light-derived cues transduced by a histidine kinase (AphA), and a response to stress mediated by homologs of RpoE, HrcA, and RsbU, a response regulator that may form a two-component regulatory circuit with AphA. These signals act to control expression of other proteins whose functions include ring biosynthesis through phenylacetate metabolism and acquisition of sulfur via reduced sulfur metabolism and CoA pathways. Details of the biosynthetic pathway and its regulation, as well as the role of TDA in maintaining the obligate symbiosis between TM1040 and its dinoflagellate host will be discussed.
ANALYSIS OF THE GENES REQUIRED FOR MOTILITY IN *SILICIBACTER SP. TM1040*

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*Silicibacter sp.* TM1040, a member of the marine *Roseobacter* clade, is an obligate symbiont of the dinoflagellate *Pfiesteria piscicida*. In order for this interaction to occur TM1040 has to be able to sense and locate *P. piscicida*. Motility is an important component of the interaction between TM1040 and *P. piscicida*. In this study, we used genomic and genetic analyses to identify the genes that are involved in the regulation and biosynthesis of the flagellum and motor. A genomic analysis revealed over 40 Open Reading Frames (ORFs) that have homology to known flagellar structural and regulatory genes. Among the ORFs encoding structural genes were 6 distinct alleles of a flagellin-encoding gene (*fliC1-6*). The regulatory genes thus far identified in the genomic analysis include homologs encoding CckA, CtrA, FlaF and FlbT. The genomic analysis was supported by a genetic approach using random transposon mutagenesis to create a bank of loss-of-function motility mutants. The nucleotide sequence surrounding the transposon in each of the genes mutated was determined and its deduced amino acid sequence compared to known flagellar proteins. The loss-of-function mutants were the result of transposon insertions in 19 structural and two regulatory genes (*cckA* and *flaF*). Significantly, the genetic analysis revealed transposon insertions in three previously genes not identified by genomic analysis. These ORFs encoded proteins with sensory and regulatory domains, e.g., histidine kinase phosphoacceptor, MarR HTH DNA-binding domain, and CheY receiver domain. Mutation in the first two genes resulted in a nonmotile phenotype suggesting that these genes and proteins play a role in up regulating flagellar gene transcription. In contrast, mutation in the gene encoding the protein with the CheY domain resulted in a higher percentage of motile cells in the population when compared to wild type, suggesting that this protein functions as a negative regulator of the flagellar regulon. In addition, the genes encoding for the proteins containing the MarR HTH DNA-binding domain and CheY receiver domain also contains a DNA binding domain supporting the idea that both proteins could be involved in transcription regulation. A model of the regulatory hierarchy of flagellar biosynthesis in *Silicibacter* sp. TM1040 is discussed.
PHYSICAL RESPONSES OF BACTERIAL CHEMORECEPTORS

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Chemoreceptors of the bacterium Escherichia coli are thought to form trimers of homodimers that undergo conformational changes upon ligand binding and thereby signal a cytoplasmic kinase. We monitored the physical responses of trimers in living cells lacking other chemotaxis proteins by fluorescently tagging receptors and measuring changes in fluorescence anisotropy. These changes were traced to changes in energy transfer between fluorophores on different dimers of a trimer: attractants move these fluorophores farther apart, and repellents move them closer together. These measurements allowed us to define the responses of bare receptor oligomers to ligand binding and compare them to the corresponding response in kinase activity. Receptor responses could be fit by a simple ‘two-state’ model in which receptor dimers are in either active or inactive conformations, from which energy bias and dissociation constants could be estimated. Comparison with responses in kinase-activity indicated that higher-order interactions are dominant in receptor clusters.
THE HYBRID FLAGELLAR MOTOR DRIVEN BY PROTON AND SODIUM ION FLUX, AND PROGRESS IN MOTOR-STEP EXPERIMENT

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The bacterial flagellar motor is a rotary molecular machine driven by ion flux across the cytoplasmic membrane. Coupling ion is via either protons or sodium ions, depending on the motor. The free energy gained from passing ions through the ion channel of the stator is converted to mechanical torque by interacting between stator units and a rotor component.

The chimeric stator, PomAPotB, works as a sodium-driven flagellar motor in Escherichia coli, which has only proton-driven motors in nature (Asai et al., 2003). MotAMotB (the wild type proton stator of E. coli) and PomAPotB were expressed in a single E. coli cell simultaneously to test whether a biological hybrid motor could be achieved. We attached a latex bead of 1μm to a truncated flagellar stub and followed the rotation of the motor by back-focal plane interferometry.

In the sodium-ion free condition, we determined the number of interacting MotAMotB units from measured speed (Ryu et al., 2000, Reid et al., 2006). After adding sodium ions, the motor speed increased in a stepwise fashion (“resurrection” by sodium ion) from the base speed produced by MotAMotB. The speed increments depended on sodium concentration. This means PomAPotB is gathering at and interacting to the rotor, which also interacts with MotAMotB. Thus we conclude we can make a biological hybrid motor driven by different energy sources.

Previously, we reported the stepping motion of the flagellar motor of chimeric PomAPotB by lowering sodium-motive force (Sowa et al., 2005). Now we are trying to devise experiments to detect steps at high temporal resolution and under low stable speed conditions. I will present recent some results of these new step experiments.

MUTUAL HISTIDINE KINASES REGULATE ENCYSTMENT IN RHODOSPIRILLUM CENTENUM

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Rhodospirillum centenum is a purple, nonsulfur, photosynthetic bacteria that exhibits a complex life cycle. When deprived of nutrients, active vegetative cells transform into dormant cysts composed of four or more spherical cells enclosed within a protective, husk-like structure. Cysts tolerate desiccation and have increased resistance to UV radiation and elevated temperature. Previous studies of mutant strains that form cysts constitutively (hypercyst strains) have identified two histidine kinases that repress encystment and one that is required to induce development. We used transposon mutagenesis to generate mutant strains that grow normally on nutrient rich media, but fail to form cysts on poor media (hypocyst strains). Analysis of these strains revealed two new histidine kinases genes, cyd1 (cyst defective) and cyd2 that are involved in regulating encystment. Strains with in-frame deletions in either cyd1 or cyd2 are incapable of initiating the process of cyst formation. Consequently, R. centenum utilizes multiple histidine kinases that function within antagonistic signal pathways to regulate the starvation response. Presumably, some kinases signal to maintain the vegetative state when nutrients are plentiful, while others are required to induce encystment under adverse conditions.
ROLE OF FLAGELLAR MOTOR PROTEIN FliN IN FLAGELLAR SPECIFIC SECRETION

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FliN is a major constituent of the C-ring in the flagellar basal body of many bacteria. It is present in more than 100 copies per flagellum, and together with FliM and FliG forms the switch complex that functions in flagellar assembly and switching. The crystal structure is known for most of FliN, and shows a saddle-shaped dimer as the asymmetric unit. Targeted cross-linking studies will be described which show that FliN is further organized into donut-shaped tetramers. The shape and size of the hypothesized tetramers are a good match for a feature seen at the bottom of the C-ring in electron microscopic reconstructions.

To identify functionally important regions on FliN, we have carried out a systematic mutagenesis of the protein surface. Non-conservative mutations were made in many positions sampling the FliN surface and effects on flagellar assembly and function were measured. Flagellar assembly was disrupted by mutations in a hydrophobic patch centered on the dimer two-fold axis. Mutant phenotypes and overexpression effects suggest that this hydrophobic patch interacts with soluble components of the export apparatus, and co-precipitation assays demonstrate an interaction with the flagellar export protein FliH. Thus, FliN may facilitate export by localizing FliH or FliH-containing complexes. A region adjacent to the hydrophobic patch was found to be important for switching; mutations in this region gave a strong CCW motor bias that was largely rescued by over-expression of the CW-signaling protein CheY. This region on FliN might contribute to the binding site for CheY on the switch.

We have also found that FliN interacts with the cytoplasmic domain of FliO. FliO has been implicated in flagellar export but its precise role is unknown. It contains a single membrane segment and might therefore interact with the other membrane-associated export proteins FliP, FliQ, and FliR, whose genes occur in the same operon with fliN and fliO. Mutant phenotypes and overexpression effects suggest that the FliN-FliO interaction may facilitate assembly of the membrane-inserted parts of the export apparatus, possibly by helping to localize FliP.
KINETIC CHARACTERIZATION OF CheZ-MEDIATED DEPHOSPHORYLATION OF CheY

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In *Escherichia coli* chemotaxis, the cell continuously regulates the level of phosphorylation of the response regulator CheY in response to an external chemical gradient. CheY is phosphorylated by the histidine kinase CheA at a rate that reflects the activity state of coupled chemoreceptors and is dephosphorylated by the phosphatase CheZ. Despite the essential role of CheZ in regulating CheYp levels, there is incomplete understanding of fundamental kinetic features of its phosphatase activity and possible regulation of that activity. We have used a combination of steady state enzyme kinetics and fluorescence intensity measurements to characterize the kinetics of the interaction of CheZ with CheYp and the ensuing dephosphorylation. The steady state rate of CheZ-dependent P$_i$ release changed sigmoidally with CheYp concentration, in agreement with pre-steady state measurements of Blat et al.\(^1\). This indicates the presence of positive cooperativity so that the activity of CheZ is suppressed at low CheYp concentrations. In contrast, the gain-of-function CheZ mutant, CheZ 21IT, did not display cooperativity and far lower CheYp concentrations were required for saturation, despite a similar $k_{cat}$ as wild type CheZ. To monitor the formation of the CheZ-CheYp complex, an environment-sensitive fluorescent probe, "badan", was covalently linked to the C-terminal cysteine of the CheZ variant, 214FC. Badan-CheZ underwent a large (>50%) fluorescence quench upon formation of the complex with CheYp. Independent measurement of the association and dissociation kinetics of the CheZ-CheYp complex using stopped flow fluorescence allowed calculation of $K_d$ for complex formation, which cannot be measured by equilibrium fluorescence methods due to the tight binding. The dissociation rate constant for the CheZ-CheYp complex was slow relative to $k_{cat}$, implying that complex formation nearly always leads to dephosphorylation. Strikingly, the rate of association of CheZ 21IT with CheYp was more than 10x faster than that for wild type CheZ. Taken together, the enzyme kinetics and binding rate measurements for wild type CheZ and the gain-of-function CheZ 21IT suggested a kinetic model for CheZ dephosphorylation of CheY and its autoregulation. Possible structural bases for the kinetic models are discussed.

E. SOLO AND E. PLURIBUS: TWO NEW “EXPERIMENTAL ORGANISMS” FOR THE QUANTITATIVE ANALYSIS OF CHEMOTACTIC BEHAVIOUR

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Biochemical changes in the chemotaxis pathway are context-specific and make sense only when one knows the chemical environment of the bacterium. A complete description therefore requires one to relate temporal changes in the concentrations of signalling molecules to spatial changes in attractants and repellents. With this in mind, we embedded a detailed, quantitative simulation of the temporal changes in chemotaxis biochemical reactions (extracted from the existing BCT program) within a graphical representation of swimming bacteria. BCT gives the correct phenotype of over 60 mutants in which components of the chemotaxis pathway are deleted or overexpressed and, after tuning the sensitivity of the response and speed of adaptation, accurately reproduces the responses to pulses and step increases of attractant. In this new program, called E. solo, one or a few bacteria are depicted at relatively high magnification, each cell having four independent flagella. Here, the environment contains a uniform concentration of aspartate, which can be adjusted in real time by the user. This program allows the analysis of such details as the frequency of tumbles and angles of turns during a tumble, adaptation times, and the small changes in direction during a run due to thermal motion. In a modified version of the program called E. pluribus, bacteria are represented at lower magnification and without flagella. Here, multiple bacteria (of the same or different genotype) are allowed to swim within a defined arena, rectangular or circular, containing a fixed gradient of aspartate of specified slope. We found that, under certain conditions, a distinctive “volcano” profile emerged in E. pluribus simulations, with peaks of cell density occurring at intermediate concentrations of attractant. Since the internal biochemistry of the bacteria is firmly based on decades of experimental data, we believe it is legitimate to treat these representations as experimental objects in their own right. The advantage is that we can expose cells of any specified genotype to precisely defined stable gradients of any required shape (including those that are difficult, or impossible, to achieve in the real world) and observe their behavioural responses.
RELATIONSHIP BETWEEN BEHAVIORAL VARIABILITY AND ADAPTATION IN BACTERIAL CHEMOTAXIS

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Bacterial chemotaxis in *Escherichia coli* is one of the best-characterized systems for the study of signal transduction. In this system precise adaptation revealed by population measurements coexists with large behavioral variability (i.e. noise) at the single-cell level. We study the relationship between adaptation and behavioral variability, at the single-cell level by recording long time-series of switching events from a single flagellum. We measure successively in the same individual cells, the amplitude of noise from non-stimulated cells by using power spectral analysis and then the relaxation time in response to a small L-aspartate stimulus. We find a linear relationship between the relaxation time and the noise that are peaking in wild-type cells. Using a simple stochastic model of the chemotaxis network, we show that such relationship between noise and relaxation time is a direct consequence of the “Fluctuation-Dissipation Theorem”. Using a high-throughput capillary array, we characterize the population chemotactic response to a gradient of L-aspartate as a function of various level of CheR-YFP and CheB-CFP fusions. We find that the response is maximal near wild-type level of [CheR-YFP]/[CheB-CFP] suggesting that the noisiest cells also exhibit the best chemotactic response. Using a computational framework called “Agentcell”, we illustrate these experimental results with digital capillary experiments.
Many bacteria use flagella operated by rotary motors to swim. These complex structures contain more than 25 different proteins that self assemble to generate torque and regulate the sense of flagellar rotation. A key molecular event during chemotaxis is the interaction between the phosphorylated response regulator CheY (CheY-P) and the flagellar switch complex, which serves to switch the direction of flagellar rotation. To understand the mechanism of flagellar switching and its relationship to signal termination we aim to determine the crystal structures of different flagellar protein complexes, in particular those contained within the switch complex: FliM, FliN and FliG. Another switch component, FliY, resembles a fusion of FliM and FliN but also has CheY-P phosphatase activity like its homologs CheC and CheX. We have cloned, expressed and purified CheY, FliM, FliN, FliG and FliY from Thermotoga maritima, Bacillus subtilis and Geobacillus stearothermophilus. Stable complexes of FliM/FliN, FliM/FliN/FliY and FliM/FliN/FliG have been produced, characterized and targeted for crystallization.
STABILITY AND FUNCTION OF HELICAL SUPERCOILS IN THE CYTOPLASMIC DOMAINS OF CHEMORECEPTORS

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The most highly conserved region of bacterial chemoreceptors is the cytoplasmic domain, which consists of an extended four-helix bundle resulting from the dimerization of two anti-parallel helical hairpins. Structural analysis has shown that these extended four-helix bundles exhibit considerable helical supercoiling, but energetic analysis of supercoils in proteins has revealed that long supercoils always consist of both stable and unstable regions (long supercoils are extremely stable, virtually never dissociate and are difficult to degrade). Thus, it is likely that the extended four-helix bundles of chemoreceptor cytoplasmic domains possess stable and unstable regions. Moreover, these regions could play important roles in signal transduction. Algorithms such as COILS, PAIRCOIL, and PAIRCOIL2 search protein sequences for regions predicted to form stable coiled coils using algorithms which utilize the propensities of individual amino acids to be found at the “a” and “d” positions in coiled coils of known structure. Algorithms such as STABLECOIL utilize the energetic contribution of individual amino acids at “a” and “d” positions to predict the locations of coiled coils in sequences based on calculated estimates of their energetic stability. Application of both the propensity and the energy algorithms to chemoreceptor cytoplasmic domains reveals that these two approaches give very different results, yielding disagreements about where coiled coil regions are located and about their relative stabilities. The two approaches thus yield two different models for the locations of stable and unstable coiled coil regions within the cytoplasmic domain, and for the relationship between coiled coil stability and signal transduction. Experiments are underway to resolve the two models and elucidate the contributions of coiled coils to cytoplasmic domain structure and function.
SITE-DIRECTED CYSTEINE AND DISULFIDE ANALYSIS OF THE HAMP DOMAIN OF Tar CHEMORECEPTOR

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The bacterial transmembrane aspartate receptor (Tar) of *E. coli* and *S. typhimurium* chemotaxis is a homodimer that assembles to form larger oligomers, most likely a trimer-of-dimers. The structures of the periplasmic, transmembrane, and cytoplasmic domains have been elucidated by various approaches. However, the structure of the highly conserved HAMP domain that links the transmembrane and cytoplasmic domains remains unknown. This structure and its conformational changes are important for understanding signal propagation. Recently, the NMR structure of an isolated archaean HAMP domain (encoded by *Af1503* and truncated from its transmembrane and cytoplasmic domains) has been reported (Hulko *et al.*, 2006). The NMR structure is a parallel four-stranded coiled coil formed by dimerization of two $\alpha$-helices from each monomer. The goal of the present work is to probe the structure of Tar-HAMP using site-directed cysteine and disulfide chemistry, in order to test the predictions of the archaean NMR structure in full-length, membrane bound Tar. Using the archaean HAMP domain as a structural model, pairs of cysteines have been targeted along the helix-helix interfaces of Tar-HAMP at positions predicted to form disulfides between subunits in the NMR structure. Currently the disulfide bond formation pattern of these di-cysteine receptors is being analyzed, and the up-to-date findings will be reported at the meeting. Resolving the Tar-HAMP domain will lead to a better understanding of chemoreceptor structure and signal transduction, shedding light on a signaling mechanism that appears to be shared with many other transmembrane receptors involved in two-component signaling pathways.
DIRECT CONFIRMATION OF THE BROWNIAN RATCHET MECHANISM OF ACTIN-BASED MOTILITY.

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The growth of networks of actin filaments is a fundamental biological process that drives a variety of cellular and intracellular motions. During motility, intracellular pathogens are propelled by actin networks through the use of nucleation-promoting factors, which trigger the formation of nascent filaments off the side of existing filaments in the network and lead to elastic attachments between the moving object and network. A Brownian ratchet (BR) mechanism has been proposed to couple actin polymerization to cellular movements, whereby thermal motions are rectified by the addition of actin monomers at the end of growing filaments. Here, by following actin-propelled microspheres using three-dimensional laser tracking, we find that beads move via an object-fluctuating BR. Velocity varies with the amplitude of thermal fluctuation and inversely with viscosity as predicted for a BR. In addition, motion is saltatory with a broad distribution of step sizes that is correlated in time. These data point to a model in which thermal fluctuations of the microsphere or entire actin network, and not individual filaments, govern motility. This conclusion is supported by Monte carlo simulations of an adhesion-based BR and suggests an important role for membrane tension in the control of actin-based cellular protrusions.
POLAR TARGETING OF FlhF, A PUTATIVE GTPASE CONTROLLING FLAGELLUM LOCALIZATION IN VIBRIO CHOLERAE

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Vibrio cholerae swims by rotating a single flagellum located at its old cell pole. Flagellum biogenesis requires correct targeting of flagellar proteins to the appropriate pole and must be closely coupled to cell division to ensure that only one flagellum is built per cell. The mechanisms controlling correct flagellum polar localisation are poorly understood. Our current work focuses on a novel flagellar protein, flhF, which has been implicated in controlling flagellum placement.

Deletion of flhF was found to severely reduce, but not abolish, V. cholerae motility. Electron microscopy revealed that flhF null cells either did not assemble a flagellum or had a flagellum that was randomly localized rather than specifically targeted to the cell pole. Cells over-expressing flhF frequently produced two flagella that were either polar or bipolar. FlhF contains a region with similarity to signal recognition particle (SRP) GTPases, and site-directed mutagenesis revealed that the GTPase motifs are critical for FlhF function. The cellular localisation of FlhF was investigated using a functional GFP fusion. FlhF-GFP was found to co-localize with the flagellum at the old cell pole and studies in the aflagellate flrA null, which lacks the master regulator of flagellar gene transcription, demonstrated that FlhF-GFP polar localization occurs independently of other flagellar proteins. Deletion studies identified a c.40 residue region of FlhF that is critical for polar targeting.
**SALMONELLA** FliL IS REQUIRED FOR SWARMING BUT NOT FOR SWIMMING MOTILITY: A SPECIALIZED MOTOR PROTEIN?

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*fliL* is the first gene in an operon that specifies members of the flagellar switch complex and the Type III secretion system. The function of this gene has been a curiosity for a long time, since it is not known to be involved in swimming motility in *S. typhimurium* or in *E. coli* (1). Interestingly, *fliL* plays a role in motility and cell differentiation in *P. mirabilis* and *Caulobacter crescentus* (2, 3). In *S. typhimurium*, FliL was shown to be a basal body protein, associated with the cytoplasmic membrane (4). These authors speculated that FliL could be located around the outer circumference of the basal-body MS ring, in the same general location as the MotA and MotB proteins, and that it might contribute to the stability of the Mot complexes. Alternatively, it might facilitate the interaction between the stationary Mot complex and the rotating MS ring. In *C. crescentus*, FliL mutants have paralyzed motors, so FliL is clearly required for motor function (Jenal et al., 1994). However, FliL may act differently in the different bacteria since its sequence is relatively poorly conserved.

The effect of a *fliL* deletion on swimming and swarming was examined in *S. typhimurium*. There was a slight effect on swimming motility in normal medium, a more pronounced effect if medium was made viscous by addition of Ficoll, but a dramatic effect on swarming motility. Although cells did not move on the surface, an interesting observation was that starting around the 4 h time point, cells showed a significant number of apparently broken flagella. Under the electron microscope, morphology of the 'broken' flagella appeared remarkably similar to that described for a point mutation in *S. typhimurium fliF*, where filaments released from the mutant cells had the hook and a part of the rod of the flagellar basal body still attached (5).

FliF is an MS-ring protein, which serves as a mounting flange for the switch and rod. Interestingly, FliL is required for FliF degradation in Caulobacter, a process associated with flagellar ejection during the transition from swarmer to stalk cells (6).

We propose that FliL has evolved to assist the Mot complex in generating extra torque (by increasing proton flow?) required to overcome friction/viscosity encountered during surface motility. Proximity of FliL to the Mot complex could also place it in the vicinity of FliF, with which it may interact directly or indirectly to stabilize the rotor complex. In the absence of FliL, the flagella may not rotate (akin the paralyzed motor phenotype in *Caulobacter*). They may become susceptible to ejection only on the surface, either because of processes specific to the inherently different physiology of surface-propagated bacteria, or because of the higher viscous load experienced by the flagella on the surface, which ultimately destabilizes FliF.

FOUR TWO-COMPONENT SIGNAL TRANSDUCTION PROTEINS FUNCTION TOGETHER TO REGULATE PROGRESSION THROUGH THE DEVELOPMENTAL PROGRAM IN MYXOCOCCUS XANTHUS

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Myxococcus xanthus is a social gram-negative soil bacterium that undergoes complex, multicellular developmental process under starvation conditions. In this development process, cells first aggregate into mounds of approximately 100,000 cells and then within these mounds, differentiate into environmentally-resistant spores. We have previously demonstrated that RedC, RedD, RedE, and RedF two-component signal transduction proteins control progression through this developmental program.

Deletion of redCDEF causes cells to aggregate and sporulate earlier than wild type and to form smaller, more numerous and more disorganised fruiting bodies. While RedC appears to be a typical membrane bound histidine kinase, RedD consists of solely of dual receiver domains. RedE is a predicted cytoplasmic histidine kinase lacking any obvious sensing domain, and RedF is a single receiver domain response regulator. Results from a combination of yeast two-hybrid analysis and Biacore surface plasmon resonance technology suggest that RedC interacts with the first receiver domain of RedD and RedE interacts with RedD’s second receiver domain. RedE also interacts with RedF. Radioactive phosphotransfer assays indicate that RedC readily autophosphorylates and the phosphate signal can be transferred to RedD. In contrast, we do not currently detect autophosphorylation of RedE, although this protein interacts with RedF in a process that is stimulated by the addition of ATP. We present our current model for signal flow between these unusual two-component proteins and relate this to control of developmental progression in M. xanthus.
THE ESSENTIAL YycG TWO-COMPONENT HISTIDINE KINASE OF *Bacillus Subtilis* CO-LOCALIZES WITH FtsZ AT THE CELL DIVISION SITE

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The YycFG two-component signal transduction system functions as a homeostatic mechanism to coordinate the expression of genes for cell wall and cell membrane constituents during growth. The signals the YycG sensor kinase recognizes to balance this expression are unknown. This kinase is membrane located and is thought to have a periplasmic PAS domain. Studies to locate the sensor kinase in the cell membrane by immunofluorescent techniques revealed the sensor kinase was not randomly distributed but accumulated at insipient septum locations between nucleoids. Since FtsZ is the well characterized initiating protein for accumulation of division proteins at the septum, we visualized the location of both YycG and FtsZ by immunofluorescence. Overlay of these results showed essentially 100% co-localization of the two proteins. As a control we immunoprecipitated FtsZ from solubilized cells and YycG was found to co-precipitate in the immune complex. These results indicate that YycG and FtsZ are either interacting at the cell division septum or both of them are part of a multi-protein complex at that site.

Further studies in depletion mutants for FtsZ or YycG revealed that YycG localization to the insipient septum required FtsZ. Thus YycG has the same properties as the multitude of division proteins known to accumulate after ring formation by FtsZ. These and additional results are consistent with the proposal that the YycG sensor kinase associates with the division apparatus for signal acquisition and serves to coordinate the formation of cell wall and other structural constituents with DNA synthesis and septation.
REGULATION OF AN ESSENTIAL SENSOR HISTIDINE KINASE BY TWO NOVEL PROTEINS: STRUCTURE AND FUNCTION OF BACILLUS SUBTILIS YYCH AND YYCI.

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The YycFG two-component system is highly conserved and essential in most low G+C Gram-positive bacteria. For this reason it has been suggested as a prime antimicrobial drug target. The genes for two proteins, YycH and YycI, are usually organized within the same operon, downstream of the gene for the YycG histidine kinase. A transposon mutagenesis study originally revealed an involvement of YycH in regulating this two-component system. Characterization of both YycH and YycI by alkaline phosphatase fusion studies confirmed predictions that both YycH and YycI were localized in the periplasm and anchored to the membrane via an N-terminal transmembrane helix. The effects of inactivation of either protein on YycF-dependent transcription was assayed using a β-galactosidase reporter strain constructed with a yoch-lacZ fusion. Deletion of either yycH or yycI or both resulted in a 10-fold elevation of YycF-dependent expression suggesting both proteins are required for regulation of the YycG sensor kinase. Structural and interaction studies were undertaken to gain insights into the nature of these YycH and YycI regulatory activities. Crystal structures of both proteins were solved and surprisingly revealed that these proteins shared the same novel fold despite an absence of sequence homology. This suggests that more examples of distantly related proteins may be found in the databases. Given that the proteins under investigation are all transmembrane proteins, interactions between the YycG sensor kinase, YycH and YycI were probed in vivo utilizing a bacterial-two hybrid system. All three components were found to tightly interact with each other. A model will be presented of this multi-protein complex that summarizes our current understanding and findings on the regulation of this important two-component system.
MotX AND MotY FORM THE T RING ON THE BASAL BODY OF Na\(^+\)-DRIVEN FLAGELLA AND ARE REQUIRED FOR STATOR FORMATION

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Bacterial flagellar motor is a rotary molecular machine that converts the electrochemical potential energy of H\(^+\) (E. coli etc) or Na\(^+\) (Vibrio etc) across the membrane to the rotational force. The flagellum consists of the filament, the hook and the basal body. In Gram-negative bacteria, the basal body is the supramolecular complex comprised of the rotor and the bushing, which consist of MS, C rings and rod and L, P rings, respectively. The four stator proteins PomA, PomB, MotX, and MotY are essential for motility by the Na\(^+\)-driven flagella of Vibrio alginolyticus because null mutants have a Mot\(^-\) phenotype. PomA and PomB are orthologues of MotA and MotB, respectively, thought to function as the Na\(^+\) channel. The functions of MotX and MotY have not yet been clarified. Recently, MotX was shown to interact with MotY and suggested to interact with PomB. It is thought that the stator assemble around the basal body, interact with the rotor and generate torque for the flagellar motor.

When we purified the flagellar basal bodies, MotX and MotY were detected in the basal body, but PomA and PomB were not. By antibody labeling, MotX and MotY were detected around the LP ring. These results indicate that MotX and MotY associate with the basal body of V. alginolyticus. The basal body had a new ring structure beneath the LP ring, which was named T ring. This structure was changed or lost in the basal body from the \(\Delta motX\) or \(\Delta motY\) strain. The T ring probably comprises MotX and MotY. In the presence of MotX and MotY, PomA/PomB complex localize to a cell pole. But, in the absence of MotX or MotY, we showed that PomA and PomB could not be localized to a cell pole. From the above results, we propose that the T ring formed by the MotX and MotY are required for incorporation and stabilization of the PomA/PomB complex in the motor complex.
FLAGELLAR TYPE III SECRETION IS DEPENDENT ON THE PROTON MOTIVE FORCE

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The flagellar type III secretion system transports a number of proteins necessary for the assembly of the flagellum across the inner membrane in an ATP-dependent manner. In this work we show that flagellar type III secretion is also dependent on the proton motive force. Abolishment of both the proton gradient \( \Delta \text{pH} \) and the membrane potential \( \Delta \Psi \) using the ionophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) inhibited secretion of the anti-\( \sigma^{28} \) factor FlgM. Secretion of FlgM could be restored by growth in medium lacking CCCP. Furthermore the secretion of FlgM was inhibited at pH 5 in the presence of 34 mM acetate indicating an important role of the proton gradient \( \Delta \text{pH} \) and/or the intracellular proton concentration. Addition of the ionophore CCCP resulted in an immediate growth arrest, but not in a significant decrease of cytoplasmic ATP levels, thus demonstrating that both the flagellar type III secretion ATPase FliI and the proton motive force are necessary for the export of flagellar type III secretion substrates.
Hook completion is the most important check point in flagellar assembly. When the hook length reaches 55 nm, the export apparatus switches its substrate specificity and starts to assemble further structure. In mutants which cannot complete hook assembly, the anti-sigma factor, FlgM, stays in the cell and the cell negatively regulates expression of genes required for further flagellar assembly. We have been trying to isolate mutants which bypass such negative regulation in the LP ring (ΔflgHI) mutant background. Flk is the first such kind of mutant we have isolated. In this study, we continued to look for these mutants. To do so, we have developed a better positive selection system. FlgM, the first substrate secreted through the flagellar type III export system after hook completion, was fused to signal peptide region deleted B-lactamase and its ampicillin sensitivity in ΔflgHI background was used for mutant selection. A second copy of flk was supplied from an arabinose-inducible promoter on the chromosome to discard flk mutants from the selection. Novel mutations other than flk were found in flgG. The mutant flgG alleles were introduced into wild-type backgrounds and the cells became weakly motile on motility agar plates. Electron micrographs showed that the flagellar structure had a longer rod, poly P ring, a shorter hook and a filament. We are currently analyzing motile revertants to understand the mechanism.
ydiV OVER-EXPRESSION IN SALMONELLA TYPHIMURIUM NEGATIVELY REGULATES FLAGELLAR GENE EXPRESSION THROUGH THE POST-TRANSCRIPTIONAL REGULATION OF flhDC

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Flagellar gene expression in Salmonella typhimurium is organized into a transcriptional hierarchy of three promoter classes, class 1, 2 and 3. At the top of the hierarchy is the class 1 flhDC operon. FlhD and FlhC (class 1 genes) act as transcriptional activators in combination with sigma 70 to transcribe the class 2 promoters. Products of class 2 transcripts are required for structure and assembly of the flagellar hook-basal body complex and the alternative transcription factor, sigma 28, which is required for class 3 promoter expression.

It was discovered that over-expression of ydiV from a tetracycline-inducible promoter resulted in a nonmotile phenotype; thus identifying ydiV as a negative regulator of flagellar gene expression. ydiV is annotated as a putative phosphodiesterase that is potentially involved in degrading cyclic-di-GMP, a bacterial signaling molecule known to regulate biofilm formation. To determine which step of flagellar gene expression is affected by ydiV, lac transcriptional and translational reporter fusions to various flagellar genes were used. Induction of ydiV resulted in the reduction in flhDC operon expression at the post-transcriptional level. Additionally, motile revertants from strains over-expressing ydiV included a mutant that mapped to the clpX locus and was shown to be a loss-of-function mutation. We are currently using transposon mutagenesis to identify additional loss-of-function mutants that alleviate the motility defect under ydiV over-expression conditions and to determine if ydiV is acting at the mRNA or protein level.
EFFECTS OF MULTIPLE PROMOTERS ON THE COUPLING OF GENE EXPRESSION TO FLAGELLAR ASSEMBLY IN \textit{SALMONELLA TYPHIMURIUM}

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In \textit{Salmonella}, the bacterial flagellum is a complex molecular machine that is constructed from at least two dozen structural proteins in a process that involves over sixty flagellar genes. These genes are expressed from three classes of flagellar promoters in a transcriptional hierarchy. This organization allows genes needed earlier in the construction of flagella to be expressed before genes needed later. Additionally, regulatory proteins like the alternate sigma factor FliA and its anti-sigma factor FlgM coordinate flagellar transcription with the stage of completion of the flagellum. Expression of these regulatory proteins from two classes of flagellar promoters may maintain the regulatory proteins at the levels needed to respond to flagellar structural intermediates. This hypothesis is being tested by isolating promoter mutants that express these regulatory proteins from only one class of flagellar promoters and then characterizing the effects these mutations have on motility and flagellar transcription. These promoter mutants should provide insight into the strategies used by \textit{Salmonella} to efficiently build flagella.
AI-2 REGULATES LuxPQ SIGNAL TRANSDUCTION BY INDUCING RECEPTOR ASYMMETRY

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Bacteria use membrane receptors to sense and respond to their surroundings, but how these environmental signals regulate receptor activity is not well understood. To determine how ligand-binding is linked to membrane receptor activity we examined the Vibrio harveyi receptor complex LuxPQ. LuxP is a periplasmic binding protein that binds the quorum-sensing signal autoinducer-2 (AI-2), a small molecule used by many bacterial species to regulate gene expression in response to cell density. This binding event modulates the activity of LuxQ, an integral membrane sensor histidine kinase—the largest family of bacterial cell-surface receptors. How AI-2 binding to LuxP regulates LuxQ kinase activity was unknown. Combined x-ray crystallographic and functional studies show that AI-2 binding stabilizes a quaternary arrangement in which two LuxPQ monomers are asymmetrically associated. The observed asymmetric configuration, rather than conformational changes within single LuxQ monomers, represses kinase activity in both receptor subunits, triggering the transition of V.harveyi into quorum-sensing mode. Further structural studies of the LuxQ cytoplasmic domain will reveal how this ligand-induced asymmetry modulates intracellular kinase activity.
ANALYSIS OF THE MOVEMENT OF A FLAGELLAR BASAL BODY STRUCTURE LABELED WITH GFP-FliG IN THE CELL MEMBRANE OF E.COLI

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In the bacterial cell membrane, many kinds of proteins such as ion channels, receptors, and sensor proteins exist. The bacterial flagellum is a supramolecular complex consisting of a basal body (rotary motor), a helical filament (propeller), and a hook (universal joint), and the cell swim toward favorable conditions by rotating the motor embedded in cytoplasmic membrane. A basal body consists of a rotor, an axial rod, stators, and bearings. Recently, we have constructed the system to observe the flagellar basal body embedded in the cytoplasmic membrane by using of the green fluorescent protein tagged to the FliG protein, which is one of the rotor components. By using this system, we observed immobile and mobile fluorescent punctate signals. It is an interesting phenomenon that a huge molecular complex like a flagellar basal body moves in the cell membrane. In this study, in order to understand the movement of the mobile fluorescent dot composed of GFP-FliG in the cytoplasmic membrane, we carried out the further analysis by using single particle tracking method.

To analyze the movement of mobile fluorescent dots, we tracked the position of center of fluorescent mass of the mobile dot every 33 msec. From the analysis using the mean squared displacement versus the time interval and the relative deviation (Kusumi et al. (1993) Biophys. J., 65, 2021-2040), some fluorescent dots were determined to be a result of simple diffusion and the other were determined to result from restricted diffusion, and their diffusion coefficients were $5.1 \pm 2.9 \times 10^{-11}$ cm$^2$/sec and $4.6 \pm 3.9 \times 10^{-11}$ cm$^2$/sec, respectively.

The fluorescent dots composed of GFP-FliG are likely to exhibit three types of mode in the bacterial cell membrane: 1) immobile mode, 2) mobile mode that behave like simple diffusion, and 3) mobile mode that behave like restrict diffusion. The factors that affect the diffusion of flagellar basal body might be present in bacterial cytoplasmic membrane, peptidoglycan layer, and/or outer membrane.
TORQUE-SPEED RELATIONSHIP OF NA⁺-DRIVEN CHIMERIC FLAGELLAR MOTOR IN ESCHERICHIA COLI

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We have demonstrated the stepping rotation of Na⁺-driven chimeric flagellar motor in E. coli using high-speed video recording and back-focal-plane (BFP) interferometry (Sowa et al., 2005, Nature). However, property of this motor, which is a key to understand the mechanism of steps in the flagellar rotation, is not fully characterized. We report here the torque-speed relationship of the chimeric motor.

After the cells were fixed on the glass surface, movement of beads adsorbed to the flagellar filament of the chimeric motor was measured using BFP interferometry. We measured the speed of rotation in several load conditions by changing the size of beads (0.3-1.0 μm). The generated torque was estimated from the rotational frictional drag coefficient and the measured speed.

The torque of the chimeric motor was constant at low speed and linearly decreased at high speed as reported for both Na⁺-driven type motor (Sowa et al., 2003, JMB) and H⁺-driven type motor (Chen and Berg, 2000, Biophys.J).

Maximum speed of the chimeric motor (~700 Hz) was ~2-fold faster than that of wild type H⁺-driven motor in E.coli, whereas maximum torque of the chimeric motor (~2000 pN.nm) was similar to that of the wild type motor. When expression of the chimeric motor was induced by application of IPTG, rotational speed increased in a stepwise manner. Unitary amount of the increased speed was 12~13-fold smaller than the maximum speed of the chimeric motor. Our results suggest that the chimeric motor can be quipped with 12~13 torque-generating units, each of which may work independently.
PROPERTIES OF NA+-DEPENDENT FLAGELLAR MOTILITY OF FACULTATIVELY ALKALIPHILIC BACILLUS PSEUDOFIRMUS OF4 AND THE BASIS FOR POOR MOTILITY AT LOW pH

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Flagella-based motility of extremely alkaliphilic Bacillus species is completely dependent upon Na+. Little motility is observed at pH values < ~ 8.0. In the current study, the number of flagella/cell was examined as a function of growth pH in the facultative alkaliphile Bacillus pseudofirmus OF4 and a derivative selected for increased motility on low agar plates. The number of flagella/cell and flagellin levels of cells grown at different pH values was shown to be independent of growth pH in both strains, but these parameters were higher in the up-motile strain. Assays of the swimming speed of the cells showed that although no motility was observed at pH < 8 in the presence of 10 mM Na+, significant motility at pH 7 was supported by much higher Na+ concentrations. Thus the poor motility of this alkaliphile at low pH, under normal growth conditions, probably reflects competitive inhibition of Na+-based motility by high [H+] rather than decreased flagella production. At pH 8-10, the swimming speed increased linearly with the logarithmic increase of Na+ concentration up to 230 mM. At every Na+ concentration, fastest swimming was observed at pH 10. Motility of the up-motile strain was greatly increased relative to wild-type on soft agar at alkaline pH whereas the swimming speed of the up-motile strain in liquid was only significantly higher than that of wild-type under conditions with increased viscosity or at extremes of pH at which wild-type motility was sub-optimal. The up-motile phenotype, with increased flagella/cell may support bundle formation that particularly enhances motility under a subset of conditions with specific challenges.
THE GLUCOSE-PHOSPHOTRANSFERASE SYSTEM (II\text{Glc}) FROM \textsc{Escherichia coli} K-12 AS A COMPLEX TRANSPORT AND SIGNAL TRANSDUCTION SYSTEM

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The glucose-specific enzyme II\text{Glc} of the PEP–dependent carbohydrate phosphotransferase system (PTS) plays a central role in \textit{E. coli}, not only in the uptake and concomitant phosphorylation of D-glucose, but also as a glucose sensor causing an accurate response in the regulation of carbon catabolite fluxes and in chemotaxis. The regulatory activities of the PTS vary with the degree of phosphorylation of its components, which in turn varies with the ratio of sugar-dependent dephosphorylation and PEP-dependent rephosphorylation. The expression of the \textit{ptsG} gene for the membrane-bound IICB\text{Glc} glucose transporter is regulated by several different regulators in a highly sophisticated manner. In cooperation with the Max-Planck-Institute in Magdeburg we developed a computer program to simulate the PTS-regulatory output signals under various growth conditions. Results from these simulation experiments indicate that small changes in the amounts of IICB\text{Glc} cause severe changes in the phosphorylation levels of the PTS-proteins, and thus in the output signal. Furthermore, we have found a novel regulator, MtfA (\textit{Mlc}-titration factor A) (Yeel, B1976), which is involved in the regulation of \textit{ptsG} gene expression. Intensive BLAST sequence similarity searches revealed that orthologs of MtfA exist in many proteobacteria of the beta- and gamma- subdivisions. Experiments for the identification and characterization of this new regulator are presented.


THE NITRATE-DEPENDENT VANISHING ACT OF THE ASPARTATE RING IN ESCHERICHIA COLI

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INTRODUCTION: When wild-type cells are inoculated into tryptone semi-solid agar that contains 50 mM sodium nitrate, the aspartate ring does not form. Although these findings emanated from our studies on energy taxis some 7 years ago, the phenomenon has never been reported, and has remained little more than a curiosity. In this study, we narrow the list of possible causes for this nitrate effect.

METHODS: We compared the colony morphology of several E. coli strains on tryptone (+/- 50 mM sodium nitrate) semi-solid agar, and included two strains [C8Asp/TL & C8Asp/23] that do not excrete aspartate. We used Tasr and Tsar chimeras that were joined near the periplasm/membrane junction to identify the general location of nitrate inhibition. We also constructed and tested Tar and Tasr mutants that exhibited altered aspartate binding.

RESULTS: The asparate ring vanished in tryptone semi-solid agar when 50 mM sodium nitrate was included. Neither Aer nor Tsr appeared to influence nitrate's effect on Tar, and both Aer and Tsr receptors were themselves immune to nitrate's effect. This made it possible to unmask the Aer ring, which ran nearly underneath the aspartate ring in strains expressing both Aer and Tar. In contrast to the results for Aer and Tsr, the effects of nitrate were more pronounced when Tar was overexpressed. It was unlikely that nitrate was somehow inducing aspartate excretion, and that this excretion had stalled outward motility, because strains that were unable to excrete aspartate also lost the aspartate ring. A Tasr chimera that contained the periplasmic region of Tar and the cytosolic region of Tsr was also sensitive to nitrate, indicating that the periplasmic binding region, rather than the cytosolic signaling region, was responsible for the nitrate effect. This was supported by experiments with a Tsar chimera that expressed the periplasmic region of the Tsr receptor and the cytosolic region of the Tar receptor; this receptor was not influenced by nitrate. As expected, both R64H and T154I altered the ability of Tar and Tasr to sense aspartate. However, aspartate sensing was not necessary for these mutant receptors to orchestrate some type of taxis on tryptone, yielding a ring, and colony sizes of ~50% that of wild-type Tar or Tasr. Surprisingly, Tar- and Tsar-R64H also coordinated sharp, defined rings on succinate plates. Tar- and Tsar-T154I retained partial aspartate sensing and were only partially inhibited by nitrate, unlike the R64H mutants, which neither sensed aspartate, nor were affected by nitrate.

CONCLUSIONS: This study confirms that one can use nitrate as an empirical method to identify the aspartate ring, or to "blind" the aspartate receptor when its ring interferes with that of another receptor (e.g., Aer). It appears that nitrate, or a nitrate-dependent metabolic product other than aspartate, competes with aspartate for the Tar receptor.
Targeted motility is one of the essential properties of many microorganisms for surviving in their environment. The human gastric colonizer Helicobacter pylori infects humans and is able to persist lifelong. The prerequisites for the successful colonization of the host niche are not fully understood. However, it is well established that motility and orientation/taxis are essential in infection and persistence. In particular energy taxis or pH-taxis was shown to be essential for infection, although H. pylori does not appear to possess Aer-like sensors or PAS domain proteins (Schreiber et al., PNAS 2004). H. pylori possesses four putative chemoreceptors with largely unknown substrate specificities. Focusing on the receptor TlpD which has, unlike the other identified sensors, no transmembrane domains, we investigated its localization, expression, function and possible sensing specificity. Although TlpD has no transmembrane domains, we could show a membrane-proximal localization of TlpD by fluorescence microscopy and confirmed this result by bacterial fractionation. In order to determine further TlpD function, we constructed a tlpD mutant in H. pylori. The behavioral pattern of the mutant was determined by temporal assay and compared with wild type and other sensing mutants in different media. In contrast to the wild type, the behavioural analysis of the mutant indicated that the ability to sense a repellent stimulus was strongly abolished. The original phenotype could be restored in the mutant by complementing tlpD in trans. We could observe a similar phenotype in a wild type strain when we uncoupled oxygen-dependent metabolism from the taxis system by adding metabolic inhibitors. Several other results supported as well a role of H. pylori TlpD in negative aerotaxis or energy taxis.

THE FIST DOMAIN: A NOVEL UNIVERSAL SENSORY DOMAIN

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The essence of signal transduction, in both eukaryotes and prokaryotes, is the conversion of a molecular signal into gene activation or other cellular responses. We are searching for universal sensory modules that detect signals in both prokaryotes and eukaryotes. Here we report the identification of one such module, the FIST domain, which is found in major signal transduction pathways in prokaryotes (one-component, two-component, and chemotaxis regulatory systems) and eukaryotes (F-box-containing components of the ubiquitin pathway). FIST-containing proteins are exclusively cytoplasmic and are proposed to detect small ligands, likely amino acids, and relay the information to pathways controlling gene expression, intracellular levels of cyclic di-GMP and flagellar motility. Analysis of gene order using the MIST database reveals that Fist-containing proteins are encoded in close proximity to amino acid permeases, asparagine synthases, peptidases and eukaryotic F-box proteins that are known to bind auxin, a plant hormone and derivative of tryptophan. This supports the hypothesis that FIST is an amino acid sensor.
A NOVEL PHOTO-SENSORY TRANSDUCTION OF MICROBIAL RHOPOSIN IN CYANOBACTERIA

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ABSTRACT

NOT AVAILABLE
PROBING COMMUNICATIONS BETWEEN DISTINCT CHEMORECEPTORS OF ESCHERICHIA COLI

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In the chemotaxis of Escherichia coli, stimuli are sensed by the chemoreceptors, also known as methyl-accepting chemotaxis proteins (MCPs), which form ternary complexes with the histidine kinase CheA and the adaptor CheW. The MCP-CheW-CheA complex clusters at a pole of a rod-shaped cell. It has become a growing consensus that communications between MCP homodimers within a cluster play a pivotal role in signal amplification and adaptation. However, it remains to be established whether (and how) attractant binding to an MCP dimer affects neighboring MCP dimers. Here, we tried to probe communications between two high-abundance MCPs, i.e. the serine and aspartate chemoreceptors (Tsr and Tar, respectively), by using in vivo disulfide crosslinking assays. We found that chemotaxis is impaired by extreme interdimer crosslinking at Cys introduced on the periplasmic surface of the Tar dimer, which is otherwise devoid of Cys. Co-expression of wild-type Tsr with the Cys-replaced Tar proteins prevented their interdimer crosslinking. The latter proteins formed interdimer crosslinked Tsr-Tar heterodimers with similar Cys-replaced Tsr, reinforcing that Tsr and Tar dimers cluster together. Moreover, depending on the position of Cys, aspartate binding to Tar increased or decreased the interdimer crosslinking of Tar-Tsr and Tsr. These results imply communications between MCP dimers, which may be involved in signal amplification.
THE FLAGELLAR MASTER REGULATOR FlhDC REGULATES OmpF

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The EnvZ/OmpR two-component regulatory system regulates the porin genes *ompF* and *ompC* in response to changes in osmolarity of the growth medium. In the commonly employed laboratory strain MC4100, it is well established that OmpF is the more abundant porin at low osmolarity. At high osmolarity, *ompF* is repressed and OmpC becomes the more abundant porin. In the present work, we examined the osmotic-dependent expression of *ompF* and *ompC* in the sequenced strain MG1655. This strain contains an insertion sequence (IS-1) in the regulatory region of the *flhD/C* promoter (Barker et al., 2004), which prevents repression by OmpR (Shin and Park, 1995). As expected, *ompF* transcription was decreased and *ompC* transcription was increased with increasing osmolarity. However, when compared to a strain in which the IS-1 element was cured, the MG1655 strain had significantly higher levels of *ompF* transcript (2.4-fold). Previous studies from our laboratory have determined that cross-talk among additional response regulators can directly activate porin gene expression (Batchelor et al., 2005; Koo et al., 2006). Thus, we explored the possibility that FlhD/C might also regulate the porin genes. We used quantitative Real-Time PCR and primer extension to investigate the effect of FlhD/C on transcription of the porin genes. FlhD/C regulated *ompF* transcription, but not *ompC* transcription. We considered the possibility that FlhD/C acted via OmpR, but Western blot analysis demonstrated that FlhD/C did not significantly affect cellular levels of OmpR. Alternative possibilities were that FlhD/C directly activated *ompF*, or that it functioned sequentially in a regulatory cascade with an unknown factor. A direct interaction between FlhD/C and the regulatory region of the *ompF* was demonstrated by an electrophoretic mobility shift assay, indicating that FlhD/C binds to the *ompF* promoter. This is the first report of a gene outside of the flagellar regulon that is regulated by FlhD/C. The precise location of the FlhD/C binding site(s) and their implication in porin gene regulation will be presented. Supported by NIH GM58746 and NSF MCB0613014 to LJK and by postdoctoral training fellowship (KRF-1005-214-F00049) from the Korean Research Foundation to JER.
PROTEIN LOCALIZATION AT THE ATTACHMENT ORGANELLE REGION OF MYCOPLASMA PNEUMONIAE VISUALIZED BY FLUORESCENT PROTEIN TAGGING.

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\textit{Mycoplasma pneumoniae} is a causative agent of pneumonia and bronchitis in humans and is one of the smallest self-replicating and cell wall-less bacteria known. Adhesion of this bacterium to human respiratory cells (cytadherence) and gliding motility are thought to be major factors of pathogenicity. These features of \textit{M. pneumoniae} are mediated by a differentiated cell terminal structure, the attachment organelle that is a membrane protrusion supported by internal cytoskeletal structures. The attachment organelle is also thought to be involved in cell division process of \textit{M. pneumoniae} and composed of a number of protein factors. However, fine structure of the organelle is not fully understood. In this study, we have designed a convenient vector system to introduce fluorescent protein fusion genes into \textit{M. pneumoniae}. By using this system, we expressed fluorescent fusions of the attachment organelle component proteins, HMW1, HMW2, HMW3, P30, P1, P90(B), P40(C), P65, P41, P24, and P200. These experiments categorized these proteins mainly into four groups. (1) P30 and P65 localized at the distal end of attachment organelle. (2) HMW1 and HMW3 localized at the middle. (3) P41, P24 and P200 localized at the proximal end. (4) HMW2 and P1 were observed at relatively broad area of attachment organelle. However, P90(B) and P40(C) proteins were not focused at the attachment organelle but were dispersed in the cell. The fluorescent protein tag might disturb native localization of these two proteins. We have also applied this technique to the other 100 proteins of interest. This showed that several of these proteins which might have functions of cytoskeleton, DNA replication or metabolic enzyme, clearly localized at the attachment organelle area. These proteins are thought to be the candidates of the component of attachment organelle.
THE crdB LIPOPROTEIN TRANSDUCES ENVELOPE STRESS SIGNALS VIA THE Che3 SIGNAL TRANSDUCTION PATHWAY IN MYXOCOCCUS XANTHUS

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*Myxococcus xanthus* is a delta-proteobacterium that moves by gliding and has the ability to form fruiting bodies when nutrient levels drop. Analysis of the *M. xanthus* genome led to the identification of 8 chemotaxis gene clusters containing genes that encode proteins whose homologs function in chemotaxis.

The *che*3 cluster encodes homologs of 2 methyl-accepting chemotaxis proteins (Mcp3A, Mcp3B) that are located in the cytoplasmic membrane. The Mcps are thought to interact with the histidine kinase CheA via 2 scaffold proteins, CrdC and CheW3. The response regulator and interaction partner of CheA in this signal transduction pathway is encoded by *crdA*. CrdA shows high homology to NtrC-like transcriptional activators and is thought to regulate the expression of various developmental genes. Adaptation to signals transduced by the Che3 system is likely to be mediated by a methyltransferase CheR and a methylesterase CheB. Mutations in these genes did not show any defects in motility but altered the timing of fruiting body and spore formation.

The first gene in the *che*3 cluster is *crdB*, a gene that shows no similarity to chemotaxis genes. Mutations in *crdB* result in reduced swarming and affect the formation of fruiting bodies. Analysis of the *crdB* sequence revealed a signal sequence and a lipobox with a signal peptidase II cleavage site. Fusions of *crdB* to *phoA* verified that CrdB is transported into the periplasm. It was also shown that CrdB is a lipoprotein using the cyclic peptide antibiotic Globomycin that specifically inhibits signal peptidase II processing of the prolipoprotein. Additionally, the CrdB C-terminus possesses a peptidoglycan binding domain. Together, the data allow us to hypothesize that CrdB functions as a sensor of envelope stress that regulates both motility and development via the Che3 signal transduction pathway.
REGULATION AND HIERARCHY OF THE FLAGELLAR SYSTEM IN *BRUCELLA*

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Flagellum is a complex rotary and filamentous molecular machine allowing motility in bacteria. While described as unflagellated, the pathogenic bacterium *Brucella* has all the genes except the chemotactic system, necessary to assemble a functional flagellum. We showed (i) flagellar genes were necessary during mouse infection (ii) the flagellar flgE proteins and flagellin were produced during log phase of growth in rich medium and, (iii) a flagellar like structure in electronic microscopy.

We also studied the flagellar regulation in *Brucella*. The expression of the bacterial flagellar genes follows a hierarchical pattern. At the top of the flagellar hierarchy we identified three genes: *vjbR* (a quorum sensing-related transcriptional regulator), *rpoE* (a sigma factor) and *ftcR* (a new flagellar master regulator present in other alpha proteobacteria). By western blotting we showed that *ftcR* and *vjbR* are necessary for FlgE and flagellin production. Electrophoretic mobility shift assay (EMSA) demonstrated that the *ftcR* protein binds to *fliF* gene promoter. FlgE and flagellin is overproduced in *rpoE* mutant. This data shows that RpoE down regulated flagellar genes probably with an unknown regulator.

In the hierarchical model of flagellar assembly, mutations of *fliF* or *flgE* normally prevent flagellin production. Unlike this model, the flagellin of Brucella is always produced in *fliF* and *flgE* mutant. In Caulobacter crescentus, the posttranscriptional regulator FlbT repress flagellin production. Surprisingly, mutation of *Brucella flbT* gene resulted in reduced synthesis of the flagellin, and *flbT* seem to act as an activator.

Taken together, these data demonstrated that *Brucella* presents an atypical flagellar system important for its virulence in the host.
LOCALIZATION FACTORS OF MCPs

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Our lab is interested in the spatial organization of the chemotaxis proteins. We have previously reported two different localizations paradigms. In *Escherichia coli*, the membrane bound methyl-accepting chemotaxis proteins (MCPs) and the soluble CheA and CheW proteins are localized in clusters at the cell poles. In *Rhodobacter sphaeroides* however, membrane bound MCPs are found at the cell poles, whereas the soluble MCPs are found in cytoplasmic clusters. *Pseudomonas aeruginosa* has 26 MCPs, and the majority of these (23) possess 1 or more transmembrane domains, while three are predicted to be soluble. *P. aeruginosa* presents a third paradigm of MCP localization. We have shown by immunofluorescence microscopy that the general MCP population is localized to the cell poles. Suprisingly, localization of an epitope-tagged soluble MCP, His-McpS, was also at the cell pole, suggesting that McpS may be in clusters with transmembrane MCPs.

Given the unexpected localization of this soluble MCP, we set out to determine the factors affecting the polar localization of McpS. In *E. coli* and *R. sphaeroides* the localization of MCPs is dependent on the presence of the cognate CheW. Using immunofluorescent microscopy, we determined that a subset of the transmembrane MCPs in *P. aeruginosa* is dependent on CheW from the chemotaxis system for polar localization. The factors affecting the localization of McpS, however, are different. To date, McpS remains localized predominately at the poles in all CheW deletion strains examined. We are using a genetic and biochemical approaches to identify the cognate chemotaxis system and potential localization factors of McpS.
EFFECTS OF ALTERING TRYPTOPHAN RESIDUES IN THE SECOND TRANSMEMBRANE HELIX (TM2) OF *E. COLI* Tar CHEMORECEPTOR

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ABSTRACT
NOT AVAILABLE
TUNING A BACTERIAL CHEMORECEPTOR WITH PROTEIN-MEMBRANE INTERACTIONS

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Chemoreceptors in *Escherichia coli* are homodimeric transmembrane proteins that convert environmental stimuli into intracellular signals controlling flagellar motion. Chemoeffectors bind to the extracellular (periplasmic) domain of the receptors, whereas their cytoplasmic domain mediates signaling and adaptation. The second transmembrane helix (TM2) connects these two domains. TM2 contains an aliphatic core flanked by amphipathic aromatic residues that have specific affinity for polar-hydrophobic membrane interfaces. We previously showed that Trp-209, near the cytoplasmic end of TM2, helps maintain the normal baseline-signaling state of the aspartate chemoreceptor (Tar) and that Tyr-210 plays an auxiliary role in this control. We have now repositioned the Trp-209/Tyr-210 pair in single-residue increments about the cytoplasmic polar-hydrophobic interface. Changes from WY-2 to WY+1 modulate the baseline signaling state of the receptor in predictable and incremental steps that can be compensated by adaptive methylation/demethylation. Greater displacements, as in WY-3, WY+2, and WY+3, bias the receptor to the “off” kinase-inhibiting state or the “on” kinase-stimulating state, respectively, to a degree that cannot be fully compensated by the adaptation system. To our knowledge, this is the first time protein-membrane interactions have been harnessed to incrementally manipulate the signaling state of a transmembrane receptor in a direct and controlled manner. Analogous aromatic residues are present in many transmembrane sensor kinases and may serve a similar function.
MECHANISM OF TRANSLATIONAL COUPLING BETWEEN THE E. COLI MOTILITY GENES motA AND motB

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The energy for flagellar rotation in most bacteria is provided by proton flow through the transmembrane channel formed by the MotA and MotB proteins. MotB in E. coli consists of 308 residues and comprises a short N-terminal cytoplasmic extension, a single transmembrane helix, and a large periplasmic domain. The latter contains a conserved sequence motif, extending from residue 210 to 228, that is found in many proteins that bind peptidoglycan and seems ideally suited to attach the Mot-protein complex to the cell wall so that it can act as the stator component of the motor. The ratio of MotA to MotB in the cell is 2:1 and MotB forms a dimer so the complex around the flagellar motor would be 4 MotA and 2 MotB. motA and motB are said to be translationally coupled due to the overlap of the start codon (AUG) of motB with the stop codon (UGA) of motA, with the resulting sequence being AUGA. Mutational analysis of motB expressed in trans to motA indicates that an apparent Shine-Dalgarno sequence upstream of motB, although only 2 bases away from the start of motB and created by residues E293 and E294 of MotA, acts as a ribosome binding site and that translation of motB in trans occurs via a de novo translational mechanism. However, utilizing alternate start codons in motA and motB and mutagenesis of the motB Shine-Dalgarno sequence in cis, analyzed by motility and β-galactosidase assays, we found that motB is translated almost exclusively by a reinitiation mechanism. The role of the ribosome in this case is not to merely open secondary structure around the start site of motB to allow for free ribosome binding, but to actually reinitiate at the motB start codon after it has completed translation of motA. In cis, there is a modest contribution by de novo translation from free ribosomes.
CHEMOTACTIC SIGNALING BY *E. COLI* TAR INVOLVES THE CHEMORECEPTOR C-TERMINUS

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The key control step in the chemotaxis of *Escherichia coli* is regulation of CheA kinase activity by a set of four transmembrane chemoreceptors. The receptor/CheA/CheW ternary complex can be viewed as an allosteric enzyme whose activity is regulated by the binding of attractant or repellent to the periplasmic domain of the receptor. Kinase activity is also modulated by reversible methylation of the receptor during the adaptation process. The extreme C-terminus of the aspartate chemoreceptor (Tar) is structurally dynamic and potentially accessible to two regions known to be important for receptor function: the HAMP linker and the adaptation sub-domain. The C-terminus of Tar has generally been regarded as a linker for the NWETF pentapeptide, which is the binding site for the CheR and CheB methylation/demethylation enzymes. However, the receptor C-terminus is also a potential contributor to transmembrane signaling. Here, we present a mutational analysis of the extreme C-terminus of Tar. The more residues that were deleted in this region, the less sensitive Tar became to inhibition by aspartate. Tar containing a deletion extending from residue 505 to the beginning of, or through, the NWETF sequence still stimulated CheA but failed to be inhibited by aspartate. Deletions starting with residue 513 increased the $K_i$ for aspartate 10 fold. Neutralization of any one of four basic residues in this region (K523A, R529A, R540A, R542A) increased CheA activation, whereas the R505A substitution decreased CheA stimulation by 40% and decreased the $K_i$ for aspartate up to 8 fold. The R505E substitution completely abolished the ability to stimulate CheA. These results implicate the receptor C-terminus in maintenance of baseline receptor activity and in attractant-induced transmembrane signaling. They also suggest how adaptive methylation might counteract the effects of attractant binding.
A STUDY OF THE ALLOSTERIC PROPERTIES OF ESCHERICHIA COLI AND SALMONELLA ENTERICA Tar

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Salmonella enterica and Escherichia coli Tar are homologous in sequence and in structural characteristics, but differ in their allosteric properties. E.coli Tar has higher affinity for aspartate and can only bind one aspartate molecule at a time while Salmonella Tar has a lower affinity for aspartate and can bind two aspartate molecules at higher concentrations of aspartate. This discrepancy in ligand binding affinities and cooperativity is puzzling since both receptors have the same exact amino acid residues bind aspartate. This indicates that it is a difference in allosteric properties of the receptors and not the binding pocket that is responsible for these differences. We hypothesize that the TM2 and the HAMP domains are the allosteric domains responsible for the differences in the allosteric properties between Salmonella and E.coli Tar. To test this hypothesis we are constructing different domain hybrids between E.coli and Salmonella Tar. The domain hybrids consist of E.coli Tar containing Salmonella TM2, HAMP and cytoplasmic domains; E.coli Tar containing Salmonella TM2 and HAMP domains; E.coli Tar containing Salmonella TM2 domain; and E.coli Tar containing Salmonella HAMP domain; E.coli Tar containing Salmonella cytoplasmic domain. Also, the same hybrids are made with Salmonella Tar as the base receptor with the allosteric domains swapped with E.coli.
AMMONIUM CHEMOTAXIS IN BACTERIA

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A diverse range of chemical compounds can act as chemical stimuli for bacterial cells including some sugars, amino acids, alcohols, inorganic ions, acids, bases and energy-linked chemicals such as oxygen and glycerol. *Escherichia coli* cells have just five chemotaxis-specific receptors, each of which therefore needs to respond to a variety of signals.

Chemotaxis towards ammonium has been previously reported in *E. coli* but understanding of the molecular mechanism of this process remains incomplete. The Tsr receptor has been implicated in the response to ammonium but it is not known whether this is the only chemoreceptor involved or exactly how ammonium is sensed. For example, some chemicals are detected via direct binding to the periplasmic sensing region of a methyl-accepting chemotaxis protein (MCP). Other stimuli are detected via the interaction of their periplasmic binding protein with an MCP and others still are sensed indirectly via their effects on the cell.

Our laboratory is interested in various aspects of nitrogen metabolism and its control with recent work focussing on the biology of the ubiquitous bacterial ammonia channel protein AmtB. We know that bacteria are able to respond to the availability of external ammonium in a variety of ways including the control of gene expression and enzyme activity. The observation that ammonium can also act as a chemoattractant provides a further example of the cellular response to this important nitrogen source.

This work aims to address the issues of precisely how ammonium is detected as a chemoattractant and whether this response relies on its transport and/or its metabolism. We have used the model organism *E. coli* to carry out a detailed investigation of the signalling pathway involved in the chemotactic response to ammonium. Responses have been quantified using a capillary assay. Using isogenic strains with defined deletions of each of the genes for the individual chemotaxis proteins we have demonstrated that the response to ammonium chloride is dependent on CheW, CheA and CheY. This suggests that a classical transmembrane chemoreceptor senses ammonium. Of the five known *E. coli* receptors two, Tsr and Tap, were found to influence ammonium chemotaxis. Studies using chimeric genes are in progress to determine which parts of these proteins act as ammonium sensors. In this context, ammonium chloride may potentially be perceived as a salt, as a base or as a nitrogen source and these alternatives are currently being explored.
R. SPHAEROIDES AND E. COLI: A COMPARISON OF POPULATION-LEVEL BEHAVIOR

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Understanding the population-level behavior of *Rhodobacter sphaeroides* is of importance not only in exploring how simple organisms can perform complex behavior, but also to elucidate its potential for bioremediation uses in the context of uranium reduction. We are interested in modeling the chemotactic behavior of *Rhodobacter sphaeroides* in an effort to identify why this species behaves differently from the better understood *Echerichia coli* in different environments. Specifically, we performed swarm-plate experiments, inoculating *R. sphaeroides* (WS8N and JPA1353ΔCheY) and *E. coli* (RP437 and RP5232ΔCheY) in agar plates with uniform concentrations of the chemoattractant L-aspartate. Separately we also measured growth rates for the bacteria in liquid cultures. From the data obtained, we examine the behavior seen via a set of partial differential equations known as the Keller-Segel equations. These equations allow us to differentiate growth effects from chemotaxis and point to sensitivities of *R. sphaeroides* as compared to *E. coli* in terms of their abilities to create and respond to gradients of chemoattractant and move within the agar media. Our models indicate that *R. sphaeroides*, despite its robust abilities to grow in varying concentrations of L-aspartate and to swim faster, does not respond to L-aspartate or move as strongly due to chemotaxis or diffusion as compared to *E. coli* in this particular agar media. This appears to be the result of differences in transport rates and abilities to swim in agar, though perhaps the chemotactic sensitivities to L-aspartate might also be a factor.
ISOLATION AND CHARACTERIZATION OF P1 ADHESIN, LEG PROTEIN OF GLIDING MOTILITY OF *MYCOPLASMA PNEUMONIAE*

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Mycoplasmas are parasitic bacteria that lack a peptidoglycan layer. *Mycoplasma pneumoniae*, causing human pneumonia, binds to a solid surface and glides at the maximum speed of 1 μm/s. *M. pneumoniae* has no homologs of genes related to known bacterial motility or conventional motor proteins, suggesting a unique motility mechanism. *M. pneumoniae* forms a membrane protrusion, so-called “the attachment organelle”. By the EM study of attachment organelle, two remarkable structures, “nap” and “rod” are observed. Nap is a spherical particle thickly clustering on the surface, and rod is an electron dense core 300 nm long in diameter. Some proteins involved in gliding have been identified, all of which are localized at the attachment organelle.

P1 adhesin, a 170-kDa protein localizes at the surface of attachment organelle and functions also as a “leg” of gliding motility. In this study, we isolated and directly characterized this protein. (1) The protein was isolated from mycoplasma cells through 4 purification steps. (2) Partial digestion analysis suggested that P1 is divided into two major domains. (3) The molecule has a spherical structure 17 nm in diameter under EM, suggesting that nap is composed of P1. (4) Analysis of amino acid sequence with P1 orthologs of 4 gliding mycoplasmas suggested that P1 has two highly conserved regions.
DegU, AN ORPHAN RESPONSE REGULATOR, CONTROLS CHEMOTAXIS, MOTILITY AND BIOFILM FORMATION IN *LISTERIA MONOCYTOGENES*

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The Gram-positive intracellular pathogen *Listeria monocytogenes* is endowed with seventeen sets of genes encoding two-component systems. *L. monocytogenes* is closely related to the Gram-positive model bacterium *Bacillus subtilis*, in which the DegS/DegU system plays a central role, controlling degradative enzyme synthesis, chemotaxis and motility, surfactin and polyketide biosynthesis as well as competence gene expression. Interestingly, although an orthologue of the DegU response regulator is present in *L. monocytogenes*, the gene encoding the cognate DegS kinase is absent. DegU negatively regulates its own synthesis in *L. monocytogenes*, in contrast to the situation in *B. subtilis*. DegU also plays a role in virulence of *L. monocytogenes*: in a murine intravenous infection model, an 11-fold increase in LD50 for the *degU* mutant was observed when compared to the parental strain.

We have shown that, in contrast to *B. subtilis*, the DegU response regulator is essential for bacterial motility and flagellar synthesis, and that this is due to the requirement of DegU for expression of several motility and chemotaxis genes, including the flaA and motAB genes which are expressed at 25°C but not at 37°C. Furthermore, expression of DegU is required for the formation of efficient biofilms by *Listeria monocytogenes* and adherence to plastic surfaces and this requirement appears to be independent from flagellar synthesis. We have inactivated the phosphorylation site of DegU *in vivo* and our results suggest a role for acetyl phosphate *in vivo* as a phosphodonor for DegU.
ROLE OF THE N-TERMINAL DISORDERED REGION IN SALMONELLA HOOK PROTEIN FlgE IN ITS EXPORT AND SELF-ASSEMBLY

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The bacterial flagellum is a motile organelle, which consists of three parts: the basal body, the hook, and the filament. The hook is a short, highly curved tubular structure made of the hook protein FlgE, connects the filament and the basal body, and functions as a universal joint to smoothly transmit torque produced by the flagellar motor to the filament.

Samatey et al. (2004) revealed the structure of FlgE missing the disordered regions of both termini by X-ray crystallography and proposed a model of the hook structure and the switching mechanism for a universal joint function. However, without these terminal regions, FlgE cannot properly polymerize into the hook either in vivo or in vitro. Thus, it still remains unknown at atomic level how FlgE self-assembles stably into the hook structure to act as a universal joint. Also, the disordered N-terminal region is found to be essential for recognition by the flagellar type III protein export apparatus. However, the signal recognized by the export apparatus is not known.

In this study, to investigate how the N-terminal disordered region of FlgE contributes to its recognition by export apparatus, to FlgE assembly and to the hook polymorphism in more detail, we constructed scanning N-terminal deletion variants of FlgE and tested their complementation ability. We examined their secretion ability, swimming behavior and hook morphology. From the results obtained, the role of the N-terminal region of FlgE in the export and self-assembly will be discussed.
EFFECT OF INTRACELLULAR pH ON FLAGELLAR MOTOR ROTATION OF *SALMONELLA* SLOW MOTILE MUTANTS

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The flagellar motor of *Salmonella* and *Escherichia coli* is a rotary nanomachine driven by the proton motive force, which is a sum of the transmembrane proton concentration gradient and the membrane potential. It has been shown that an increase in the intracellular proton concentration abolishes flagellar motor function, suggesting that the stator complex has an intracellular proton-binding site where cytoplasmic proton kinetically interferes with the motor rotation. Thus, the absolute concentration of protons in the cytoplasm is likely to be one of the factors that determine the rotation rate of the motor.

MotA and MotB form a complex consisting of four copies of MotA and two copies of MotB and acts as the stator that conducts protons and couples proton flow to motor rotation. Both protonation and deprotonation of Asp33 of MotB cause conformational changes of the cytoplasmic loop of MotA, which may drive flagellar motor rotation. Two proline residues conserved in MotA (Pro173 and Pro222) are postulated to be significant for the conformational change of the stator complex that may facilitate proton translocation. However, the molecular mechanism of energy coupling between proton influx and flagellar motor rotation still remains largely unknown.

In this study, to investigate how proton dissociation from the stator to the cytoplasm drives flagellar motor rotation, we measured flagellar motor rotation of *Salmonella* wild-type strain by bead assays at external pH over a range from 5.5 to 8.0 with a 20 mM sodium benzoate buffer. When the external pH was shifted from 7.0 to 5.5, the rotation speed of wild-type flagellar motor sharply decreased. We are currently measuring the rotation speed of a slow motile *motA*(P173A) mutant. We will discuss the effect of intracellular pH on the torque generation process.
The bacterial flagellum has a type III protein export apparatus as part of its basal body structure. It selectively translocates flagellar axial proteins into the central channel of the flagellum for their self-assembly at the distal, growing end of the filament. The mechanism of export is not understood except that the energy released by ATP hydrolysis by FliI is used for the export process.

FlhA is a component of the export apparatus. It consists of an N-terminal transmembrane domain (FlhA\textsubscript{TM}, MW: 34.5 kDa) and a C-terminal cytoplasmic domain (FlhA\textsubscript{C}, MW: 40.5 kDa). We previously reported a 2.8Å crystal structure of FlhA\textsubscript{C}, which is composed of an elongated linker region continued from FlhA\textsubscript{TM} and four globular subdomains (A\textsubscript{C}D1, A\textsubscript{C}D2, A\textsubscript{C}D3 and A\textsubscript{C}D4). Since this domain structure suggested that FlhA\textsubscript{C} could go through a relatively large conformational change, we have carried out molecular dynamics simulation. The simulation shows that FlhA\textsubscript{C} periodically repeats conformational changes between an open and a closed state through two hinges, one between A\textsubscript{C}D1 and A\textsubscript{C}D3, and the other between A\textsubscript{C}D3 and A\textsubscript{C}D4. Point mutations in key residues involved in the interactions between the subdomains failed to restore the motility of a flhA-null mutant. These results suggest that the large conformational fluctuation observed, on the order of ~ 10 nano seconds, is important in the function of FlhA\textsubscript{C}. The crystal structure, together with the result of multicopy effects of the N-terminal half of FlhA\textsubscript{C} on the motility of wild-type cell and previous studies, suggest that the linker region and A\textsubscript{C}D1 hold soluble components of the export apparatus, probably FliI, the ATPase.
The bacterial flagellum, a motile organelle made of a rotary motor and a helical propeller, is composed of almost 30 different proteins. For flagellar self-assembly, the axial component proteins are transported through the central channel of the growing flagellum to the distal end where they polymerize. The flagellar type III protein export apparatus composed of six transmembrane and three soluble proteins mediates protein export driven by the energy of ATP hydrolysis. FlhA (75 kDa) is the largest membrane component with a large cytoplasmic domain, FlhAc, which interacts with soluble components including the ATPase FliI. The ATPase regulator FliH is required for the efficient FlhAc-FliI interaction. The crystal structure of FlhAc shows a four-domain structure (Saijo-Hamano et al, unpublished). We isolated temperature sensitive mutants (ts-FlhA) with a mutation in domain D1 and six suppressor mutants (sts-FlhAc). In our previous studies, we characterized the difference in the thermal stability between ts-FlhAc and sts-FlhAc, which clearly indicated that the loss of function is due to unfolding. We have now carried out limited proteolysis at 30 degree and 42 degree and found that these mutants are degraded into small fragment quickly at both temperatures and domain D1 is relatively unstable. We have also isolated FlhA(V404M) as a suppressor for FliH null mutation and solved the crystal structure. On the basis of structural comparison with the wild-type FlhAc, we will discuss how the FlhAc-FliI affinity is increased by this mutation.
ROTATION MEASUREMENT OF THE BACTERIAL PROTON-DRIVEN FLAGELLAR MOTOR USING 40 nm FLUORESCENT BEADS

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Bacteria such as *Escherichia coli* and *Salmonella* can swim in liquid environments by rotating their flagella, each of which consists of a thin helical filament attached via a universal joint called the hook to the basal body, which as a rotary motor. The flagellar motor embedded in the cell surface transforms the protonmotive force, an inward-directed electrochemical potential gradient of protons across the cytoplasmic membrane, into torque.

The molecular mechanism of torque generation remains unknown. Since the filament is very long and flexible, it is difficult to observe the dynamic behavior of the flagellar motor itself at high resolution by observing and measuring the filament rotation. We have set up an optical nanophotometry system capable of determining the two-dimensional position of a 40 nm fluorescent bead attached to the motor at sub-millisecond temporal resolution. A 40 nm fluorescent bead was attached to the straight hook of a filament-less flagellar motor of *Salmonella* cells so that the dynamic behavior of the motor under a low-load condition can be probed at high fidelity. The angular velocity measured at high temporal resolution showed relatively large fluctuations while the average speed of motor rotation did not change much from revolution to revolution. However, we have not been able to find steps in rotation even at low speed rotation, unlike the recent observation of step-wise rotation of a sodium-ion driven hybrid motor of *E. coli* (Sowa et al., 2005).

In this study, we have improved the nanophotometry system by implementing a more stable stage and a dye-laser illumination system so that the system is capable of determining the position of a 40 nm fluorescent bead at a spatial resolution of 1 nm and a temporal resolution of 0.2 millisecond. Based on estimation from the radius of the circular trajectory of the bead, this system should be able to visualize step motions of 7-8 nm and even sub-steps of 3-4nm of motor rotation if present.
ATPASES OF THE TYPE III PROTEIN EXPORT SYSTEMS

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The type III protein export systems are widely used by Gram-negative bacteria. The flagellar protein export apparatus is required for flagellar assembly and motility, and the virulence-associated type III secretion systems (T3SSs) deliver virulence protein factors into eukaryotic host cells. In each apparatus, a multi-ring anchors the apparatus to the bacterial envelope, and a tube-like extracellular structure facilitates the translocation of the secreted proteins from the bacterial cytosol. An ATPase that is believed to be in close association with the basal body drives the protein translocation.

The specific ATPases of the flagellar protein export apparatus and T3SSs of \textit{Salmonella} are FliI and InvC, respectively. These ATPases show a significant primary sequence similarity with the catalytic $\beta$ subunit of F\textsubscript{0}F\textsubscript{1}-ATPases. FliI and InvC form hexagonal ring-shaped structures with a central channel as other Walker type ATPases do. Although the structure and the central role of FliI and InvC are alike, little is known about their enzymatic properties.

In this meeting, we will report enzymatic properties of wild-type FliI, an interesting mutant FliI we found recently. We will also compare enzymatic parameters of FliI and InvC.
INTENSIVE RAMAN OPTICAL ACTIVITY OF L-TYPE STRAIGHT FLAGELLAR FILAMENTS OF SALMONELLA

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The flagellar filament of Salmonella is a polymer of a single protein, flagellin. There are different helical and straight shape filaments from wild type and mutants. The difference between two types of straight filaments, L- and R-type straight, is the inclination of the flagellin monomer arrangements. Since Raman and Raman Optical Activity (ROA) spectroscopies are powerful techniques for studying the solution structure of biomacromolecules, these methods were employed for the structural characterization of L- and R-type straight and normal helical flagellar filaments.

We previously reported that the intensive ROA signals were obtained only from the L-type filaments and that these signals were disappeared and reduced upon depolymerization and shorting of the filaments respectively. On the other hand, there were some signals observed even in the R-type and normal filaments at higher concentration. From Raman spectra of the different shapes of flagellar filaments and monomers, there were differences between polymer and monomer of flagellin.

In this meeting, we report that the signals were disappeared when the L-type filaments were shortened further. Furthermore, the intensive signals of the L-type filaments were not observed at low concentration. Effects of concentration and length of the flagellar filaments on the ROA signals will be discussed.
ISOLATION AND CHARACTERIZATION OF VARIOUS SHAPE FLAGELLAR FILAMENT MUTANTS AND THOSE PSEUDOREVERTANTS OF *SALMONELLA*

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A cell of *Salmonella typhimurium* swims by rotating its flagellar filaments. A wild-type cell carries a left-handed helical filament that is called a normal filament. On the other hand, various mutants carrying different helical shapes were isolated. There are curly, coiled, semi-coiled and two kinds, L- and R-type, of straight filaments known. Transformations of filament helical shape among normal, semi-coiled and curly were observed during tumbling of the cell. Structure of R-type straight flagellar filament has been described, but no other shape was reported. To investigate mechanism of transformation and structure of other flagellar filaments, we isolated mutants with different shapes of flagellar filaments and their revertants which recovered their swarming abilities. From sequencing analysis of original mutations for straight shape mutants, there are several mutation sites which have never been reported. From them, there were about 100 revertants isolated. Some of them are true revertants having the original amino acid residue at mutation site, but others are pseudorevertants carrying another mutations. Among them, there are second site mutations which are known as carrying curly or straight type flagellum. We will discuss about shape of mutant flagellar filaments and distribution of original and second site mutations.
AN INVESTIGATION OF THE SOUBLE CHEMORECEPTOR HlyB FROM HELICOBACTER PYLORI

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The pathogenic bacterium Helicobacter pylori lives in the stomach lining of over half the world’s population. In most cases, infection is asymptomatic or causes very mild gastritis. However, a significant proportion of infected people develop severe gastritis, ulcers, and stomach cancer. The mechanism by which H. pylori infects its human hosts are unknown, as are the factors which determine the severity of infection. One known factor in the pathogenicity of H. pylori is its ability to move via a polar cluster of flagella. The chemotaxis system in H. pylori is still poorly understood. It has the classic CheW (receptor-kinase coupling), CheA (kinase), and CheY (response regulator) chemotaxis signal transduction system, as well as a remote homolog of the CheY-phosphatase CheZ. However, H. pylori appears to lack the CheR (methylase) and CheB (demethylase) proteins involved in signal adaptation in E. coli, but has three extra CheW-CheY hybrid proteins called CheVs. The H. pylori genome contains four chemoreceptors: three traditional membrane-bound receptors (TlpA, TlpB, and TlpC), and one soluble receptor (HlyB, also known as HylB and TlpD). One of the membrane-bound receptors, TlpB, has been shown to sense pH, triggering movement away from acidic conditions. What the other 3 chemoreceptors sense is still unknown.

Our group is investigating the protein HlyB, H. pylori’s soluble chemoreceptor. We have found that the C-terminal portion of this protein contains a motif present in many putative proteins, including many chemoreceptors. The function of this motif, however, is unknown. Few soluble chemoreceptors have been characterized, and little is known about their function and localization within the cell. We have generated knockout mutants of this gene in H. pylori, and are analyzing the mutants in several chemotaxis assays. We have also performed a cell-fractionation experiment, and via Western Blotting of the cell fractions have demonstrated that HlyB is found both free within the cytoplasm and associated with the membrane. This membrane association is demolished in the absence of CheA or the other chemoreceptors, and reduced in the absence of CheW. We have also found that the membrane-bound and cytoplasmic populations of HlyB run at slightly different sizes on an SDS-PAGE gel, indicating that there may be some modification of this protein between these two regions of the cell. Our results thus suggest that H. pylori’s cytoplasmic chemoreceptor is localized to the membrane via its interactions with other chemotaxis proteins.
Tap mediates chemotaxis to pyrimidines in Escherichia coli

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In Escherichia coli, chemotaxis is accomplished via signal transmission between receptor complexes and flagellar-motor complexes. The signal arises when a chemoafferctor or its periplasmic protein complex binds to a transmembrane methyl-accepting chemotaxis protein (MCP). MCPs have an N-terminal periplasmic ligand-binding domain and a conserved C-terminal signaling domain, which sends the signal to downstream chemotaxis proteins. There are five MCPs in E. coli, with Aer mediating aerotaxis and the other four MCPs (Tar, Tsr, Trg and Tap) mediating chemotaxis to sugars, amino acids, and dipeptides. We showed for the first time that E. coli RP437 is attracted to the pyrimidines thymine, uracil, and cytosine, which it can use as sole nitrogen sources. The chemotactic response to pyrimidines was constitutively expressed under all tested growth conditions. To identify the chemoreceptor that mediates pyrimidine taxis, MCP knockout mutants were tested in chemotaxis assays. Tap− mutants showed no response to any of the pyrimidines, while all the other single MCP knockout mutants were still attracted to pyrimidines. These results suggest that Tap, which is known to be involved in dipeptide chemotaxis, is also required for chemotaxis to pyrimidines. Tap was expressed from a multicopy plasmid in an E. coli strain lacking all five of the native MCPs and chemotaxis of the strain was tested. The strain with Tap as the only MCP was only very weakly chemotactic to pyrimidines and dipeptides, most likely due to the absence of high-abundance chemoreceptors. Chimeric chemoreceptors (Tapsr and Tsrap) were constructed by switching periplasmic and cytoplasmic domains of Tap and Tsr. Tapsr and Tsrap were individually expressed in the MCP− strain and chemotaxis of these strains was analyzed. Tapsr was able to mediate chemotaxis towards pyrimidines and the dipeptide Pro-Leu, while the reciprocal chimeric receptor Tsrap was unable to function. Our results indicate that the periplasmic domain of Tap is responsible for detecting pyrimidines, and the Tsr signaling domain is sufficient to confer Tap with the ability to mediate efficient chemotaxis.
DELETION AND SUPPRESSION ANALYSIS OF THE Aer F1 DOMAIN

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The Aer transducer guides *E. coli* to optimal oxygen environments. The Aer molecule has an MCP-like signaling domain, but a unique N-terminal organization that includes a cytoplasmic PAS-domain (residues 1-120) and a membrane-embedded segment (residues 168-205). The F1 region (residues 121-167) connects the PAS and membrane anchor domains, but its function is enigmatic. F1 sequences are not conserved among Aer homologs, although they tend to be devoid of acidic residues and rich in hydrophobic and basic amino acids. Moreover, F1 function is relatively insensitive to single amino acid replacements, suggesting that it has a relatively sequence-independent role. To further explore F1 function, we constructed and characterized a series of 7-residue deletions, spaced every three residues across the F1 region of Aer.

All F1 deletions abrogated Aer function and reduced Aer steady-state expression levels to ~10% of the wild-type. However, a PAS mutation (S28G) known to counter similar instabilities in many other mutant Aer proteins restored function to some of the F1 deletion mutants. Deletions located in F1 residues 146-160 regained Aer function at both 24°C and 30°C; those located in an adjacent segment (residues 132-145) regained function at 24°C, but not at 30°C. The S28G mutation increased the steady-state level of most F1 deletion proteins about 3-fold, but this effect was not strictly correlated with restoration of function. Deletions at both ends of the F1 segment (residues 129-135 and residues 157-166) were not functionally suppressed by S28G, despite comparable increases in the steady-state levels of the deletion proteins.

The S28G suppressor appears to have two modes of action when combined with F1 deletions. It reduces proteolysis of all nascent F1-deletion molecules and it enables some of the mature mutant molecules to function. Interestingly, the F1 deletions in segment 132-145, regain function at 30°C in the presence of a high-abundance MCP, either Tsr or Tar. MCP-dependent functional rescue of F1 deletion mutants probably involves formation of mixed trimers of dimers, which could further stabilize the mutant Aer dimers or somehow facilitate their ability to generate oxygen-dependent signals.

These results define three functionally distinct segments in the F1 region. Residues 121-131, play a role both in Aer maturation and in the function of the mature Aer protein. This segment adjoins the PAS domain and may interact with it structurally. Similarly, residues 161-166 seem to play a role in both the maturation and function of Aer molecules, because their defects do not respond to the S28G suppressor. The central portion of F1, residues 132-160, is evidently not critical for aerosensing and output control in mature Aer molecules, but rather seems to play a role in Aer synthesis and maturation because lesions in this segment make Aer molecules susceptible to proteolytic degradation, but suppressible by S28G.
INPUT/OUTPUT CONTROL LOGIC IN THE AEROTAXIS TRANSDUCER Aer

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The *Escherichia coli* Aer protein, which mediates aerotactic behavior, has a cytoplasmic PAS domain at its N-terminus that contains an FAD prosthetic group for sensing oxygen-related redox changes. PAS signals in turn regulate the C-terminal MCP-like signaling domain of Aer to control the flagellar motors, but the route and mechanism of input/output control in the Aer molecule are not well understood. We investigated these issues by constructing and characterizing an Aer molecule (AerΔ[1-111]) deleted for the PAS domain. We found that:

1. The steady-state expression level of AerΔ[1-111] was 20-30% of wild-type Aer, reflecting enhanced proteolytic destruction during synthesis. Moreover, about 80% of the completed AerΔ[1-111] subunits were also rapidly degraded, suggesting that the PAS domain plays an important role in maturation of the Aer molecule. The cellular content of mature, membrane-anchored (i.e., stable) AerΔ[1-111] subunits could be raised to wild-type levels by increasing their rate of synthesis to offset the higher turnover rate.

2. Cells containing wild-type levels of AerΔ[1-111] exhibited no episodes of CW flagellar rotation, suggesting that the Aer signaling domain normally requires PAS input to activate the CheA kinase. Mutational changes in the Aer signaling domain or adjoining HAMP domain that cause CW-biased output in full-length Aer molecules had no effect on AerΔ[1-111] output. However, AerΔ[1-111] chimeras containing a heterologous MCP signaling domain (from Tsr or Tar) with an extreme modification state (QQQQ) were able to generate CW output signals, demonstrating that the Aer signaling domain has intrinsically low CW-signaling potential.

3. The junction of the HAMP and proximal signaling domains seems the most likely target for CW PAS input signals, but a residue-by-residue mutational analysis of this region [L251-I257] failed to reveal residues that were especially critical for Aer function. Conceivably, PAS could control Aer output state through a non-specific structural interaction, for example, one that encloses the HAMP domain to constrain its range of dynamic motion.

4. In complementation tests, AerΔ[1-111] subunits abrogated the function of wild-type Aer, suggesting that AerΔ[1-111]/Aer-WT heterodimers are nonfunctional. Thus, Aer input/output signaling might involve collaboration between the PAS domains of an Aer dimer.

We conclude that aerotactic stimuli control Aer signal output by modulating the intensity of a PAS-dependent CW input signal. The control target for that PAS signal remains to be identified.
IDENTIFICATION AND CHARACTERIZATION OF RapA RESIDUES INVOLVED IN SPECIFIC INTERACTION WITH ITS SUBSTRATE Spo0F~P OR ITS INHIBITOR PhrA IN BACILLUS SUBTILIS

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The Rap family of proteins in Bacillus subtilis includes 11 members among which the RapA, RapB and RapE are known to target the sporulation phosphorelay by dephosphorylating the intermediate response regulator Spo0F~P. Phosphatase activity of these Rap proteins is generally regulated at two levels: by transcriptional regulatory control of protein synthesis and by inhibition of their activity by specific pentapeptides generated from a precursor encoded by the phosphatase regulator phr genes after a complex export-import maturation pathway. Rap proteins are structurally characterized by six tetratricopeptide repeats (TPR). TPRs are structural domains of 34 amino acids that form a pair of antiparallel \( \alpha \)-helices generally occurring in tandem arrays, with a packing angle of 24° between the two helices of each TPR. Multiple TPR domains stack on top of each other generating a spiral superstructure of antiparallel \( \alpha \)-helices. The pattern of sequence conservation imposed by the amphipathic \( \alpha \)-helices of a TPR mediates interactions both within the TPR and between adjacent pairs, in addition to providing residues for interaction with other proteins.

In order to find residues involved in specific interaction between RapA and its substrate Spo0F~P and its inhibitor PhrA we carried out a random mutagenesis study on the whole protein as well as site-directed mutagenesis on residues postulated to be in an inner surface possibly interacting with Spo0F and/or PhrA. Residues were identified that, in \textit{in vivo} and \textit{in vitro} analyses, affected RapA interaction with either Spo0~P or the PhrA pentapeptide. This allowed us to define two possible surfaces involved in protein::protein or protein::peptide interaction within RapA.
BISON: BIO-INTERFACE FOR THE SEMI-GLOBAL ANALYSIS OF NETWORK PATTERNS

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The investigation of gene regulation involves an understanding of the regulatory network as well as protein function. We developed a tool that evaluates patterns based on extensive network and annotation information. This tool provides a bridge between current strategies of either investigating a small number of interactions between regulatory proteins and regulated genes, or using high-throughput approaches in combination with computational techniques that address large networks. The tool can be used for a wide variety of applications in biology, medicine, or agriculture. It is global in its design, yet specific with respect to the questions that a biology user may want to ask.

Our tool uses our own data mining engine, integrated into current visualization and navigation techniques. It was developed in the Java 5.0 programming language. The tool is divided into a graphical visualization part and a text database interface for analysis and search and takes input provided by our own external Perl library for frequent pattern mining. Network data were obtained from public databases, as well as experiments of our own and other researchers (1-4). The total number of interactions is 6,227 between 186 regulators and 1,934 targets. Annotation data were obtained from the E. coli Genome project, Pfam and our own annotations obtained with HMMER hidden Markov model software (5-7). All input data are collected in a single data directory. We named the tool BISON (Bio-Interface for the Semi-global analysis Of Network patterns).

The functionality of BISON will be discussed in the context of the transcriptional network of regulation in the enteric bacterium Escherichia coli. Examples will be provided for the kind of questions that can be answered with BISON. Screen shots will guide the reader through the two analyses that BISON provides, gene-centered and pattern-centered analysis.

References

PUNCHING HOLES IN *E. COLI*

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We are developing an in vitro system to study the bacterial flagellar motor. Tightly-focused femtosecond laser pulses are used to vaporize a submicrometer-sized hole in the wall of filamentous *Escherichia coli*. Since all three layers of the wall are damaged, the hole should be stable and not reseal. If we then placed a punctured cell across a membrane dividing two volumes, one side will correspond to the exterior of the cell and the other side (where the bacterium is pierced) will correspond to the interior. We are working on experimentally realizing this by partly introducing a filamentous bacterium inside a micropipette. Focusing our laser inside the micropipette should allow us to pierce the bacterium, thereby granting us access to the inside of the cell and the control over the proton-motive force. Having a working flagellar motor whose rotation speed can be monitored outside the micropipette will then provide an ideal in vitro assay to study the motor’s physical and chemical characteristics. Encouraging preliminary results will be presented. This work was started by SR as a postdoctoral fellow in Howard Berg’s laboratory (in collaboration with Aravinthan Samuel) and is now continuing at Laval University.
ROLE OF CHEMORECEPTOR PROTEINS IN *SINORHIZOBIUM MELILOTI* CHEMOTAXIS AND SYMBIOSIS

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The genome of the symbiotic soil bacterium *Sinorhizobium meliloti* contains eight *mcp* genes (*mcpS-Z*) and one transducer-like gene (*icpA*) to sense the concentration of attractants and to transmit this information to the flagellar motor. Seven of the MCP proteins are localized in the cytoplasmic membrane via two membrane spanning domains, whereas McpY and IcpA are distinguished by their cytoplasmic localization. Using GFP as a reporter we show the formation of chemoreceptor clusters at one pole of the cell. The polar localization of all transmembrane receptors and of the cytosolic McpY requires the presence of heterologous MCP proteins, CheW and CheA. In contrast, cytosolic IcpA clusters localized independent on other chemotaxis components at the pole of the cell.

Deletion of any one of the receptors caused impairments in the chemotactic response towards most organic acids, amino acids and sugars in a swarm plate assay. The data imply that chemoreceptor proteins in *S. meliloti* can sense more than one class of carbon source and suggest that many or all receptors work as an ensemble. Tactic responses were virtually eliminated for a strain lacking all nine receptor genes. Receptor deletions variously affected free swimming speed and attractant-induced chemokinesis. Noticeably, cells lacking *mcpU* swam 9% slower than the wild type. As consequence and unlike *E. coli* MCPs, kinase activity of CheA is inhibited in the presence of McpU causing an increase in swimming speed. Cells lacking one of the two soluble receptors were impaired in chemokinetic proficiency by more than 50%. We propose that the internal sensors IcpA and McpY monitor the metabolic state of *S. meliloti*. *S. meliloti* induces nodule formation in the rootlets of its host plant *Medicago sativa* for nitrogen fixation. The symbiosis between bacteria and host plant is established in a sequence of events, initiated by bacterial adhesion to plant root hairs. To ensure a successful symbiotic interaction, bacteria are guided to the rhizosphere by a multitude of compounds released into the soil by plants. Among these secreted substances are numerous carbon sources which are utilized by soil microorganisms, as well as a number of different signaling substances such as the *nod* gene inducer, luteolin. *S. meliloti* wild-type cells exhibit a strong chemotactic reaction towards seed and root exudates from alfalfa, whereas the response of a strain lacking all nine receptor genes is strongly diminished. The contribution of individual receptors to symbiosis was tested in a competition assay. When alfalfa seedlings were co-inoculated with *S. meliloti* wild-type and chemoreceptor mutant cells, most individual receptor knock-out strains were outcompeted by their wild-type parent. These results indicate, that chemotaxis is an important trait for the symbiotic fitness in this plant symbiont.
DIRECT INTERACTIONS BETWEEN AN UNUSUAL CheW PROTEIN AND THE DYNAMICALLY LOCALIZED RESPONSE REGULATOR RomR IN MYXOCOCCUS XANTHUS

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In *Myxococcus xanthus* morphogenetic cell movements constitute the basis for the formation of spreading vegetative colonies and fruiting bodies in starving cells. *M. xanthus* moves by gliding using two different engines: At the leading pole, type IV pili pull the rod shaped cell forward. At the lagging pole, nozzle like structures have been suggested to push the cell forward by slime secretion, resulting in the so-called adventurous motility (A-motility). At regular intervals cells reverse their direction of movement. Regulation of the reversal frequency is essential for the formation of spreading colonies as well as fruiting bodies. The molecular mechanism underlying a cell reversal involves the rearrangement of the gliding engines from pole to pole. The RomR protein, which is a response regulator with a C-terminal output domain consisting of a proline-rich region and a charged tail, is required for A-motility and fruiting body formation. RomR localizes in an asymmetric bipolar pattern with a large cluster localizing to the lagging cell pole. In parallel with a cellular reversal, the large RomR cluster switches to the opposite pole suggesting that RomR marks at which pole the A-engine is active. The dynamic RomR localization depends on the Frz chemosensory signal transduction system. Here, we have focused on the identification of proteins that interact with RomR.

RomR homologs were found in several but not all δ-proteobacteria. In all the species where *romR* is conserved, it is flanked by a gene encoding a protein containing two CheW domains (*romA*) suggesting that RomA and RomR are involved in the same process. Homology models of the two CheW domains in RomA showed that both domains contain two SH3-like domains, which interact with proline-rich targets. Inactivation of *romA* does not interfere with gliding motility whereas fruiting body formation and sporulation is severely compromised. We demonstrate a direct interaction between RomA and RomR by *in vitro* cross-linking and in the yeast two-hybrid technology. Using the latter we have narrowed down functionality and interaction specificity to defined protein domains.

During fruiting body formation the intercellular C-signal induces a decrease in the cellular reversal frequency by inhibiting the activity of the Frz system. We hypothesized that the C-signal by modulating the activity of the Frz system regulates localization of RomR and that this regulation would depend on RomA. We report the first data on the connection between the C-signal and RomR localization and its dependence on RomA.
FORCE GENERATION BY DEPOLYMERIZATION: THE FUNCTION OF PilB AND PilT IN TYPE IV PILI FUNCTION IN *MYXOCOCCUS XANTHUS*

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Type IV pili (T4P) are bacterial appendages responsible for a type of locomotion referred to as gliding motility or twitching motility. Generation of motive force by T4P depends on a cycle of events including polymerization/assembly of pili, attachment to a surface and retraction by depolymerization. Of these events, only retraction generates motive force. *Myxococcus xanthus* moves by gliding and has two distinct motility systems. The A-system is likely powered by slime extrusion and allows the movement of single cells. The S-system depends on T4P and is only functional when cells are within contact-distance of each other. In *M. xanthus* T4P are located at one cell pole at a time. Periodically, cells reverse their direction of gliding, presumably by switching the poles at which the two gliding systems are active. Thus, in *M. xanthus* T4P are regulated at two levels: Polymerization/depolymerisation and polar localization. The mechanisms of assembly and disassembly of T4P remain unknown. Likewise, the mechanism involved in regulating at which pole T4P are made remains unknown. Here, we investigate the function of the PilB and PilT proteins of *M. xanthus* in T4P function and in the polarity switching mechanism.

PilB is essential for pilus assembly and PilT for pilus retraction. The sequences of PilT and PilB are similar to proteins of the AAA+ family of ATPases suggesting that PilB and PilT could energize T4P assembly and disassembly. To test this hypothesis we substituted conserved residues in the Walker A and Walker B boxes of PilB and PilT. The mutant proteins were not able to restore T4P dependent motility in ΔpilB or ΔpilT mutants. Transmission electron microscopy revealed that Walker A and Walker B mutants of PilB are not piliated whereas the Walker A and Walker B mutants of PilT mutants are piliated. PilB and PilT proteins purified from *E. coli* showed ATPase activity *in vitro*. Cell fractionation experiments showed that PilB and PilT are localized to the cytoplasm. To address the spatial localization of PilB and PilT, GFP fusions to PilB and PilT were constructed. GFP-PilB showed predominantly unipolar localization whereas GFP-PilT showed a mixture of unipolar and bi-polar localization. T4P co-localize with unipolarly localized GFP-PilB and GFP-PilT.

Based on our findings we suggest that PilB and PilT are ATPases and that ATPase activity of PilB is necessary for assembly of T4P and retraction depends on the ATPase activity of PilT. In addition, our data suggest that polarity switching of T4P in *M. xanthus* involves the pole-to-pole transfer of PilB and PilT.
A SYSTEMS BIOLOGY APPROACH IN THE ANALYSIS OF DEVELOPMENTALLY REGULATED ORPHAN HISTIDINE PROTEIN KINASES IN *MYXOCOCCUS XANTHUS*

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Under starvation conditions, *Myxococcus xanthus* initiates a developmental program that culminates in the formation of spore-filled fruiting bodies. Fruiting body morphogenesis depends on the temporal and spatial coordination of two morphogenetic processes, aggregation of cells into fruiting bodies and sporulation of cells inside the fruiting bodies. This coordination needs to integrate sensory input from inter- and intracellular signalling pathways. Two component systems (TCS) are critical in these signalling pathways. The genetic organization of TCS genes in *M. xanthus* is highly unusual with 58% of the 142 histidine protein kinase genes and 51% of the 130 response regulator genes being orphans. A large fraction of the TCS proteins involved in fruiting body formation is encoded by orphan genes. This suggests that TCS involved in fruiting body formation are organized in signalling networks with an unprecedented level of complexity.

FruA is an orphan response regulator that has a pivotal role in the C signal transduction pathway. Transcription of *fruA* is induced during development and genetic evidence suggests that the activity of FruA is controlled by phosphorylation. However, the cognate histidine protein kinase(s) of FruA remains unidentified. To identify the cognate histidine protein kinase(s) of FruA, we carried out a systematic analysis of histidine protein kinase genes that share key characteristics with the *fruA* gene, i.e. they are orphan, developmentally regulated at the transcriptional level and deficient in development in a null mutant. Moreover, yeast two-hybrid analysis was preformed to investigate the potential interaction between FruA and these developmentally regulated orphan histidine protein kinases. Global gene expression profiling experiments using DNA microarrays and quantitative real time PCR revealed that 28 out of the 83 orphan histidine protein kinase genes are developmentally regulated at the transcriptional level. Four of these kinases (*sdeK, espA, espC* and *mokA*) have previously been characterized and inactivation of these genes causes developmental defects. So far, in frame deletions have been created in thirteen out of remaining 24 histidine protein kinase genes. Ten of these mutants have defects in fruiting body formation. To date, yeast two-hybrid analysis have suggested interactions between five of these kinases and FruA. Based on our current data, we propose that FruA may have more than one cognate kinase and that these kinases may activate FruA by phosphorylation simultaneously or individually over time.
ACTIVATION OF A CONTACT-DEPENDENT INTERCELLULAR SIGNALLING SYSTEM BY
REGULATED PROTEOLYSIS

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In response to starvation, *M. xanthus* initiates a developmental program that culminates in the formation of the multicellular, spore-filled fruiting bodies. During this development program, proteases have essential functions in the synthesis of the extracellular A- and C-signals, which are required for fruiting body morphogenesis. The cell surface-associated C-signal becomes important for fruiting body formation after 6 hrs of starvation and induces multiple responses including aggregation of cells into fruiting bodies, sporulation and expression of developmentally regulated genes that are induced at 6 hrs or later. Here, we have focused on synthesis of the C-signal.

Synthesis of the C-signal depends on the *csgA* gene. *csgA* encodes two proteins: a 25 kDa species, which corresponds to full-length CsgA protein (p25) and a 17 kDa protein (p17). We have previously shown that the C-signal is identical to p17 and that p17 corresponds to the C-terminal 17 kDa of p25. Moreover, we have provided biochemical evidence that p17 is produced by proteolytic cleavage of p25. Proteolytic cleavage of p25 depends on a serine protease (PopC for protease of C-signal precursor), which is developmentally regulated. Based on the observation that p25 as well as p17 are associated with the outer membrane, we speculate that PopC is likely to be secreted. To understand how synthesis of p17 is regulated, we have focused on the identification of the *popC* gene.

Based on the above-mentioned information, we developed a three-tiered strategy to identify *popC* candidates. First, 146 genes likely to encode protease in the *M. xanthus* genome were identified. Second, among these genes 32 likely to encode secreted serine proteases (including 10 trypsin-like proteases, 10 subtilisin-like proteases, eight HtrA homologs and four homologs of Rhomboid proteases) were identified. Based on the biochemical characteristics of these four protease families, the HtrA and Rhomboid homologs were unlikely to be identical to PopC. Finally, DNA microarray expression data from developing cells showed that two of the subtilisin-like proteases were up-regulated during development. To test whether any of these two proteases could be identical to PopC, we inactivated the two corresponding genes by insertion mutagenesis. Inactivation of *MXAN0206* results in developmental defects and reduced accumulation of p17 *in vivo*. Moreover, total cell extract from the MXAN0206 mutant displays strongly reduced levels of p25 cleavage *in vitro*. *MXAN0206* encodes a 51 kDa protein including a C-terminal subtilase-like protease domain with the characteristic catalytic triad of subtilases. An active site mutant of *MXAN0206* displays the same developmental defects as a mutant with an insertion in *MXAN0206*. MXAN0206 accumulates in vegetative cells; however, MXAN0206 is only detected in the medium in starving cells. Our data strongly suggest that *MXAN0206* encodes PopC and that regulation of MXAN0206/PopC secretion represents a crucial step in the regulation of MXAN0206/PopC activity.
GENE EXPRESSION NOISE IN THE E.COLI CHEMOTAXIS PATHWAY

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Networks of interacting bio-molecules have to function in a noisy environment using imperfect components. Particularly in gene regulation and signal transduction, one expects a strong selective pressure towards networks being intrinsically robust against perturbations such as fluctuations in protein concentration, also called gene expression noise. We used E.coli chemotaxis, which has one of the best-studied simple signalling systems, as a model for a quantitative analysis of gene expression noise and studied the origins of this individuality and the robustness of the E.coli chemotaxis signaling network to such noise. Hierarchical regulation of transcription of receptor and chemotaxis genes is relatively well understood. To analyze the propagation of the gene expression noise in this hierarchy, we quantify single-cell expression of fluorescent protein reporters at all levels of regulation using imaging and FACS. We found transcriptional regulation of chemotaxis genes at the lowest level of the hierarchy to be the main origin of noise. In addition, we are applying time-lapse imaging to study the time scale of the fluctuations in single-cell protein levels and observe the order of switching of the genes in the hierarchy. In addition, we combined an experimental analysis of chemotaxis at different levels of gene expression with computer simulations to demonstrate that the chemotaxis signaling network is designed to be robust against the observed gene expression noise. We believe that the underlying principles will be of general importance for biological systems.
AUTOPHOSPHORYLATION BY NATIVE AND SOLUBLE FORMS OF NITRATE RESPONSIVE SENSORS NarX AND NarQ FROM ESCHERICHIA COLI K-12

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In *Escherichia coli*, the dual interacting two-component regulatory systems NarX-NarL and NarQ-NarP control expression of genes encoding anaerobic respiratory enzymes. The NarX and NarQ proteins are histidine protein kinases that autophosphorylate in response to the signal ligands, nitrate and nitrite. The phosphorylated sensors serve as substrates for the activation (phosphorylation) of the response regulators NarL and NarP. In the absence of signal, the NarX and NarQ proteins stimulate dephosphorylation of NarL and NarP, respectively. NarX and NarQ are paralogous sensor proteins that share considerable overall similarity. The NarX and NarQ transmitter domains are members of the HPK7 sequence family as defined by Grebe and Stock (Grebe and Stock, 1999). However, their physiological functions differ in their responses to ligand and their regulation of target operon expression. Here, we report our analysis of the autokinase activities of both sensors. The autophosphorylation of truncated soluble forms of NarX and NarQ fused to maltose binding protein (MBP-NarX and MBP-NarQ) were investigated over a range of ATP and ADP concentrations. MBP-NarX autokinase activity was strongly inhibited by ADP, whereas MBP-NarQ was much less sensitive to ADP. Similar observations were made for the native forms of NarX and NarQ proteins enriched in membrane vesicles. Thus, these paralogous sensors display a striking difference in this fundamental property. In addition, native NarX displays an enhanced rate of autophosphorylation in response to nitrate, whereas the rate of native NarQ autophosphorylation was not influenced by signal ligand. Thus, the two sensors exhibit substantial differences also in signal perception.
STRUCTURAL INSIGHTS INTO A NEW MODE OF DNA BINDING BY THE BACTERIAL RESPONSE REGULATOR AgrA FROM STAPHYLOCOCCUS AUREUS

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*Staphylococcus aureus* is a significant human pathogen that causes a wide range of maladies ranging from minor skin infections to life threatening conditions such as toxic shock syndrome and myocarditis. The regulation of the genes that modulate the virulence response in *Staphylococcus* has been shown to be in part coordinated by the quorum sensing accessory gene regulator (*agr*) system. It has been shown to be a global regulator of virulence as it controls the expression of more than twenty extracellular and cytoplasmic accessory genes. The *agr* system contains two different transcription units that are under the control of the P2 and P3 promoters. The P2 operon encodes an autoinducing peptide (AgrD), a membrane associated protein that serves to modify AgrD and help transport it outside the cell (AgrB), and a two-component system consisting of a sensor histidine kinase (AgrC) and its cognate response regulator (AgrA). The P3 transcript encodes a regulatory RNA molecule (RNA III) that controls downstream gene expression. The transcription factor AgrA functions as part of a feed-back loop whereby it controls expression of the P2 and P3 transcripts.

The domain architecture of AgrA consists of an amino-terminal CheY-like receiver domain and a carboxy-terminal effector DNA binding domain termed the LytTR domain. Phosphorylation-dependent conformational changes in the receiver domain affect the protein’s ability to regulate transcription. The LytTR domain is part of a well-defined family of proteins and is usually found in transcription factors that regulate important functions in pathogenic organisms. The DNA binding site for the LytTR domain has been determined to be a conserved sequence containing two direct 9-base pair repeats separated by a 12-base pair spacer. Based on sequence homology the LytTR domain is not a member of any known DNA binding motif and represents a new class of DNA binding domains.

We have determined the X-ray crystal structure of the LytTR domain region of AgrA (AgrAc) bound to its DNA binding site in the P2 promoter. The structure of AgrAc consists of ten anti-parallel β-strands arranged in three sheets with a small connecting helix. The sites of interaction of AgrAc with the 16-bp DNA occur between conserved residues in the loops and specific conserved bases in the DNA sequence. The structure of AgrAc and its mode of binding appear to be unique and represent a new DNA recognition motif.
IDENTIFICATION AND CHARACTERIZATION OF Tsr MUTANTS THAT ARE DEFECTIVE IN TRIMER OF DIMERS FORMATION

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E. coli chemoreceptors appear to be organized in trimer-of-dimer arrangements that may be important to their signaling activities. We have developed an in vivo crosslinking competition assay to identify mutant chemoreceptors with defects in trimer formation. In the competition assay, serine receptors (Tsr) are expressed at low or high level in cells expressing chromosomal levels of an aspartate receptor bearing a cysteine reporter near the trimer axis (Tar•C). The cells are then treated with the tri-functional maleimide-targeted crosslinker TMEA and the extent of Tar•C crosslinking is measured in the presence of low and high levels of the Tsr “competitor.” Trimer-proficient Tsr molecules cause a dramatic decrease in Tar•C crosslinking products when expressed at high levels, whereas trimer-defective Tsr molecules do not interfere with the formation of Tar•C crosslinking products. Using this competition assay, we identified several trimer-defective Tsr mutants based on their inability to dilute crosslinking yields of the Tar•C reporter molecules. Quantitative analysis of the competition assay allowed us to identify Tsr variants that are completely unable to interact with Tar-C dimers in mixed trimers, as well as ones that seem to be incorporated into mixed trimers as single dimers but which show difficulty in contributing two dimers to the same trimer. To further characterize the trimer formation ability of the Tsr mutants, we introduced a cysteine reporter into each of them and analyzed their direct TMEA-crosslinking behavior when expressed as the only receptor in the cell or together with Tar•C. Direct crosslinking assays of the Tsr mutants were generally consistent with the behavior predicted from the competition assay: some Tsr mutants showed a clear decrease in their ability to get crosslinked and some showed the expected bias in forming mixed crosslinking product/s with Tar-C. However, the competition assay seemed to be more sensitive to trimer formation defects, presumably due to the fact that direct crosslinking can potentially trap transient, unstable species.
TRIMERS OF DIMERS AND THE METHYLATION RESPONSE OF CHEMORECEPTORS IN E. COLI

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The chemotaxis machinery of E. coli modulates the frequency of directional changes in swimming cells through gradient-sensing receptors that govern the activity of the associated CheA kinase. An attractant increase causes rapid inhibition of CheA and a slower increase in the net methylation level of the stimulated receptor molecules, culminating in sensory adaptation and a return to pre-stimulus CheA activity upon reaching a methylation level that offsets the ligand occupancy state of the receptor population. In this study, we investigated the role that chemoreceptor trimers of dimers might play in this methylation response, using two different experimental set-ups:

1. trimer-defective Tsr molecules in cells lacking CheA and CheW
2. wild-type Tsr in cells with high levels of CheW, which disrupts trimer formation

We found that several trimer defective mutants had elevated methylation levels in the absence of stimulus, as compared to wild-type Tsr. Similarly, wild-type Tsr showed an increase in unstimulated methylation level as cellular CheW level was increased. In both cases, a serine stimulus often caused a further increase in methylation level. However, some trimer defective mutants were unmethylated, and remained so after a serine stimulus, implying a serious structural disruption in the receptor dimer. Taken together, these results suggest that the formation of trimers of dimers is not essential for the methylation response to attractants. However, the assembly of chemoreceptor dimers into trimers seems to limit the accessibility of some receptor methylation sites to the methyltransferase CheR.
CRYO-ELECTRON TOMOGRAPHY OF *E. coli*: STRUCTURE AND FUNCTIONAL ANALYSIS OF CHEMOTAXIS RECEPTOR ASSEMBLIES

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Bacteria respond to changes in their chemical environment by activating an assembly of proteins that collectively represent the bacterial chemotaxis apparatus. In *Escherichia coli*, a specialized family of methyl-accepting chemotaxis proteins (chemoreceptors) bind extracellular ligands and initiate a complex signal transduction pathway, which ultimately controls the direction of flagellar rotation. The cytoplasmic C-terminal signaling domains of the chemoreceptors form stable ternary complexes with the histidine autokinase CheA and the adapter protein CheW, which is thought to couple CheA to the chemoreceptors. Using cryo-electron tomography, we are investigating the architecture of higher order assemblies formed by chemotaxis receptors both in isolation and in complex with CheA and CheW. In wild-type cells, receptors, CheA and CheW form extended arrays in the polar region of intact *E. coli* cells. We have directly visualized these arrays and have determined their location relative to other landmarks in the cellular landscape. By combining cryo-electron microscopy with physiological assays, we have demonstrated that the extent of these arrays correlates with the ability of the cells to perform chemotaxis. These studies also provide powerful approaches for the determination of chemoreceptor density maps under near physiological conditions within the cell membrane using local averaging methods, and for the interpretation of these maps using known atomic structures of the individual signaling components. In related studies, we are comparing variations in structural organization of differentially methylated assemblies formed by interacting receptors both in the presence and absence of CheA and CheW. Together these studies are beginning to provide molecular snapshots of the complex machinery involved in bacterial chemotaxis.
LOW FLAGELLAR MOTOR TORQUE AND HIGH SWIMMING EFFICIENCY OF
CAULOBACTER CRESCENTUS SWARMER CELLS

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The water born bacterium Caulobacter crescentus has a single flagellum at its swarmer cell stage. It swims efficiently, which may allow the species to thrive in the fresh water environment where the nutrient level is often low. Caulobacter crescentus is also chemotactic, and the mechanism of its chemotactic behavior must be very different from that of E. coli, which achieves its directional response through the regulated run-and-tumble behavior. The exact mechanism how C. crescentus achieves chemotaxis is not well understood at the present time. Since the flagellar motor of Caulobacter crescentus is very similar in structure from that of E. coli, we set to compare whether the torques they each generate are different, and if so, whether the difference are relevant to their rather distinct swimming behavior.

We determined the torque of the flagellar motor of Caulobacter crescentus for different motor rotation rates by measuring the rotation rate and swimming speed of the cell body and found it to be remarkably different from that of other bacteria, such as E. coli and V. alginolyticus. The average stall torque of the Caulobacter flagellar motor was about 350 pN nm, much smaller than the values of the other bacteria measured. Furthermore, the torque of the motor remained constant in the range of rotation rates up to those of freely swimming cells. In contrast, the torque of a freely swimming cell for V. alginolyticus is typically less than 20% of the stall torque. We derive from these results that the C. crescentus swarmer cells swim more efficiently than both E. coli and V. alginolyticus. Our findings suggest that C. crescentus is optimally adapted to low nutrient aquatic environments.

THE Aer F1 DOMAIN - PROXIMITY, LOCATION AND DETERMINANTS FOR MEMBRANE INSERTION

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INTRODUCTION. Aer is an aerotaxis, energy and redox sensor that contains an N-terminal PAS sensing domain and a C-terminal signaling domain separated by a membrane anchor. The intervening F1 and HAMP domains play roles in signal transduction and are necessary for protein stability. The HAMP domain consists of a four-helix bundle that may be involved in direct signal transduction from the PAS domain, however the structure of F1, and its exact role in signaling, is unknown. In this study, the proximity of cognate F1 residues, the location of F1 within a trimer of dimers, and the requirement for F1 in membrane insertion, was investigated.

METHODS. To determine the proximity between cognate F1 and F1’ within an Aer dimer, we introduced single cysteines at each residue from 120 to 166. We expressed these constructs in BT3312 (aer tsr) and treated whole cells with the oxidant copper phenanthroline (10 min, 23°C). Residues that crosslinked at 23°C were re-analyzed at lower temperatures to limit lateral diffusion in the membrane. Longer bifunctional (BMOE, 8.0 Å; 10 min, 23°C) and trifunctional (TMEA, 10.3 Å; 15 min, 23°C) sulfhydryl reactive probes were also tested.

N-terminal Aer truncations with deletions in F1 (Aer[127-506], Aer[135-506] and Aer[154-506]) were tested for their ability to resurrect the function of a null aerotaxis mutant (Aer-Q248R) by intragenic complementation. Proper membrane insertion of the truncated proteins was tested by measuring protein solubility in membrane vesicles after the addition of a mild detergent.

RESULTS. All 46 single cys Aer mutants supported aerotaxis in succinate swarm plates. Only four crosslinked at 23°C (A121C, A135C, G136C and R137C), of which three also crosslinked at both 10°C and 4°C (A121C, A135C and R137C). The Aer-F1 domain begins after the PAS domain and is predicted to consist of an amphipathic helix, followed by a loop that connects F1 to the membrane anchor. The crosslinking data suggested that i) cognate F1 domains do not form an interactive face along their length and ii) F1 is probably not a continuous helix. Crosslinking of contiguous residues 135 to 137 indicates that F1 and F1’ contact or crossover in this region, where the predicted helix-loop transition occurs. The longer BMOE probe (8 Å) crosslinked many additional residues, reflecting the relative distance between cognate F1 domains. The trifunctional TMEA probe trapped trimers of Aer-R137C, suggesting that the F1 domains may be located in the center of a trimer of dimers, while the PAS domains are arranged peripherally.

Previously we reported that heterodimers missing one PAS domain support aerotaxis, while heterodimers missing contiguous PAS and F1 domains produce non-aerotactic swarms. Since F1 may have been required for membrane insertion, rather than function, three longer truncations were made (see methods). These truncated proteins produced functional heterodimers in intragenic complementation assays. Moreover, Aer[154-506] homodimers inserted into the membrane correctly, while homodimers of the mutant missing the entire PAS and F1 domains (Aer[165-506]) did not. This suggests that residues in the F1 domain between 154 and 165 are required for correct membrane insertion of Aer.
A FRACTIONAL DIFFUSION MODEL FOR CHEMOTAXIS

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Quantifying the path taken by a motile bacterium can be used to gather information about its chemotactic response. We utilized video tracking of bacterial swimming behavior, and from that, obtained x and y coordinate positions for time t. Using a fractional diffusion model for the position (x, y) we developed a method for quantifying behavioral differences between wild type and mutants. We use the convention \[ \langle R^2(t) \rangle \propto t^\alpha \] for the model where \( R(t) \) is the distance from the origin at time t, \( \alpha \) is the scaling exponent and the brackets indicate an average. The value of \( \alpha \), which ranges from one to two characterizes the swimming behavior of the bacteria. If \( \alpha \) is close to one the percent of time the bacterium spends tumbling is large. If \( \alpha \) is close to two, the bacterium tumbles rarely and almost always swims. We have tested this model on the chemotactic bacterium Helicobacter pylori. For H. pylori the value of \( \alpha \) for wild type ranges from 1.0~1.2, while a mutant missing the chemotaxis regulator CheY has \( \alpha \) values ranging from 1.8~1.9. The larger alpha value of cheY mutants imply they tumble less than their wild-type parents. The value of \( \alpha \) can also be used to identify attractants that change swimming behavior. Simulations of the stochastic process will be shown, as well as different methods for calculating \( \alpha \).
A MODEL ORGANISM DATABASE (MOD) THAT INCORPORATES WIKIPEDIA PRINCIPLES

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The Model Organism Database (MOD) has become a nearly ubiquitous component of the scientific 'cyberinfrastructure', but the exact purpose of a MOD, and its relationship to the community it serves, is not well defined. After much deliberation and frank discussion, we designed and implemented xanthusBase (http://www.xanthusbase.org) as the official MOD for the social bacterium Myxococcus xanthus. When appropriate, the design of xanthusBase incorporates open-source software, leveraging the cumulative experience made available through the Generic Model Organism Database (GMOD) project, MediaWiki (http://www.mediawiki.org), and dictyBase (http://www.dictybase.org), to create a MOD that is both familiar and navigable. To provide xanthusBase with a unique purpose, we incorporated a Wikipedia-style curation model which exploits the internet's inherent interactivity, and thus enables M. xanthus and other myxobacterial researchers to contribute directly toward the ongoing genome annotation. In many respects, the xanthusBase project represents an ideal proof-of-concept for a MOD based on Wiki principles.
REGULATION OF THE METHYLTRANSFERASE ACTIVITY OF FrzF DURING SOCIAL MOTILITY AND DEVELOPMENT IN MYXOCOCCUS XANTHUS

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The methyltransferase, FrzF, belongs to the Frz signal transduction pathway, which regulates both vegetative swarming and developmental aggregation by controlling the reversal frequency of cells. FrzF regulates the activity of the receptor FrzCD, a methyl accepting chemotaxis protein homolog (MCP), by methylation (1). Deletion of frzF results in a defect in swarming motility on nutrient rich media and incapacity to aggregate into fruiting bodies in nutrient limiting conditions. Unlike any characterized methyltransferase, FrzF contains three tetratricopeptide repeat (TPR) domains. TPR domains in other systems have been shown to mediate protein-protein interactions (2). To investigate the role of FrzF in sensing the input signal that leads to methylation of FrzCD, we are trying to identify proteins that might interact with the two domains of FrzF, the methyltransferase domain and the TPR containing domain. We hypothesize that the methyltransferase activity of FrzF is subject to feedback regulation by FrzE, a histidine kinase-response regulator fusion protein (3). To test this hypothesis we are analysing if the activity of FrzE influences the activity of FrzF in vitro and if the methylation state of FrzCD is altered in FrzE mutant strains.

References:


LOCALIZATION OF FrzCD IN *MYXOCOCCUS XANTHUS*

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FrzCD, the cytoplasmic receptor for the *Myxococcus xanthus* Frz chemosensory system, shows homology to the C-terminal region of Methyl-accepting Chemotaxis Protein (MCPs). However, in contrast to most MCPs, it lacks the periplasmic sensing domain, the transmembrane domain, and the HAMP linker region typically found at the N-terminus of these receptors (1). In this study, we used fluorescence microscopy to examine the localization of FrzCD in cells. We found that FrzCD is not freely diffused in the cytoplasm, but is localized in discrete (punctate) clusters that form a spiral pattern. This unusual localization is maintained in the absence of the CheA homologs, FrzE or CheA4, or 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 (2, 3). The unique N-terminal region of FrzCD appears to play a role in FrzCD localization, as *frzCD*Δ6-130, a mutant that expresses a FrzCD protein lacking the N-terminal region of FrzCD, showed altered FrzCDΔ6-130 localization. GFP fusions showed that the protein is dynamic when cells move on agar surfaces. The novel dynamic localization pattern of FrzCD may provide new insights into the functioning of this receptor.

References


REGULATION OF GLIDING MOTILITY AND FRUITING BODY FORMATION BY RECEPTOR METHYLATION IN MYXOCOCCUS XANTHUS

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*M. xanthus* coordinates movement of thousands of cells into fruiting bodies during starvation, and cell swarms towards attractants in the presence of nutrients. This coordination is regulated by the Frz signal transduction pathway. The Frz system controls the direction of cell movement and FrzF, a methyltransferase homolog, plays an important role in this regulation.

A deletion of *frzF* results in loss of methylation of FrzCD (methyl accepting chemotaxis protein homolog). In addition, *frzF* mutant cells are unable to swarm on nutrient rich media, or form fruiting bodies during starvation. Unlike characterized methyltransferases, FrzF contains three tetratrico peptide repeats (TPR) domains. Theses TPR domains are required for proper fruiting body formation, but are only partially needed for swarming on rich media.

To understand the role of methylation on the activity of the Frz pathway we developed an *in vitro* methylation assay in which, the cytoplasmic receptor, FrzCD, can be methylated by FrzF in the presence of the methyl donor S-adenosyl methionine. Interestingly, we have found that FrzF lacking its TPR domains hyper-methylates FrzCD as compared to full-length FrzF as determined by mass spectrometry. These results suggest that the TPR domains may be playing an inhibitory role on FrzF methylation *in vitro*. Current research is aimed at determining the sites of FrzCD methylation throughout *M. xanthus*’s life cycle.