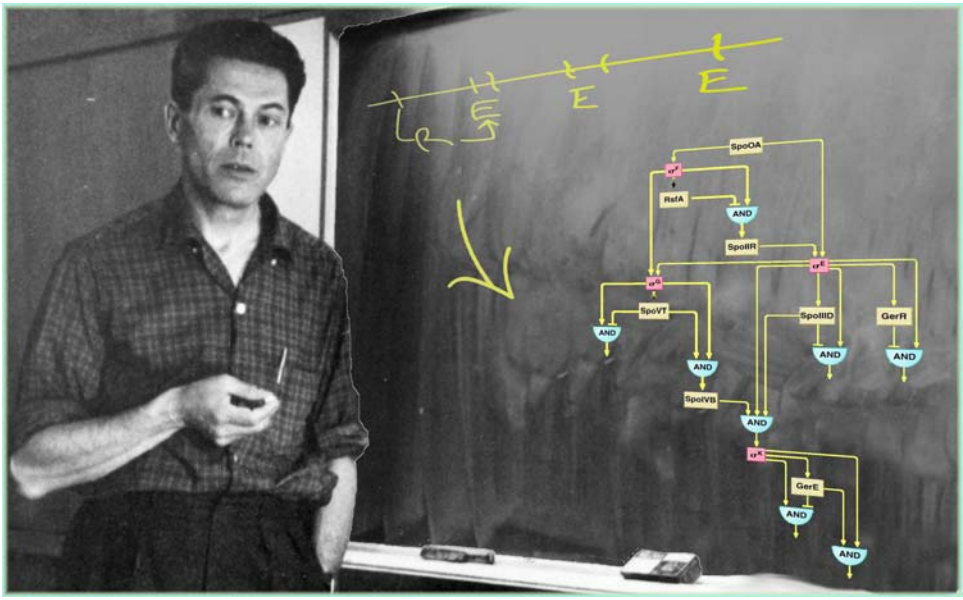
Abstracts of papers presented
at the 2010 meeting on

MOLECULAR GENETICS OF BACTERIA & PHAGES

August 24–August 28, 2010



Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

Abstracts of papers presented
at the 2010 meeting on

MOLECULAR GENETICS OF BACTERIA & PHAGES

August 24–August 28, 2010

Arranged by

James Hu, *Texas A&M University*
Petra Levin, *Washington University*
Malcolm Winkler*, Indiana University*

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

This meeting was funded in part by the **National Institute of Allergy and Infectious Diseases**, a branch of the **National Institutes of Health;** and the **National Science Foundation.**

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Front cover: To celebrate the 100th anniversary of the birth of Jacques Monod on Feb 9, 1910, we illustrate how Monod's ideas about regulation continue to influence our thinking about biological regulation. The illustration by Daniel Renfro modifies the famous photo of Monod and Leo Szilard at CSHL taken by Esther Bubley for Time magazine. Szilard and the material on the blackboard removed (Sorry, Dr. Szilard) and the drawings were replaced by a sketch showing how Monods basic idea of regulatory genes and relationships underlies the systems biology of a complex regulatory circuit from *B. subtilis*.

Back cover: Cartoon by Martin Pollock drawn in 1953 depicting Monod mentoring Mel Cohn under the smiling face of Max Delbrück. Cohn describes the left hand side: "we had destroyed all existing so-called facts, replacing them with nothing he was willing to believe (*Faits confirmés*), and we had produced nothing but wild theories." Reproduced from The Operon, Second Edition (p.7).

MOLECULAR GENETICS OF BACTERIA & PHAGES

Tuesday, August 24 – Friday, August 28, 2010

Tuesday	7:30 pm	**1** Development, Cell Signaling and Cell-Cell Interactions
Wednesday	9:00 am	**2** Molecular Evolution, Systems Biology and Genetics
Wednesday	2:00 pm	**3** Poster Session I
Wednesday	4:30 pm	Wine and Cheese Party *
Wednesday	7:30 pm	**4** Bacterial Cell Surfaces
Thursday	9:00 am	**5** Bacteriophage Biology
Thursday	2:00 pm	**6** Poster Session II
Thursday	4:00 pm	Hatch Echols Tennis Tournament
Thursday	7:30 pm	**7** Mechanism and Regulation of Transcription
Friday	9:00 am	**8** Bacterial Metabolism and Physiology
Friday	1:30 pm	**9** Translation and Posttranscriptional Regulation
Friday	5:00 pm	KEYNOTE SPEAKER
	6:00 pm	Banquet
Saturday	9:00 am	**10** DNA Replication, Recombination and Transposition

Poster sessions are located in *Bush Lecture Hall*

* *Airslie Lawn*, weather permitting

Mealtimes at Blackford Hall are as follows:

Breakfast 7:30 am-9:00 am

Lunch 11:30 am-1:30 pm

Dinner 5:30 pm-7:00 pm

Bar is open from 5:00 pm until late

Printed on 100% recycled paper.

PROGRAM

TUESDAY, August 24—7:30 PM

SESSION 1 DEVELOPMENT, CELL SIGNALING AND CELL-CELL INTERACTIONS

Chairperson: **M. Laub,** Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge

Michael Laub.
Presenter affiliation: Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts.

Functional analysis of AtxA, the major virulence gene regulator of *Bacillus anthracis*
Troy G. Hammerstrom, Yeonhee Kim, Edward P. Nikonowicz, Theresa M. Koehler.
Presenter affiliation: University of Texas Health Science Center-Houston, Houston, Texas. 1

The *escherichia coli* extracellular death factor EDF induces the endoribonucleolytic activity of MazF
Hanna Engelberg-Kulka, Maria Belizky, Haim Avshalom, Idan Yelin.
Presenter affiliation: Hebrew University-Medical School, Jerusalem, Israel. 2

Regulation of secretion of the PopC protease
Anna Konovalova, Lotte Sogaard-Andersen.
Presenter affiliation: Max-Planck Institute for Terrestrial Microbiology, Marburg, Germany. 3

The role of the SipW signal peptidase of *Bacillus subtilis* in stimulating gene expression in response to surface attachment
Rebecca L. Terra, Cao Guoqiang, Beth A. Lazazzera.
Presenter affiliation: University of California-Los Angeles, Los Angeles, California. 4

Stringent response regulation of biofilm formation in *Vibrio cholerae*
Huajun He, David M. Raskin.
Presenter affiliation: The Methodist Hospital Research Institute, Houston, Texas. 5

WEDNESDAY, August 25—9:00 AM

SESSION 2 MOLECULAR EVOLUTION, SYSTEMS BIOLOGY AND GENOMICS

Chairperson: **M. Giglio,** University of Maryland School of Medicine, Baltimore

WEDNESDAY, August 25—2:00 PM

SESSION 3 POSTER SESSION I

WEDNESDAY, August 25—4:30 PM

Wine and Cheese Party

WEDNESDAY, August 25—7:30 PM

SESSION 4 BACTERIAL CELL SURFACES

Chairperson: **R. Young,** Texas A&M University, College Station

THURSDAY, August 26—9:00 AM

SESSION 5 BACTERIOPHAGE BIOLOGY

Chairperson: **G. Christie,** Virginia Commonwealth University, Richmond

THURSDAY, August 26—2:00 PM

SESSION 6 POSTER SESSION II

Redundant regulation by transcription factors in *Escherichia coli*
Joseph T. Wade, Anne M. Stringer.
Presenter affiliation: Wadsworth Center, Albany, New York. 109

Bypass of the essential PcsB cell division protein by a carboxyl-terminal frame-shift Mutation in DivIVA requires the carboxypeptidases DacA and newly identified DacB in serotype 2 D39 *Streptococcus pneumoniae*
Skye M. Barendt, Kimberley E. Kopecky, Lok-To Sham, Ho-Ching T. Tsui, Kyle J. Wayne, Malcolm E. Winkler.
Presenter affiliation: Indiana University Bloomington, Bloomington, Indiana. 110

A mutation in the inhibitory periplasmic protein CpxP facilitates the degradation of the bundle-forming pilus by the chaperone-protease DegP in enteropathogenic *Escherichia coli*
Julia L. Wong, Tracy L. Raivio.
Presenter affiliation: University of Alberta, Edmonton, Canada. 111

It's a feast or a famine—Can *Escherichia coli* O157:H7 survive the rigors of starvation and go on to live another day?
Ron N. Xavier, Hugh Morgan, Ian R. McDonald, Helen Withers.
Presenter affiliation: University of Waikato, Hamilton, New Zealand; AgResearch MIRINZ, Hamilton, New Zealand. 112

THURSDAY, August 26—4:00 PM

Hatch Echols Tennis Tournament
at CSHL Tennis Courts

THURSDAY, August 26—7:30 PM

SESSION 7 MECHANISM AND REGULATION OF TRANSCRIPTION

Chairperson: **B. Nickels,** Rutgers University, Piscataway, New Jersey

NanoRNAs prime transcription initiation in vivo
Bryce E. Nickels, Seth R. Goldman, Josh S. Sharp, Irina O. Vvedenskaya, Simon L. Dove.
Presenter affiliation: Rutgers University, Piscataway, New Jersey. 113

SESSION 8 BACTERIAL METABOLISM AND PHYSIOLOGY

Chairperson: **A. Wolfe,** Loyola University, Chicago, Illinois

FRIDAY, August 27—1:30 PM

SESSION 9 TRANSLATION AND POSTTRANSCRIPTIONAL REGULATION

Chairperson: **R. Britton,** Michigan State University, East Lansing

FRIDAY, August 27—5:00 PM

KEYNOTE SPEAKER

Susan Gottesman
National Cancer Institute

"Beyond transcription—Complex regulatory networks for responding to changing environments" 140

FRIDAY, August 27

BANQUET

Cocktails 6:00 PM Dinner 6:45 PM

SATURDAY, August 28—9:00 AM

SESSION 10 DNA REPLICATION, RECOMBINATION AND TRANSPOSITION

Chairperson: **D. Rudner,** Harvard Medical School, Boston, Massachusetts

David Rudner.
Presenter affiliation: Harvard Medical School, Boston, Massachusetts.

The nucleoid occlusion factor SlmA is a DNA-activated FtsZ antagonist
Hongbaek Cho, Heather R. McManus, Simon L. Dove, Thomas G. Bernhardt.
Presenter affiliation: Harvard Medical School, Boston, Massachusetts. 141

MatP, the septal ring and replication control the segregation of the Ter macrodomain of the *E. coli* chromosome
Romain Borne, Romain Mercier, Axel Thiel, Frédéric Boccard, Olivier Espéli.
Presenter affiliation: CNRS, Gif sur Yvette, France. 142

FUNCTIONAL ANALYSIS OF ATXA, THE MAJOR VIRULENCE GENE REGULATOR OF *BACILLUS ANTHRACIS*

Troy G Hammerstrom[1], Yeonhee Kim[2], Edward P Nikonowicz[3], Theresa M Koehler[1]

[1]University of Texas Health Science Center - Houston, Department of Microbiology and Molecular Genetics, Houston, TX, 77030, [2]National Institute of Health, KCDC, Division of High-risk Pathogen Research, Seoul, 122-701, South Korea, [3]Rice University, Department of Biochemistry & Cell Biology, Houston, TX, 77251

Expression of the anthrax toxin and capsule genes by *Bacillus anthracis*, the causative agent of anthrax, requires the presence of AtxA, a soluble 56-kDa protein produced throughout growth of the bacterium in batch culture. *B. anthracis atxA*-null mutants are highly attenuated in animal models for anthrax. Despite the importance of AtxA in pathogenesis, the molecular function of the protein remains unknown. Our work focuses on determining the molecular mechanism by which the protein controls toxin and capsule gene transcription. The AtxA protein is predicted to possess amino-terminal winged helix (WH) and helix-turn-helix (HTH) DNA-binding domains, two carboxy-terminal phosphotransferase system regulation domains (PRDs) and an EIIB-like motif. We have generated several loss-of-function and gain-of-function alleles of *atxA* using site-directed and random mutagenesis. Using an IPTG-inducible expression system which allows over-expression of AtxA up to 25-fold above the native level of the protein, we assessed the activity of *atxA* mutant alleles by quantifying expression of a toxin gene promoter – reporter gene fusion ($P_{\text{lethal factor}}$ – *lacZ*). Loss-of-function mutations included disruptions in the HTH and in the putative phosphorylation sites within the PRDs. These mutations decreased AtxA activity without affecting AtxA protein stability. Gain-of-function mutations mapping to one of the predicted HTH motifs and to a residue within the second PRD increased AtxA activity 2-3 fold. Currently, we are mutating each of the six cysteine amino acids to assess the role of each residue on AtxA function. Additionally, we determined that AtxA function is unchanged during growth in glycerol versus glucose. These data indicate that despite the presence of PRD motifs, AtxA activity is unaffected by a PEP-dependent phosphotransferase system (PTS) sugar compared to a non-PTS sugar. In future experiments we will utilize the IPTG-inducible system to over-express AtxA with a C-terminal hexa-histidine tag to purify AtxA alleles for *in vitro* DNA-binding experiments.

THE ESCHERICHIA COLI EXTRACELLULAR DEATH FACTOR EDF INDUCES THE ENDORIBONUCLEOLYTIC ACTIVITY OF MAZF

Hanna Engelberg-Kulka, Maria Belizky, Haim Avshalom, Idan Yelin

Hebrew University-Medical School, Microbiology and Molecular, Jerusalem, 91120, Israel

In recent years, a great deal of attention has been focused on the abundance of toxin-antitoxin (TA) gene modules in chromosomes of many bacteria, including pathogens. The most studied of these modules is E.coli mazEF in which mazF encodes a stable toxin, MazF, and mazE encodes a labile antitoxin, MazE, which prevents the lethal effect of MazF(1). E. coli mazEF is a stress-induced TA module that mediates cell death in the bacterium (1-3). The E. coli toxin MazF is a sequence-specific endoribonuclease that preferentially cleaves single-stranded mRNAs at ACA sequences (4).
We have recently reported that E. coli mazEF-mediated cell death is a population phenomenon requiring the E. coli quorum sensing factor EDF (Extracellular Death Factor) (5,6). Structural analysis revealed that EDF is the linear peptide Asn-Asn-Trp-Asn-Asn: that is required for triggering mazEF-mediated cell death (5).
Here we asked: Does EDF specifically affect the endoribonucleolytic activity of MazF? We found that, in vitro, EDF significantly amplifies the endoribonucleolytic activity of MazF. In addition, EDF also overcomes the inhibitory activity of the antitoxin MazE over the toxin MazF. EDF sequence was found to be important for both functions. Furthermore, our results revealed a direct interaction of EDF with MazF. These findings are particularly intriguing, since quorum sensing molecules are generally known to monitor gene expression rather than an enzymatic activity.

1)Aizenman et al., PNAS. 93, 6059-6063. (1996).
2)Hazan et al., J. Bacteriol. 186, 3663-3669 (2004).
3)Godoy et al., The EMBO J. 25, 868-879 (2006).
4)Zhang et al., Mol. Cell 12, 913-923.(2003)
5)Kolodkin-Gal et al., Science 318, 652-655 (2007) .
6)Kolodkin-Gal and Engelberg-Kulka , J. Bacteriol. 190, 3169-3275 (2008).

REGULATION OF SECRETION OF THE POPC PROTEASE

Anna Konovalova, Lotte Sogaard-Andersen

Max-Planck Institute for Terrestrial Microbiology, Ecophysiology, Marburg, 35043, Germany

In response to starvation M. xanthus initiates a developmental program that culminates in the formation of spore-filled fruiting bodies. The C-signal is an intercellular signal that induces and coordinates aggregation of cells into fruiting bodies and the subsequent sporulation of cells inside fruiting bodies. The regulated accumulation of the C-signal is essential for the coordinating function of the C-signal. The C-signal is a 17 kDa protein (p17) that is produced by regulated proteolysis of the 25 kDa CsgA protein (p25). p25 accumulates in vegetative as well as in starving cells; however, p17 accumulation is restricted to starving cells. We identified the PopC protease as directly responsible for cleavage of p25. PopC accumulates in the cytoplasm of vegetative cells and is selectively secreted during starvation coinciding with the generation of p17. Therefore, p25 and PopC are only present in the same cell compartment in starving cells. Thus, p25 cleavage is restricted to starving cells by a compartmentalization mechanism that depends on selective secretion of PopC in response to starvation. Here, we focus on elucidation of the molecular mechanisms involved in regulation of PopC secretion.

Initiation of development is M. xanthus is triggered by accumulation of intracellular starvation signal (p)ppGpp. PopC secretion as well as p17 generation is abolished in a relA mutant, which is unable to produce (p)ppGpp. This finding indicates that activation of PopC secretion is a part of starvation induced response and depend on accumulation of (p)ppGpp. It was also observed that vegetative cells treated with chloramphenicol and subsequently exposed to starvation are still capable of secreting PopC suggesting that the secretion system responsible for PopC secretion is already present in vegetative cells and activation of secretion is independent of translation. PopC does not contain a signal peptide suggesting that it is secreted via a Sec/Tat-independent pathway. A systematic analysis of the M. xanthus genome identified several putative type I secretion systems, two putative type III secretion systems and one putative type VI secretion system. Inactivation of these secretion systems did not interfere with PopC secretion. Therefore, we performed a random transposon mutagenesis to identify proteins important for PopC secretion. We identified several mutants, which are unable to secrete PopC in response to starvation. Interestingly, all mutations map to genes encoding hypothetical proteins. The function of these proteins in PopC secretion will be discussed.

To identify proteins interacting with PopC to inhibit secretion in vegetative cells or stimulate secretion in starving cells we focused on the PopD protein, which is encoded in an operon with popC. We were able to show that PopD and PopC proteins interact directly. popD is essential in an popC+ genetic background but not in a popC mutant and , surprisingly, not in a csgA mutant. Moreover, PopC secretion occurred more rapidly in a popD mutant. These results suggest that PopD may act as a negative regulator of PopC secretion in vegetative cells.

THE ROLE OF THE SIPW SIGNAL PEPTIDASE OF BACILLUS SUBTILIS IN STIMULATING GENE EXPRESSION IN RESPONSE TO SURFACE ATTACHMENT

Rebecca L Terra, Cao Guoqiang, Beth A Lazazzera

University of California, Los Angeles, Microbiology, Immunology & Molecular Genetics, Los Angeles, CA, 90095

In *Bacillus subtilis*, the type I signal peptidase SipW is known to be required for the proteolytic removal of the signal sequence from two exported proteins YqxM and TasA. TasA is the major protein component of the extracellular matrix of biofilms formed by *B. subtilis*. Here, we present evidence that SipW has a second function independent of processing YqxM and TasA and that this second function is specifically required for cells to form a biofilm on a solid surface. While cells lacking SipW could only attach as single cells to a solid surface, cells lacking YqxM and TasA are able to form a biofilm on a solid surface, albeit of altered structure. In contrast, cells lacking either SipW or YqxM and TasA were phenotypically indistinguishable at forming air-liquid interface biofilms. To further understand the role SipW has in surface-adhered biofilm formation, we characterized suppressor mutations that restored biofilm formation to a strain lacking SipW. These mutations mapped to the gene for SinR, a transcriptional repressor of the biofilm matrix operons *epsA-O* and *yqxM-sipW-tasA*. These data suggested a model in which the role of SipW on a solid surface is to antagonize SinR activity and up-regulate biofilm matrix gene expression. To test this, we monitored *eps* and *yqxM* operon expression by fusing the promoters of these operons to the gene for GFP. Expression was then monitored in single cells by flow cytometry for planktonic and air-liquid interface biofilm cells and fluorescence microscopy for surface-adhered cells. While cells lacking SipW showed a profound defect in induction of the *eps* and *yqxM* operons when the cells were grown attached to a solid surface, there was no affect of SipW on expression of these operons when the cells were grown as air-liquid interface biofilm cells or planktonic cells. These data indicate that there are different regulatory pathways for inducing biofilm matrix gene expression during solid-surface versus air-liquid interface biofilm formation and that SipW is part of the regulatory pathway to control matrix gene expression in cells sensing a solid surface.

STRINGENT RESPONSE REGULATION OF BIOFILM FORMATION IN *VIBRIO CHOLERAE*

Huajun He, David M Raskin

The Methodist Hospital Research Institute, Infectious Disease, Houston, TX, 77006

Vibrio cholerae is the causative agent of the severe diarrheal disease cholera and can survive and flourish in diverse environments. Normally an aquatic organism, some strains of *V. cholerae* are able to pass through the gastric acid barrier of the stomach and colonize the small intestine, where it produces virulence factors responsible for disease. Biofilm formation is a key factor in *V. cholerae* environmental survival and persistence, as well as host colonization. We examined the role of (p)ppGpp in biofilm formation. Mutants that are deficient in (p)ppGpp synthesis have a reduced capacity to form biofilms. Biofilm genes are regulated by the transcriptional activators *vpsR* and *vpsT*, which are transcriptionally regulated by a number of environmental signals. There are three (p)ppGpp synthases in *V. cholerae*, RelA, SpoT and RelV and each had a discrete impact on *vpsR* and *vpsT* expression. All three synthases were necessary for *vpsR* transcription, with RelV having the strongest impact. RelA was the only synthase that was necessary for *vpsT* expression. We also found that (p)ppGpp regulation of *vpsR* and *vpsT* was partially mediated through *rpoS*, the stationary phase sigma factor. We showed that (p)ppGpp synthesis occured during biofilm formation primarily through RelA. These results suggest that (p)ppGpp has a significant role in regulation of biofilm and understanding how the three (p)ppGpp synthases are regulated during the *V. cholerae* life cycle will be important to understanding spread of cholera disease.

SEQUENCE DETERMINANTS FOR SMALL RNA REGULATION OF VIRULENCE BY THE *VIBRIO CHOLERAE* QUORUM SENSING SYSTEM

Xiaonan Zhao, Brian K Hammer

Georgia Institute of Technology, School of Biology, Atlanta, GA, 30332

Vibrio cholerae, the causative agent of the fatal diarrheal disease cholera, uses quorum sensing (QS) to control the transcription of four non-coding small RNAs (sRNAs) called Qrr1-4 (for quorum regulatory RNAs). In response to changes in extracellular signal molecules that accumulate proportionally with the bacterial population density, these sRNAs regulate biofilm and virulence gene expression by altering the translation of mRNA targets to which they are predicted to bind. All sequenced pathogenic *Vibrios* encode multiple Qrrs that contain an absolutely conserved 21 nt region, which is predicted to imperfectly base pair at the ribosome binding site (RBS) of multiple mRNA targets to regulate expression post-transcriptionally. Previous genetic studies support the hypothesis that these 21 nt comprise an interaction region (I-region) essential to repress the translation of the QS master regulator, HapR, and the sRNA-activator, LuxO, which are both critical for proper QS control. However, computational predictions suggest that different nucleotides in the Qrr I-region participate in base-pairing with *hapR* and with *luxO*. To determine the contribution of each nt within the I-region to mRNA binding, a Qrr expression plasmid (pQrr) was constructed that properly regulates plasmid-borne *hapR-gfp* and *luxO-gfp* fusions in *E. coli* and *V. cholerae*. Reporter gene fusions that monitor Qrr-dependent HapR and LuxO activities (*luxC-lux* and *qrr4-lux*, respectively) are also properly controlled by pQrr in *V. cholerae*. Preliminary results with these *in vivo* reporter systems show that deletion of the I-region abolishes the inhibitory function of the sRNAs for both mRNA targets. Using the predictive computational algorithm RNAup, we have designed single nt substitutions in both the Qrr I-region and the mRNA of *hapR* and *luxO* to test experimentally Qrr/mRNA interactions with the *in vivo* system we have developed. To systematically analyze the nt determinants for sRNA/mRNA interaction, we have also constructed, and are currently screening *in vivo* with our gene fusions, a plasmid-borne sRNA library ($pQrr^{lib}$) randomized within the I-region. By complementing our *in silico* predictions and *in vivo* results with *in vitro* RNA binding studies also underway, we will be able to dissect the molecular mechanisms of sRNA/mRNA interactions that coordinate extracellular chemical signaling to virulence in this human pathogen.

EXPRESSION OF THE TYPE III-SECRETED EFFECTOR *ESPS* OF *CITROBACTER RODENTIUM* IS REGULATED BY THE *CROIR* QUORUM SENSING SYSTEM.

Kevin J Roberts[1], Gordon Dougan[2], George P Salmond[1]

[1]University of Cambridge, Biochemistry, Cambridge, CB2 1QW, United Kingdom, [2]Wellcome Trust Sanger Institute, Microbial Pathogenesis , Cambridge, CB10 1, United Kingdom

Citrobacter rodentium is a Gram-negative member of the Enterobacteriaceae that is commonly used as a model organism to study attaching and effacing (A/E) lesion formation in its natural host, the mouse. *C. rodentium* and the related human A/E pathogens, enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC), encode a Type III secretion (TIIIS) system located on a pathogenicity island entitled the locus of enterocyte effacement (LEE). The TIIIS system is responsible for the delivery of effector proteins direct to the host cytoplasm and is required for the formation of A/E lesions and for virulence. EspS was recently identified as a novel *C. rodentium* effector protein that is translocated into mammalian cells by the LEE-encoded TIIIS system and may contribute to full colonisation in the mouse. Quorum sensing (QS) is a form of bacterial cell-to-cell communication using diffusible chemical signals to regulate gene expression in a cell density-dependent manner. Many Gram-negative bacteria synthesise and respond to N-acyl-homoserine lactone (AHL) signalling molecules, altering gene expression accordingly. *C. rodentium* encodes an AHL synthase (CroI) which produces N-butanoyl-L-homoserine lactone (BHL) as its major product and also a LuxR-family protein (CroR), a BHL receptor and transcriptional regulator. Here we demonstrate, using an *espS::lacZ* fusion, that *espS* expression is up-regulated in a *croI* mutant under laboratory conditions. *espS* expression was restored to wild type levels upon addition of exogenous BHL, expression of *croI in trans* or by the introduction of a *croR* mutation. Thus, *espS* is a member of the *croIR* regulon of *C. rodentium*, linking QS, via the elaboration of a Type III effector, to murine pathogenesis.

A NEW FAMILY OF SECRETED BACTERIAL PROTEINS MEDIATES INTERACTIONS WITH EUKARYOTIC CELLS

Sudharsan Sathyamurthy, Magdalena Krol, Tom A Strickland, Claire R Wright, Jon R Sayers

University of Sheffield Medical School, Infection & Immunity, Sheffield, S102RX, United Kingdom

We have been studying small proteins secreted by a range of Gram negative bacteria. Erythrophilin, the prototype of this family, is secreted by *Neisseria meningitidis* and other members of the genus. As these bacteria can only survive within a human host we reasoned that the secreted proteins might interact with human proteins or cells. A yeast 2 hybrid screen suggested that the protein might bind a human membrane protein found in red blood cells. We found that it is capable of binding to and deforming red blood cells. Using transfected mouse cells we have confirmed the identity of the human receptor for this protein. We are in the process of analyzing a knock-out mutant strain of *Neisseria* to see how it behaves in comparison with its wild type parent.

It initially appeared that our erythrophilin protein was unique to the *Niesseria*, however, we have recently discovered that it is more widespread than we thought. Sequence analysis of the rapidly expanding bacterial genome databases has allowed us to identify homologous proteins encoded by human pathogens such as *Haemophilus influenzae*, *Providencia stuartii*, *Vibrio cholerae*, *Salmonella enterica*, and animal pathogens such as *Mannheimia haemolytica* (infects cattle and sheep), *Pasteurella dagmatis* (infects domestic pets), and a pathogen infecting catfish, *Edwardsialla ictaluri*. Several other species of bacteria also appear to encode a related protein such as *Curvibacter* (an endosymbiont of hydra), *Azorhizobium caulinodans* (plant-associated nodulating nitrogen fixing bacterium), and *Roseovarius* species which are associated with dinoflagellates. All of these proteins appear to possess a signal peptide and a common structure. As many of these organisms are either closely associated with or found only in association with a eukaryotic host, we speculate that this family of proteins is involved in mediating bacterial-host interactions. We have expressed and purified a number of erythrophilin-like recombinant proteins encoded by various pathogens and are carrying out wide ranging studies in order to characterize this interesting new family of bacterial proteins. The results of our structural, genetical, biochemical and cell biological experiments will be presented.

REFERENCE GENOMES AT THE HUMAN MICROBIOME PROJECT (HMP) DATA ANALYSIS AND COORDINATION CENTER (DACC)

Michelle Giglio[1], Heather Creasy[1], Joshua Orvis[1], Jonathan Crabtree[1], Konstantinos Liolios[2], Amy Chen[3], Amrita Pati[3], Konstantinos Mavrommatis[3], Victor Felix[1], Najveet Singh[4], Todd Desantis[4], Rob Knight[5], Gary Andersen[4], Victor Markowitz[3], Nikos Kyrpides[2], Jennifer Wortman[1], Owen White[1]

[1]University of Maryland Sch. of Med., Inst. for Genome Sciences, Baltimore, MD, 21029, [2]Joint Genome Institute, Genome Biology, Walnut Creek, CA, 94598, [3]Lawrence Berkeley National Lab, Computational Research, Berkeley, CA, 94720, [4]Lawrence Berkeley National Lab, Earth Sciences, Berkeley, CA, 94720, [5]University of Colorado, Chemistry and Biochemistry, Boulder, CO, 80309

The Human Microbiome Project (HMP) is an NIH Roadmap initiative that is collecting and analyzing information regarding the microbial communities of the human body. The goal of the HMP is to provide resources and tools to enable researchers to determine how differences in microbial communities contribute to human health and disease. This effort is producing huge quantities of data of numerous types including nucleotide sequence, metadata, and analysis results. Sequencing efforts are focused in three areas: sequencing of ~1000 reference genomes isolated from the human body, metagenomic sequencing of DNA samples from five HMP body sites in 300 healthy individuals, and metagenomic sequencing of samples from subjects suffering from particular diseases along with healthy controls. The reference genomes perform an important function in the analysis of the metagenomic sequences by providing guideposts in a sea of unknown sequences. Sequences from the metagenomic samples can be assigned to phylogenetic and functional groups through homology to the reference genomes. Responsibility for the coordination of the information produced from the HMP falls on the Data Analysis and Coordination Center (DACC). All publicly released information and analyses produced from the HMP are available on the DACC's comprehensive web resource (http://hmpdacc.org). Information that can be found on the DACC web site includes: all sequence data that has been released to the public via the DACC's download site, analysis results, a repository of HMP SOPs, and new software developed as part of the HMP. The web analysis toolsets for the DACC are based on those of the Integrated Microbial Genomes resource for both single genomes and metagenomes (img/hmp and img/m). Analyses for over 200 complete reference genome projects is currently available on the IMG HMP site (http://www.hmpdacc-resources.org/img_hmp) while information on the ~1,400 organisms on the reference genomes list can be found on the HMP Project Catalog (www.hmpdacc.org/project_catalog.html).

MINING THE E. COLI GENOME FOR THE STRESS-INDUCED-MUTAGENESIS GENE NETWORK

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Under growth-limiting stress, bacteria, yeast, and human cells display elevated mutation rates, termed stress-induced mutagenesis, under the control of stress responses. These mechanisms may increase a cell's or organism's ability to evolve specifically when it is maladapted to its environment, i.e., is stressed. Although previous work identified several genes, proteins, pathways and stress responses required for stress-induced mutation in E. coli, the total scope of the gene/protein network required for this mutation response was unknown. We undertook a transposon mutagenesis and genetic screen to identify the genes required for stress-induced mutagenesis in E. coli. The screen uses papillation of starving colonies to blue (based on lac– to Lac+ frameshift reversion of a constitutively transcribed lac gene) during carbon-starvation stress. We have identified genes involved in transcriptional regulation, metabolism, cell division, replication, DNA repair, and a large number in the electron-transport chain and ubiquinol biosynthesis. The mutation-defective phenotypes of some electron-transport/ubiquinol mutations can be reversed by mutation in arcB, which upregulates RpoS activity (which is required for stress-induced mutagenesis), implying that the electron-transport proteins may function in mutagenesis as the upstream signalers of the RpoS stress response during starvation. Supporting this interpretation, we find reduced expression from RpoS-regulated promoters in the ubiD and nuoC mutants in stationary phase. We also found 12 new genes of unknown function, most of which are conserved in bacteria, and a few of which are conserved to human. This network also contains regulators of the following stress responses: SOS DNA-damage, rpoS general stress, rpoE membrane unfolded-protein, phoU phosphate-transport, and others, some identified previously and some not. This network of genes will guide more detailed characterization of the mechanisms of stress-induced mutagenesis in E. coli and other organisms.

INVERTING A PARADIGM: MOLECULAR CHARACTERIZATION OF *ESCHERICHIA COLI* INSERTION MUTANTS ALTERED IN THEIR RESPONSE TO CHEMICALS

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Currently, cellular responses to chemicals are most often analyzed by whole transcriptome methods that are limited by the inability to separate the causal from the coincidental. Moreover, classically time-consuming mapping is undertaken only for the most interesting of mutations. Transposition and targeted insertional mutagenesis provide alternative approaches for identifying tolerance specifying genes. Technical advances now allow facile and detailed characterization of the output of genetic selections and screens. This was illustrated by the precise identification 107 butanol hypersensitive and thirteen butanol tolerant variants in a screen of an 11,000 plus member *Escherichia coli* transposon library. Phage 29 DNA polymerase mediated genomic amplification preceding sequencing of each chromosome-transposon junction resulted in the near complete molecular characterization of these genetic screens; improved ease and lower cost of sequence analysis suggest that such approaches will be applied more often at the earliest stages of genetic characterization. These analyses implicated stress responses and the cell surface as important determinants of *E. coli* tolerance to 1-butanol; this multifaceted response to 1-butanol was modulated in part by insertions within or polar upon *spoT* specifying a ppGpp synthetase while a knockout of *acrB*, encoding an efflux pump component, also conferred 1-butanol tolerance. Inactivation of a *spoT* homolog in *Lactobacillus plantarum* also lead to a butanol tolerant phenotype.

ARE TRANSPOSONS SELFISH?

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Transposases are the most abundant gene in the world, and are generally thought to be selfish DNA - just replicating for their own sake. Most genomes have transposons, and and they can move between hosts with relative ease. Typically, transposons are thought to be selfish genes, only replicating for their own survival. However, their vast abundance suggests that there are positive benefits to their presence that are being selected for. In addition to transposons, bacterial genomes are under assault from a range of invading DNA - especially phages and plasmids. For example, most bacterial genomes that have been sequenced contain at least one prophage. These are ticking time bombs, waiting to kill their unwitting host, and transition from a quiescent lysogenic state to a violently explosive lytic life cycle. We suggest that transposons aid their bacterial hosts by inactivating invading prophages and allowing the bacteria to clear the infection from their genome. The positive selection for transposons may ultimately lead to the death of transposons.

ECONOMICAL EVOLUTION: MICROBES OPTIMIZE THE SYNTHETIC COST OF EXTRACELLULAR PROTEINS

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Protein evolution is not simply a race towards improved function. Because organisms compete for limited resources, fitness is also affected by the relative economy of an organism's proteome. Indeed, many abundant proteins have a relatively high content of amino acids that are metabolically less taxing for the cell to make, thus reducing cellular cost. However, not all abundant proteins are economical and many economical proteins are not particularly abundant. Here we examined protein composition and found the relative synthetic costs of amino acids constrain the composition of microbial extracellular proteins. In *Escherichia coli* extracellular proteins contain, on average, fewer energetically-expensive amino acids independent of their abundance, length, function, or structure. Economic pressures have also strategically shaped the amino acid composition of multi-component surface appendages such as flagella, curli, Type I pili; and extracellular enzymes including Type III effector proteins and secreted serine proteases. Furthermore, *in silico* analysis of *Pseudomonas syringae, Mycobacterium tuberculosis, Saccharomyces cerevisiae* and over 25 other microbes spanning a wide range of GC-content revealed a broad bias towards more economical amino acids in extracellular proteins. Thus economy may address the compositional bias seen in many extracellular proteins and deliver further insight into the forces driving their evolution. Microbes secrete proteins to perform essential interactions with their environment such as motility, pathogenesis, biofilm formation and resource acquisition. However, because microbes generally lack protein import systems, secretion is often a one way street. Consequently, extracellular proteins present a greater burden on the cell as their amino acids cannot be re-utilized during translation. We hypothesize evolution has in turn optimized extracellular proteins to reduce their synthetic burden on the cell.

SELF-TARGETING BY THE PROKARYOTIC CRISPR IMMUNE SYSTEM: GENE REGULATION OR AUTOIMMUNITY?

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CRISPR/Cas is a recently discovered prokaryotic immune system, which is based on small RNAs ("spacers") that restrict phage and plasmid infection. It has been hypothesized that CRISPRs can also regulate self gene expression by utilizing spacers that target self genes. By analyzing CRISPRs from 330 organisms we found that one in every 250 spacers fully matches self-DNA, and that such self-targeting occurs in 18% of all CRISPR-bearing organisms. However, we revealed that self-targeting spacers display complete lack of evolutionary conservation across species. We found that the acquisition of self-targeting spacers is often a relatively recent evolutionary event, and further found evidence pointing at the fact that the self-targeting spacers or even the entire CRISPR/cas locus may have become inactivated. This suggests that self-targeting is a consequence of *autoimmunity* rather than gene regulation. We suggest that accidental incorporation of endogenous DNA as new spacers is the cause of this autoimmunity, and that the rate of such insertions is at least 0.4%. Thus, coding for CRISPR/*cas* might incur a fitness cost. To prevent the negative effects associated with autoimmunity, inactivation of CRISPR or the self-targeting spacer must occur. This view may explain the patchy distribution of degraded and functional forms of CRISPR systems across prokaryotic domains.

THE E. COLI -PHAGE LAMBDA PROTEIN INTERACTION NETWORK

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Despite decades of intense research on phage lambda, few systematic studies have been undertaken to illuminate the global impact of the pathogen on its hosts. Only recently some papers (e.g. Osterhout, 2007) were published employing microarray techniques to unravel the hosts' response to phage infection and induction. However, to our knowledge a comparable global approach has not been attempted for E. coli -phage lambda protein-protein interactions (PPIs).
Here we describe yeast two-hybrid screens of all phage lambda proteins against each other as well as against a complete E. coli K12 (W3110) ORF-library employing our well-established and previously successful high-throughput screening system (Uetz, 2006; Titz, 2008). This resulted so far in the identification of 66 lambda-lambda, as well as 131 lambda-host PPIs. While some of these interactions such as gpA – gpNu1 heterodimer formation have been found previously, most others are new.
In conjunction with previous studies, these data will not only lead to a more detailed knowledge of lambda's ability to assemble its proteins into a mature particle but also shed light on the role of host proteins in the infection and replication process. Hence we integrated structural, transcriptional, as well as other information into a network of phage-host interactions.

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DISCOVERY OF NOVEL PATHWAYS USING COMPARATIVE GENOMICS

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We present a computational method for discovery of novel pathways from annotated genomes. We present candidate pathways from *E. coli* and seek collaborators to investigate them. The method relies on sequence information, and on patterns of presence or absence of genes across hundreds of genomes. Even though the main goal of the method is to discover new pathways, it can also be used, for example, to find new protein complexes or other kinds of functional groups, and, in some cases, find functional gene groups for genes of interest.

The proposed method consists of three steps. Step 1 generates a functional similarity score for each pair of genes of interest using the gene neighbor method. Given two genes, this method generates a score that reflects the frequency with which the best homologs of those genes are close to one another in 343 reference genomes. When this score exceeds a certain threshold we declare the two genes to be functionally related. Step 2 finds groups of genes where the member genes are functionally related to each other. This step results in a set of candidate functional groups. When the group list is large, step 3 selects the highest confidence groups by considering the number of organisms in a reference list of bacterial genomes for which the group is significantly enriched.

We tested the accuracy of our method in *Escherichia coli* K-12 using 922 protein complexes and 300 pathways curated from the literature into EcoCyc version 13.1. We find that, from all the candidate groups of size 4 or larger generated by our method, 35% of them are correct. We define a candidate to be correct if at least 70% of its genes are in a known pathway or protein complex. Correctness decreases to 28% if the third filtering step is not used. The program finds 41 of the 261 pathways and complexes in EcoCyc that include at least 4 genes. We declare a pathway or complex as found by our method if at least 70% of its genes are included in some candidate. Examples of pathways and complexes for which a perfectly matching candidate is found are the histidine and tryptophan biosynthesis pathways, and the ATP synthase complex.

From the 65% of candidates that do not match a known EcoCyc pathway or complex, some are likely to be novel functional groups. For example, one predicted *E. coli* group contains fixA, fixB, ydiQ, ydiR, ygcR, and ygcQ. FixA and fixB are known to be required for anaerobic carnitine reduction, ydiQ and ydiR are predicted subunits of an acyl-CoA dehydrogenase, and ygcR and ygcQ are predicted flavoproteins. Taken together, this may be a previously uncharacterized electron transfer pathway. These genes occur in three different places in the *E. coli* genome, and thus the group is not likely to be obvious via a simple examination of the genome.

OLIGONUCLEOTIDES STIMULATE GENOMIC ALTERATIONS OF *LEGIONELLA PNEUMOPHILA*

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Genetic variation generates diversity in all kingdoms of life. The corresponding mechanisms can also be harnessed for laboratory studies of fundamental cellular processes. Here we report that oligonucleotides (oligos) generate mutations on the *L. pneumophila* chromosome by a mechanism that is independent of RecA. In addition to single nucleotide changes, oligos can stimulate excision of ≥ 2.1 kb of chromosomal DNA and insertion of ≥ 18 bp. The DNA replication process likely contributes, either directly or indirectly, since oligo-induced mutagenesis requires as few as 21 nucleotides of homology, is strand-dependent, and is most efficient in exponential phase. However, in contrast to λ-Red recombination, oligos that are non-extendable by DNA polymerase are also mutagenic. Oligos with non-homologous flanking on both 5' and 3' termini decrease, but do not abolish, mutagenesis, suggesting the cell may process these overhangs. Compared to *E. coli*, *L. pneumophila* encodes fewer canonical single-stranded exonucleases, and inactivation of its RecJ and ExoVII nucleases substantially increases its frequency of oligo-induced mutagenesis. Oligo modification with phosphorothioate bonds and Locked Nucleic Acid (LNA) bases further increases the frequency of mutagenesis. We exploit this endogenous activity to generate chromosomal deletions by excising a counter-selectable cassette and to insert an epitope into a chromosomal coding sequence.

In addition to use as a genetic tool, oligo-induced mutagenesis may have evolutionary implications. *L. pneumophila* is a parasite of amoebae and protozoa that can obtain exogenous DNA via natural transformation. Accordingly, the ability of short regions of homology to mediate incorporation of divergent DNA sequences, including those that encode eukaryotic-like motifs, may contribute to the extraordinary genome plasticity and evolution of pathogenic *L. pneumophila*.

RIBONUCLEASE P: WHY IS IT ESSENTIAL IN *E. COLI* ?

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The endoribonuclease RNase P is an essential enzyme involved in multiple aspects of RNA metabolism in *Escherichia coli*. Its primary function has always been assumed to be the generation of mature 5' termini on all 86 *E. coli* tRNAs. However, with the identification of polycistronic tRNA transcripts (i.e. *valV valW* and *leuQ leuP leuV*) that require RNase P to separate the pre-tRNAs (1) and the report that tRNAs with immature 5' ends are functional *in vivo*, it was suggested that the essential function of RNase P might be related to its requirement for processing of specific polycistronic tRNA transcripts (2). Since there are multiple copies of most tRNAs, we investigated whether RNase P might be responsible for the primary processing of all members of a particular tRNA family. Here we report that the processing of all seven valine tRNAs, which are present in three polycistronic operons (*valV valW*, *valU valX valY lysV* and *lysT valT lysW valZ lysY lysZ lysQ*), requires RNase P to generate the pre-tRNAs that are subsequently converted into mature species. As previously reported, the *valV valW* operon is separated by RNase P (1). In the case of the *valU* operon, RNase P separates all four pre-tRNAs. Processing of the *lysT* operon is more complex in that RNase P separates the two valine tRNAs from the downstream lysine species but RNase E is used to generate the lysine pre-tRNAs. We are currently attempting to see if the failure to generate any functional valine tRNAs in an RNase P mutant is the cause of cell inviability.

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A HIGH–THROUGHPUT SCREEN FOR SMALL MOLECULE INHIBITORS OF TOXT, A VIRULENCE GENE ACTIVATOR IN *VIBRIO CHOLERAE*.

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Vibrio cholerae is a water-borne pathogen that can colonize the gut and secrete cholera toxin (CT) which causes diarrhea, dehydration, and, if left untreated, death. Production and secretion of CT is regulated by a cascade of transcriptional activation, such that CT is only transcribed when Vibrio senses it is in the gut of a host. ToxT is considered the master regulator of virulence in this pathway, because it directly activates CT transcription as well as over 20 more virulence genes. In order to gain a deeper understanding of how the cascade regulates virulence, we developed a GFP-based assay for use in a high-throughput screen to search for small molecule inhibitors of toxT transcription. The primary screen, in which over 63,000 compounds and 11,000 natural product extracts were tested, revealed 512 potential hits. Of these, 175 were confirmed active and behaved in a dose-dependent manner. Of the top 21 compounds, 17 decreased CT secretion. The mechanisms of inhibition for the top 3 compounds are currently being determined.

NOVEL INTER-RELATIONSHIPS BETWEEN RNA DEGRADATION AND FACTOR-DEPENDENT TRANSCRIPTION TERMINATION IN *E. COLI*

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Two of the important enzymes for RNA degradation in *E. coli* are RNase E and RppH. The former is an endonuclease of length 1061 amino acids comprised of a catalytic domain in its N-terminal half (NTH) and a "degradosome-assembly" domain in its C-terminal half (CTH). The endonucleolytic activity of RNase E is activated by the presence of a monophosphoryl (mono-P) group at the 5'-end of its RNA substrate, and RppH has been identified as an RNA 5'-pyrophosphohydrolase that stimulates RNase E-mediated RNA degradation by generating 5'-mono-P ends on RNAs. Although RNase E (*rne*-encoded) is essential, its CTH is dispensable for viability. Δ*rppH* strains are also viable.

Factor-dependent transcription termination involves, among others, the products of the *rho* and *nusG* genes, which are essential in "wild-type" *E. coli* strains such as MG1655. These proteins mediate the termination of nascent untranslated transcripts, and thus contribute to the phenomenon of transcriptional polarity. Earlier results from our laboratory had suggested that inefficient transcription termination in *rho* and *nusG* mutants is associated with an increased propensity for formation of R-loops (RNA-DNA hybrids) in the genome (Harinarayanan and Gowrishankar, 2003; Gowrishankar and Harinarayanan, 2004).

In this study, we have identified novel inter-relationships between *rne* and *rppH* on the one hand, and *rho* and *nusG* on the other. Thus, several phenotypes of the *rho* and *nusG* mutants, including lethality with the R-loop-dependent plasmid pACYC184, are suppressed by *rne*-ΔCTH mutations. Conversely, the *rho* and *nusG* mutations suppress the synthetic lethality of *rne*-ΔCTH Δ*rppH* derivatives, provided that the strains are also *rnhA*+ (that is, RNase H1-proficient). In a "reduced-genome" *E. coli* strain MDS42 in which Δ*nusG* is not lethal, it could be shown that Δ*nusG* also rescues the inviability associated with *rne*-ΔCTH Δ*rppH*. An RNase E variant with a combined deletion of its CTH and an R169Q substitution in its "5'-sensor-domain" is also associated with inviability, unless the strain carries a *rho* or *nusG* mutation. These results are discussed in terms of a model in which (i) RNase E carries out its essential mRNA degradative function by either of two pathways that are CTH-dependent or 5'-end-dependent, respectively; and (ii) R-loop-dependent mRNA degradation by RNase H1 serves as a mechanism to bypass the essentiality of RNase E in the *rho* and *nusG* mutants.

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CHARACTERISATION OF *HDA* SUPPRESSOR MUTANTS (HSM´S)

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In nearly all bacteria, duplication of the chromosome is coordinated by DnaA. In Escherichia coli, DnaA maintains an appropriate copy number of chromosomes by ensuring that replication occurs simultaneously from all origins present in the cell and only once per cell cycle. Initiation of replication occurs when the amount of DnaAbound to ATP has reached a certain threshold. After initiation of replication the level of DnaA-ATP is decreased by a process called RIDA (Regulatory inactivation of DnaA. The major elements of the RIDA process are the DnaN sliding clamp of DNA polymerase III and a DnaA paralogue called Hda. Hda is recruited by DnaN at the replicating origin and stimulates the conversion of the active ATP-bound form of DnaA to the inactive ADP-bound form which prevents further replication initiation. Accordingly, deletion of the hda gene leads to over-initiation of DNA replication and cell death. A number of hda suppressor mutations (hsm´s) have been isolated in our laboratory. Among these are two mutants in the dnaA gene (hsm2 and 4),a point mutation in the iscU gene (hsm5) and a C-terminal deletion of the flavine reductase (hsm6). Here we characterize the mechanisms by which these mutations alter the initiation of replication in order to counterbalance to the hyperactivity of DnaA in an hda mutant background.in an hda mutant background.

GENETICAL ENGINEERING OF THE GP37, GP38 AND GP12 T4 BACTERIOPHAGE GENES INVOLVED IN THE PHAGE-HOST RECOGNITION WITH ESCHERICHIA COLI

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With the advent of pathogenic bacteria mass-resistance strains to most antibiotics, bacteriophage therapy is regaining attention. Bacteriophages do provide some solutions but their use is limited by a rapid development of resistance in bacterial target populations and the ineffectiveness of selecting new phage strains from nature. Therefore, we decided to genetically engineered bacteriophages in order to change their host range, while maintaining their capacity to replicate. We have developed a nested PCR-based technology, named TAPE, to extensively and stochastically modify the gp37, gp38 and gp12 T4 lytic bacteriophage genes that encode proteins involved in interactions with its host Escherichia coli. The technology utilizes interplay between error-prone and high fidelity PCR reactions: random point mutations are introduced into DNA sequences, while the identity of pre-defined inner segments is preserved in order to keep the functional characteristics of the protein. The modifications were introduced by decreasing the fidelity of the Taq polymerase during DNA synthesis or by random incorporation of nucleotide analogues. This is followed by a series of selective high-fidelity reactions that reconstruct a wide spectrum of variant forms of the genetic region of interest, while automatically eliminating the products containing mutations within the domains selected to remain constant. Our data demonstrated that this technology was very efficient to obtain functional variants of the three wild-type T4 bacteriophage genes. Such engineered genes variants are inserted into the T4 wild-type genome and modified T4 phage particles are produced to obtain a very large functional lytic phage banks (cf. Poster, Production of Engineered and Functional T4 Bacteriophages Bank that Target Pathogenic Gram Negative Bacteria).

THE REPLICATION INITIATION PROTEINS DNAD AND DNAB ASSOCIATE WITH DNAA AT ITS SECONDARY TARGETS IN *BACILLUS SUBTILIS*

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Initiation of DNA replication requires binding of the initiator protein, DnaA, to the chromosomal origin of replication, *oriC*. In *Bacillus subtilis* and other low G+C content Gram-positive bacteria, the primosomal proteins DnaD and DnaB, in conjunction with loader DnaI, help load helicase (DnaC in *B. subtilis*) at *oriC*. DnaA also acts as a transcription factor at several sites outside of the *oriC* region. Prompted by the association of DnaA with these secondary sites and the previously demonstrated dependence on DnaA of the replication initiation proteins DnaD and DnaB for their association with *oriC*, we determined the genome wide co-localization of these factors in vivo in *B. subtilis*. We found that DnaD and DnaB, but not helicase, are recruited de novo to the secondary targets of DnaA. The order of recruitment of DnaD and DnaB to the secondary loci is independent of *oriC* yet demonstrates a comparable hierarchy to that of *oriC*. The presence of DnaD and DnaB at the secondary targets of DnaA in the absence of helicase loading is suggestive of a role in modulating a general activity of DnaA.

IN-VIVO, SINGLE-MOLECULE CHARACTERIZATION OF THE MINCDE SYSTEM'S LOCALIZATION AND DYNAMICS

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In bacterial cell division, the accurate placement of the septal ring at midcell is of inherent importance. One important system in *E. coli* that performs this task is the MinCDE system, which prevents peripheral division complex formation through the pole-to-pole oscillation of MinC, a known FtsZ antagonist. It was proposed that this oscillatory redistribution is driven by the molecular interactions intrinsic to MinD and MinE, namely cooperative membrane-bound self-assembly and regulation of membrane association-dissociation via ATP binding and hydrolysis. However, the molecular details of this proposal are difficult to verify *in vivo* using conventional ensemble-based methods due to the highly dynamic nature of the system. In this work we employ single-molecule techniques to identify structural details of the previously reported cytoskeleton-like, helical framework of the Min proteins with 30-nm spatial resolution, and their membrane-associated dynamics with millisecond time resolution. We provide evidence elucidating the molecular mechanisms responsible for the self-organized, oscillatory behavior of the MinCDE system that ultimately govern the proper placement of the septum.

SEQA CONTRIBUTES TO CONTROL THE AMOUNT OF DNAA MOLECULES AVAILABLE FOR INITIATION OF REPLICATION

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The initiation of replication in bacteria is a complex and highly regulated process centered around the control of DnaA activity. This is particularly manifested in fast growing bacteria such as *Escherichia coli* where multiple rounds of replication are taking place, yet initiation of replication occurs simultaneously at all origin of replication and only once per cell cycle. The means by which DnaA amount and activity is regulated in order to facilitate this degree of regulation is not fully understood, but thought to rely on mechanisms that act post-initiation while newly replicated origins are sequestered by the Dam/SeqA system. During Sequestration i) the absolute amount of operative DnaA molecules in the cell is reduced by a titration mechanism and by cessation of *de novo* DnaA synthesis and ii) the activity of DnaA is reduced by Regulatory Inactivation of DnaA, also called RIDA. DnaA is an ATPase that is active in its ATP-bound state. Only in its ATP-bound state is DnaA capable to fire the initiation of replication. The major elements of the RIDA process are DnaN and Hda. Hda is recruited by DnaN at the replicating origin and stimulates the conversion of the active ATP-bound form of DnaA to the inactive ADP-bound form which prevents further replication initiation. As a consequence, deletion of the *hda* gene leads to a higher proportion of DnaA molecules bound to ATP, thus over-initiation of DNA replication and cell death.
In order to discover new factors involved in the control of the DnaA-ATP/DnaA-ADP ratio, we sequenced the genomes of spontaneous *hda* suppressor mutants. One of these carries a mutation in the promoter region of SeqA. Our results point to a possible role for SeqA in the control of a free pool of DnaA molecules.

THE TRNASE COLICIN D UNDERGOES ENDOPROTEOLYTIC CLEAVAGE DURING ITS IMPORT INTO THE CYTOPLASM OF TARGET CELLS

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Colicin D is a tRNase which kills target cells by cleaving the four tRNA-Arg isoacceptors. Colicin D producer cells are resistant to their own and exogenous toxins because they constitutively express an immunity protein (ImmD). The ImmD protein is tightly bound to the C-terminal catalytic domain of colicin D during secretion. To enter the periplasm Colicin D parasitizes the outer-membrane receptor FepA and the energy transducing TonB pathway. Several colicins are known to remain in contact with their receptor, when their nuclease domain enters the cytoplasm and their Imm protein is released. This suggests that an endoproteolytic cleavage may occur prior to or during the translocation across the cytoplasmic membrane. There is genetic evidence that colicin D undergoes proteolytic processing, so that only the C-terminal domain penetrates the cytoplasm. The inner membrane signal peptidase LepB was shown to be specifically required for cell killing by colicin D. We showed *in vitro* that, in the presence of both LepB and the outer-membrane protease OmpT, colicin D is cleaved at a unique site, located just upstream of the tRNase domain. However, this cleavage appears to have no relationship to colicin import, since the inactivation of the ompT structural gene has no effect on colicin toxicity. Interestingly, we detected *in vivo* a different colicin D cleavage product carrying the catalytic domain. As expected, OmpT is dispensable for the *in vivo* cleavage. In contrast, there is no *in vivo* cleavage in the absence of LepB or another inner membrane peptidase FtsH. It is known that FtsH is essential for cell killing by all nuclease colicins. Our results support the possibility that the colicin processing is an essential step common to the translocation of other nuclease colicins.

REGULATION OF THE *STM2209-STM2208* OPERON OF *SALMONELLA ENTERICA* BY DAM METHYLATION AND OXYR

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DNA adenine methyltransferase (Dam methylase) regulates multiple cell processes, such as DNA mismatch repair, initiation of chromosome replication, chromosome segregation, and transcription of certain genes. Our work deals with one particular locus of *Salmonella enterica* serovar Typhimurium under Dam methylation control: the *STM2209-STM2208* transcriptional unit.

STM2209-STM2208 is a *Salmonella*-specific bicistronic operon that encodes inner membrane proteins. Transcription of *STM2209-STM2208* is subjected to phase variation in wild type *Salmonella*. Expression is very low in the majority of cells (OFF state). However, a bacterial subpopulation undergoes phase variation switch, and expresses the operon (ON state). Switching frequencies are approximately 6×10^{-4} (OFF → ON) and 4×10^{-2} (ON → OFF) per cell and generation.

In *Salmonella dam* mutants there is no phase variation, as all cells are expressing the operon (ON), and *STM2209-STM2208* expression is locked in the ON state. Hence, *STM2209-STM2208* may be a novel example of a phase variation locus subjected to epigenetic transcriptional control. Such a control appears to be exerted at the *STM2209-STM2208* UAS, and involves a symmetrical element containing 4 GATC sites.

A genetic screen revealed that a transposon insertion in the *oxyR* gene completely abolished *STM2209-STM2208* expression, locking the phase variation system in the OFF state. Hence, the LysR-type factor OxyR is an activator of *STM2209-STM2208*. Indeed, two putative OxyR-binding motifs are present in the UAS, overlapping with two of the GATC sites. Our current hypothetical model for transcriptional regulation of *STM2209-STM2208* involves prevention of OxyR binding by Dam methylation.

CIS-ACTING ELEMENTS CONTROLLING EXPRESSION OF THE MASTER VIRULENCE REGULATOR *ATXA* IN *BACILLUS ANTHRACIS*

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Expression of the *Bacillus anthracis* anthrax toxin and capsule genes is controlled by the regulatory protein AtxA. A *B. anthracis* strain devoid of *atxA* is attenuated in a murine model demonstrating the necessity of *atxA* expression for virulence. Multiple studies suggest that precise control of the timing and steady state level of *atxA* expression is critical for optimal expression of AtxA target genes. In previous work, the transition state regulator AbrB was demonstrated to repress *atxA* transcription by binding to specific sequences upstream of the transcript start site. Here we show that *atxA* promoter activity is dependent upon a promoter consensus sequence for the housekeeping sigma factor SigA. Site-directed mutagenesis of the sequence results in loss of *atxA* promoter activity. We also report that an uncharacterized *trans*-acting protein(s) binds specifically to *atxA* promoter sequences and impacts promoter activity. Results of electrophoretic mobility shift assays, using an *atxA* promoter probe and *B. anthracis* crude extract, indicate that a soluble *trans*-acting protein(s) interacts specifically with the *atxA* control region. Treatment of the extract with proteinase K results in degradation of total protein and a diminished DNA-shift. Results of 3' deletion analysis of the *atxA* control region demonstrate that sequences downstream of the transcription initiation site are required for binding of the protein(s) and regulation of *atxA* promoter activity. Promoter activity increased 7-fold when sequences from +13 to +31, relative to the transcription start site (+1), were deleted. Site-directed mutagenesis of the putative 18-nt binding region revealed sequences from +14 to +22 are important for binding of the *trans*-acting protein(s). Promoter activity increased 4-fold when sequences from +14 to +22 were mutated. We propose a model whereby a *trans*-acting protein(s) binds to the *atxA* promoter in an AbrB-independent manner and that multiple levels of *atxA* regulation ultimately impact expression of the *B. anthracis* virulence genes.

IRON REGULATION OF AMYLOIDOGENIC CURLI FIBERS

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Amyloids are protein aggregates that are historically associated with human disorders such as type II diabetes, Parkinson's disease, and Alzheimer's disease. Recently a growing class of functional amyloids has been described. Unlike disease-associated amyloids, functional amyloids are synthesized by intricate cellular pathways and are not harmful to the organism that makes them. Curli are functional amyloids produced by *Escherichia coli* and other enteric bacteria. The curli biosynthesis genes are encoded on two divergently transcribed operons, *csgDEFG* and *csgBA*. CsgD is a transcriptional activator of the *csgBA* operon, which encodes the curli fiber subunits, and *csgD* expression is induced under various environmental stresses such as iron starvation. Iron provides a fascinating link between functional amyloids and disease-associated amyloids. Disease-associated amyloid aggregates are often linked with high local iron levels, and our data show that curli expression and iron homeostasis are intricately connected. In broth, *E. coli* forms a pellicle at the air/liquid interface, and addition of an iron chelator enhances pellicle morphology and induces *csgBA* expression. Furthermore, high iron represses transcriptional expression of both *csgDEFG* and *csgBA* and subsequently reduces CsgA protein levels on solid media. Iron also regulates the *E. coli* red, dry and rough (RDAR) morphotype, a complex colony formation that develops when enteric bacteria are plated under curli-inducing conditions. High iron levels induce RDAR formation, and RDAR is associated with colony expansion on agar plates. In this way iron acts as a chemotactic agent for solid surface motility. Our continuing studies are revealing the signaling pathways that link iron regulation and curli production and the role of curli fibers in *E. coli* motility.

AUG TRIPLETS IN THE *AROL* UNTRANSLATED REGION INFLUENCE AROL EXPRESSION AND BIND RIBOSOMES INDEPENDENTLY OF A SHINE-DALGARNO SEQUENCE IN *ESCHERICHIA COLI.*

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The *Escherichia coli aroL* gene encodes shikimate kinase II, an enzyme involved in the biosynthesis of aromatic amino acids. The *aroL* mRNA contains an untranslated leader region of 124 nucleotides which includes a canonical Shine-Dalgarno (SD) sequence upstream to the *aroL* start codon. The *aroL* mRNA contains three AUG triplets (AUG-1, AUG-2 and AUG-3) within the untranslated leader located 59, 64, and 79 nucleotides from the transcriptional start site, respectively; none are preceded by a SD sequence. Ribosome-binding assays (toeprints) support tRNA-dependent binding of 30S subunits to the *aroL* start codon and the upstream AUG triplets. The third codon of the AUG-1 reading frame is a stop codon. The AUG-2 reading frame encodes a putative 21 amino acid peptide, with a stop codon overlapping the *aroL* start codon, suggesting translational coupling of *aroL* to the AUG-2 reading frame. AUG-3 is located at codon 6 of the AUG-2 reading frame. Translation initiating from each AUG triplet was measured using translational fusions to the *lacZ* reporter gene. Fusion of AUG-2 to *lacZ* produced approximately 30% of the activity measured when the *aroL* coding sequence was fused to *lacZ*. AUG-1 and AUG-3 fusions to *lacZ* did not support significant activity.

Site-directed mutagenesis of the upstream AUG triplets to AUC had varying effects on expression from the downstream *aroL* start codon. While mutagenesis of AUG-1 to AUC had a negligible effect, mutagenesis of AUG-2 to AUC reduced *aroL* expression by approximately 30%, supporting the translational coupling of *aroL* to AUG-2's open reading frame. The greatest reduction in *aroL* expression, approximately 75%, was observed when all three upstream AUG triplets were mutated to AUC.

Although AUG-2 and AUG-3 triplets bind 30S subunits, they appear to do so in the absence of a canonical SD sequence. Current data (toeprint assays and *lacZ* fusion reporter assays) suggest that the sequence important for ribosome binding to AUG-2 resides 18-27 nucleotides upstream of AUG-2. Furthermore, ribosome binding to AUG-3 appears be dependent on sequence upstream as well as downstream of the AUG-3 triplet. The *aroL* SD sequence does not appear to play a role in ribosome binding to the upstream AUG-triplets. Research is underway to identify all signals required for ribosome binding to the upstream AUG triplets and to understand how the signals influence the ribosome-mRNA interaction.

A WIDESPREAD FAMILY OF POLYMORPHIC CONTACT-DEPENDENT TOXIN DELIVERY SYSTEMS IN BACTERIA

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Bacteria have developed mechanisms to communicate and compete with one another in diverse environments. A new form of intercellular communication, contact-dependent growth inhibition (CDI), was discovered recently in *Escherichia coli*[1]. CDI is mediated by the CdiB/CdiA two-partner secretion system. CdiB is an outer membrane protein that facilitates secretion of the CdiA 'exoprotein' onto the cell surface. An additional immunity protein (CdiI) protects CDI+ cells from autoinhibition [1,2]. The mechanisms by which CDI blocks cell growth and CdiI counteracts this growth arrest are unknown. Moreover, the existence of CDI activity in other bacteria has not been explored. Here we show that the CDI growth inhibitory activity resides within the carboxy-terminal region of CdiA (CdiA-CT), and that CdiI binds and inactivates cognate CdiA-CT, but not heterologous CdiA-CT. Bacteria deleted for the CdiA-CT and CdiI coding regions are unable to compete with isogenic wild-type CDI+ cells, indicating that CDI systems confer a significant growth advantage. Bioinformatic and experimental analyses show that multiple bacterial species encode functional CDI systems with high sequence variability in the CdiA-CT and CdiI coding regions. CdiA-CT heterogeneity implies that a range of toxic activities are utilized during CDI. Indeed, CdiA-CTs from uropathogenic *E. coli* and *Dickeya dadantii* were found to have different nuclease activities, each providing a distinct mechanism of growth inhibition. The polymorphic nature of CDI suggests that these systems constitute an intricate immunity network with implications for bacterial competition.

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IDENTIFYING AND CHARACTERIZING THE TOXT AND H-NS BINDING SITES IN THE CHOLERA TOXIN PROMOTER OF *VIBRIO CHOLERAE*

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Vibrio cholerae is a gram-negative curved rod that causes the severe diarrheal disease cholera. After ingestion by the host, the bacterium produces virulence factors including cholera toxin (CT), which is directly responsible for the large volumes of water loss in the intestine during acute cholera. The regulatory network controlling virulence gene expression is complex and responds to various environmental signals and transcription factors. Ultimately ToxT, a member of the AraC/XylS transcription regulator family, is responsible for activating transcription of the virulence genes.
V. cholerae virulence gene promoters all contain one or more copies of the toxbox, a 13 base pair DNA sequence that ToxT recognizes. The 5' half of the toxbox sequence is well conserved and contains an invariant tract of four consecutive T nucleotides, whereas the 3' half of the toxbox sequence is not highly conserved other than being A/T rich. The binding of ToxT to toxboxes is required to activate the transcription of virulence genes and these binding sites have been characterized in several virulence gene promoters. However, the toxboxes required for activating transcription from the cholera toxin promoter have not been identified. The cholera toxin promoter contains a series of heptad repeats (GATTTTT) each of which matches the 5' half of the toxbox consensus sequence and is a potential binding site for ToxT. Using site-directed mutagenesis and high resolution Copper-Phenanthroline footprinting, we have determined which of these heptad repeats are required for CT expression. The A/T rich regions of the cholera toxin promoter also provide binding sites for H-NS, a global transcriptional repressor in gram-negative bacteria. The current model suggests that H-NS is de-repressed by ToxT to activate transcription of the CT genes, *ctxAB*. Our goal is to understand the interplay between ToxT and H-NS and their interaction with the *ctxAB* promoter.

THE COMPLETE GENOME SEQUENCES OF *HELICOBACTER PYLORI* STRAIN SS1: SEQUENCING A PARTIALLY CLONAL POPULATION

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The pathogenic bacterium *Helicobacter pylori* lives in the stomach lining of more than half of the world's population, where it causes ulcers and stomach cancer. The genome of *H. pylori* is highly variable, resulting in many different strains with unique infectious properties. Although over 9 strains have already been completely sequenced, none are capable of infecting mice, a common animal model for studying *H. pylori*'s pathogenicity.

We have sequenced the genome of *H. pylori* strain SS1, the most-characterized mouse-infecting strain, using 454 and SOLiD mate-pair technology. We performed the sequencing on a partially clonal culture that was many generations removed from single colony isolation, which is representative of *H. pylori* cultures used in laboratory experiments. Our extremely high fold-coverage (>100x for 454, >400x for SOLiD) and use of mate-pair data has allowed us to not only determine the consensus genome sequence, but also to observe the genetic changes which have occurred in subpopulations of the sequenced culture.

Surprisingly, the genome of *H. pylori* strain SS1 appears relatively stable, despite considerable laboratory passage and the well-documented interstrain variability of this organism. However, movement of a transposon (a homolog of IS607) among four insertion sites is occurring within the sequenced culture. The large depth of read coverage also allows an estimation of the frequency of SNPs among subpopulations: 8 SNPs are found in over 25% of the population, and 40 have a frequency above 5%. In addition, we have discovered that SS1 has a major rearrangement of the *cag* pathogenicity island, a set of genes crucial for *H. pylori*'s ability to cause severe disease – a result which may shed light both on the strain's rare ability to infect mice, and its observed deficiencies in *cag* function.

The sequencing of a partially clonal population of H. pylori SS1 not only provides the consensus genome sequence for an organism used frequently in research, but also informs researchers of the types of genetic variations to expect within the cultures used in their laboratory experiments. Genomes are not static, and consensus sequences fail to capture the variation present in cell cultures. This new technique exposes a genome in flux, and creates a new window into the population dynamics of this common bacterial pathogen.

PHANTOME (PHAGE ANNOTATION TOOLS AND METHODS): A PLATFORM FOR PHAGE ANNOTATION AND COMPARATIVE GENOMICS

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Having a consistent and accurate phage gene nomenclature is critical to phage research, and discriminating viral versus microbial genes will help all researchers seeking to understand microbial genomes and metagenomes. Here we present PhAnToMe (PHage ANnotation TOols and MEthods), a new platform for phage genome annotations and comparative phage genomics. PhAnToMe relies on the SEED database to store and compare phages and prophage genomes, develop a controlled vocabulary and a consistent nomenclature for phage genes, and create new tools for reliable prophage identification. PhAnToMe provides high-quality annotations to all phage and prophage genomes and dozens of existing phage metagenomes. Currently, PhAnToMe comprises the sequences, annotations, and metadata of 637 phage genomes in addition to the > 1,000 prophages integrated within bacterial and archaeal genomes.

Over 40 subsystems have been developed for most phage functional and structural modules, and these allow the classification of phage proteins into families based on a combination of computational and human expert-based methods. PhAnToMe Labs, our test bed for new research ideas, includes tools to find DNA repeats, map phages to metagenomes (and vice-versa), and find phage signature genes. Using PhAnToMe researchers may analyze the public genomes or bring their own sequences for analysis and annotation.

Finally, the tools and high-quality annotations developed in this project are being used for annotating user-uploaded phage sequences. The annotation pipeline includes gene calling, RNA finding, gene annotation, and preliminary reconstruction of structural and functional modules in uploaded phages. Future developments include a tool to predict whether a phage is virulent or temperate, lysin gene prediction tools, and tools for phage analysis in ecosystems.

IDENTIFYING FACTORS THAT INHIBIT BACTERIAL AMYLOID ASSEMBLY

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Amyloid formation has traditionally been described as the aggregation of off-path or misfolded proteins. Amyloid deposits are associated with many neurodegenerative conditions, including Alzheimer's, Parkinson's, and Huntington's diseases. Protein folding and amyloid formation/prevention is often mediated by molecular chaperones. Recently, a new class of "functional amyloids" has emerged. Unlike disease-associated amyloids, functional amyloids are assembled through a dedicated and highly controlled pathway. Our lab studies the biogenesis of curli amyloid fibers by bacteria. Curli are cell surface-associated protein polymers assembled by many enteric bacteria that aid in biofilm formation and host colonization during bacterial infection. The high degree of regulation and control of curli biogenesis enables the bacterium to avoid premature amyloidogenesis and amyloid-related cytotoxicity within the cell. I have evidence that periplasmic chaperones can prevent curli subunit aggregation. Periplasmic protein extracts from curli specific gene deletion strains have chaperone-like activity in vitro. Induction of periplasmic chaperone activity is curli specific and depends on the presence of either the major or minor curli subunits, CsgA or CsgB. These data suggest the involvement of molecular chaperones in preventing premature amyloid formation in the periplasm during curli biogenesis. Molecular chaperones therefore add another layer of control to what is already known about the directed assembly of curli.

CHARACTERISATION OF THE CONSERVED HYPOTHETICAL *YGFYX* GENES IN *SERRATIA*

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Bacteria of the genus *Serratia* are opportunistic human, plant and insect pathogens. *Serratia* sp. ATCC 39006 secretes plant cell wall degrading enzymes and produces the secondary metabolites, carbapenem and prodigiosin. Reverse transcription PCR indicated that *ygfXY* is transcribed as a bicistronic message. Clean marker-less mutations were made in *ygfY*, *ygfX* and both *ygfYX* and the phenotypic effects examined. Both genes have a role in prodigiosin biosynthesis at least in part at the level of transcription. Furthermore, mutation of *ygfY* caused pleiotropic effects, affecting potato virulence, exoenzyme production, carbapenem biosynthesis, motility and cell length. YgfY was shown to be a cytoplasmic protein and YgfX is anchored in the inner membrane via two transmembrane helices. Truncation, site-directed mutagenesis and domain-swap experiments have mapped important functional regions of YgfX and shown that it not only needs to be localised to the membrane but amino acids in the periplasmic loop and cytoplasmic domain are essential for function. Furthermore, bacterial two-hybrid analysis has demonstrated that YgfX interacts with itself, potentially as a dimer or in a multimeric state. These studies have begun to elucidate the physiological and molecular details of these conserved proteins.

STRUCTURE–FUNCTION RELATIONSHIP BETWEEN THE BACTERIOPHAGE CTXΦ WITH VIBRIO CHOLERAE TYPE IV PILI

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Type IV pili (Tfp) are dynamic protein-based organelles expressed on the surface of many pathogenic bacteria. The expression of these surface structures are associated with several phenotypes such as the initial interaction between bacteria and host, uptake of DNA from the environment, autoagglutination and biofilm formation, surface movement, and sensitivity to bacteria-specific viruses, bacteriophages. The interaction between bacteria and bacteriophages are particularly important in the pathogenicity of some variants of E. coli and in V. cholerae as it is the bacteriophages that encode the genetic material for shiga toxin (stx) and cholera toxin (CT) production in these bacteria, respectively. Despite the fact that interactions between Tfp and the different bacteriophages have implication in important infectious diseases, the mechanism for their interactions remain poorly understood.
In V. cholerae it is the bacteriophage CTXφ that encode the gene for CT expression. CT is the primary factor for V. cholerae-induced diarrhea and thus has a major impact on disease prevalence of cholera epidemics. In our research we are interested in studying the structure-function relationship between CTXφ and the toxin coregulated pilus (Tcp). An imaging approach is presented using immuno-fluorescent microscopy (IFM) and immuno-transmission electron microscopy (TEM) to visualize the interactions between Tcp and CTXφ. In doing so we are using a CTXφ hybrid with f-phage, previously shown to infect Tcp expressing V. cholerae strains. The binding interaction of the phage hybrid with a wild type and a Tcp negative mutant strain of V. cholerae are compared. To better understand the intrinsic phage interacting factors of Tcp, various mutants of the TcpA, the major subunit of Tcp, are analyzed in our assay. These TcpA mutants are known to cause variant pilus structures with differential phage infection efficiency. Furthermore, phage binding to purified pili might give some insight into bacterial cell independent binding interactions and such data will also be presented.

IDENTIFICATION OF NEW IRON-REGULATED GENES IN *CAULOBACTER CRESCENTUS* USING A TRANSPOSON MUTANT LIBRARY

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Bacteria respond to iron limitation coordinating expression of many genes and most of them are under control of the transcriptional regulator Fur. In order to identify genes belonging to the *Caulobacter crescentus* iron stimulon, we screened a library of mini-Tn*5lacZ* insertion mutants for *lacZ* fusions regulated by iron availability. Five mutant strains with iron-responsive beta-galactosidase activities were isolated (3/2G, 34/8G, 58/11H, 60/1E and 51/1A) and unique transposon insertions in each clone were confirmed by Southern blot. Cloning and sequencing of the transposon/chromosome boundaries allowed identifying the transposon insertion sites into four distinct genes, which encode three conserved hypothetical proteins and a TonB dependent receptor. In the three strains showing the highest induction under iron limitation (3/2G, 34/8G and 58/11H), transposon insertions occurred in two genes encoding conserved hypothetical proteins belonging to the widespread Pfam family PF10670. The presence of a signal peptide and two predicted transmembrane helices suggest that these two paralogous proteins can be addressed to the bacterial envelope. To further characterize the expression pattern of these two genes, their promoters regions were cloned in a *lacZ* reporter plasmid and the constructions were introduced into the wild type and a null *fur* mutant strains. Beta-galactosidase activity assays indicated that the operons containing these two genes were induced under iron limitation and were derepressed in the *fur* mutant strain. Predicted Fur binding sites in these regulatory regions were confirmed by gel mobility shift assays using purified Fur protein. Thus, we identified new iron-regulated genes in *C. crescentus* and demonstrated that at least two of them are part of the Fur regulon. Our results suggest that two members of this widespread family of proteins with unknown function (PF10670) could be involved in transport or signaling in response to iron limitation.

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NONSTOP MRNA DECAY INITIATES AT THE RIBOSOME.

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The translation machinery deciphers genetic information encoded within mRNAs to synthesize proteins needed for various cellular functions. Defective mRNAs that lack inframe stop codons trigger nonproductive stalling of ribosomes. We have investigated how cells deal with such defective mRNAs, and present evidence to demonstrate that RNase R, a processive 3'-to-5' exoribonuclease, is recruited to stalled ribosomes for the specific task of degrading defective mRNAs. The recruitment process is selective for nonstop mRNAs and is dependent on the activities of SmpB protein and tmRNA. Most intriguingly, our analysis reveals that a unique structural feature of RNase R, the C-terminal lysine-rich (K-rich) domain, is required for both ribosome recruitment and targeted nonstop mRNA decay activities of the enzyme. These findings provide new insights into how a general RNase is recruited to the translation machinery and highlight a novel role for the ribosome as a platform for initiating nonstop mRNA decay.

DIVERSITY OF TYPE II BACTERIAL TOXIN-ANTITOXIN SYSTEMS

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Type II toxin-antitoxin (TA) systems are in general composed of two proteins, a stable toxin that targets an essential cellular process and a labile antitoxin that inhibits the deleterious activity of the toxin. TA systems are organized in operon, in which the antitoxin gene precedes that of the toxin. Expression of the operon is regulated negatively by the antitoxin alone or in complex with the toxin. The characterized type II toxins target two cellular processes: DNA replication and translation of mRNAs.
Type II TA systems are currently divided into 10 families in which each family of toxins is associated to a specific family of antitoxins. However, few examples of hybrid systems composed of toxins and antitoxins belonging to different families have been recently described in the literature.
A bioinformatics approach developed in our lab led to the discovery of novel hybrid systems. We validated experimentally 6 of these novel hybrids in *E. coli*. These systems originated from various bacterial species, distantly related to *E. coli*. We are currently analysing the mode of toxicity of 6 toxins.
Our work shows that multiple associations between toxins and antitoxins do exist. Evolutionary aspects will be discussed.

PRIMING OF TRANSCRIPTION INITIATION WITH NANORNAS IN VIVO

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Transcription initiation in all cells is presumed to occur “de novo”, i.e. RNA polymerase (RNAP) is presumed to initiate RNA synthesis using free NTPs alone. However, prokaryotic and eukaryotic RNAP can utilize small (~2-4 nt) RNA oligomers to prime transcription initiation in vitro, raising the possibility that small RNA transcripts could also serve as primers for transcription initiation in vivo. To investigate the hypothesis that ~2-4 nt RNA transcripts, “nanoRNAs”, can prime transcription initiation in vivo we engineered Pseudomonas aeruginosa cells in a manner that allowed accumulation of nanoRNAs in a controlled manner. By use of deep sequencing we establish that increasing the concentration of nanoRNAs leads to widespread use of nanoRNAs by RNAP to prime transcription initiation. By use of microarray analysis we further show that widespread nanoRNA-dependent priming is coupled with global alterations in gene expression. These data suggest that transcription initiation in vivo is not strictly "de novo" and, furthermore, that nanoRNAs may represent a new class of regulatory small RNAs.

STRUCTURE/FUNCTION ANALYSIS OF RBGA, AN ESSENTIAL GTPASE REQUIRED FOR RIBOSOME BIOGENESIS IN *BACILLUS SUBTILIS*.

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Ribosome biogenesis GTPase A (RbgA) is an essential GTPase required for biogenesis of the 50S subunit in *Bacillus subtilis*. RbgA contains two structural domains; an N-terminal GTP-binding domain and a C-terminal domain that contains a putative RNA binding domain with structural similarities to the ANTAR domain of AmiR.
Research conducted previously has shown that cells depleted of RbgA do not form a mature 50S subunit but instead form a novel intermediate that migrates at 45S in sucrose gradients. This 45S intermediate lacks three ribosomal proteins, L16, L27 and L36.

A genetic system was developed to study the effect of site-directed mutations in RbgA by placing the wild type gene under the control of an IPTG inducible promoter and a mutated gene under the control of xylose inducible promoter. Using this system we screened 48 mutations and identified 19 mutations in different functional domains that affect cell growth. Mutations targeting conserved residues within the putative ANTAR domain rendered RbgA non-functional. The mutated proteins were tested for their ability to bind to the ribosome subunits in an *in vitro* binding assay. Only one mutant in the ANTAR domain disrupted binding to the ribosomal subunit. This may indicate multiple sites on RbgA are involved in interaction with the ribosome.

In order to test if expression of mutated RbgA protein *in vivo* led to incorporation of L16, ribosomal intermediates were isolated from strains expressing mutated RbgA protein and analysed for the presence of ribosomal protein L16. All intermediates tested so far are similar to the 45S intermediate and lack the L16 protein. Our results indicate that hydrolysis of GTP could be driving a conformational change in the 45S intermediate, leading to the addition of the ribosomal proteins and exit of RbgA from a mature 50S subunit. We are currently using biochemical approaches to identify whether these mutants are stimulated in their GTPase activity in the presence of the ribosome. A model for RbgA function in biogenesis of the 50S subunit will be presented.

THE TRANSCRIPTIONAL FEEDBACK IN THE Σ^E RESPONSE OF *ESCHERICHIA COLI*

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Bacterial genomes rapidly adjust to stress environments by sensing external conditions and converting that information into transcriptome changes. Group 4σ factors, the extracytoplasmic function σ factors, are one system that relays such information. The Group 4σ's have a common activation pathway whereby inducing signals cause derepression of σ by anti-σ, leading to σ-dependent transcription at regulon genes. A representative Group 4σ is the *Escherichia coli* heat shock σ factor, σE. σE is normally sequestered in the IM by its anti-σ, RseA. Activation signals, such as the unfolding of outer membrane porins (OMPs), induce cleavage of RseA through the protease DegS. Subsequent degradation of RseA frees σE to transcribe regulon genes. Interestingly, Group 4σ's are also regulated on the transcriptional level: a Group 4σ's regulon often contains both σ and anti-σ as members, creating a complex circuitry of interlocked positive and negative feedback loops at the transcription and the protein-protein interaction level. For σE, the effect of these feedback loops is further emphasized by the fact that σE and RseA are adjacent and polycistronic. While the mechanism of DegS activation and RseA degradation is been extensively studied, the role of transcriptional feedback in σE and Group 4σ signaling has not well understood. To understand how σE response is mediated by its intertwined transcriptional feedback loops, we have constructed several promoter mutants in the σE locus. We have removed positive feedback by changing the promoter in front of σE to an IPTG-regulatable promoter so that σE levels can be tightly controlled. We have also altered the negative feedback by deleting the σE-dependent promoter regulating RseA. Analysis of these promoter mutants serially or in combinations will elucidate the contributions from the transcriptional network to kinetics, amplitude, and duration of the σE response. In preliminary experiments, it appears that positive autoregulation of σE is not required to recapitulate the characteristic heat shock response. Tellingly, even without positive autoregulation, heat-shock dynamics are robust across a large range of σE transcript (and correspondingly, protein) levels, suggesting that the key requirement for robust σE response may be the linking of σ and anti-σ transcripts in the polycistron. Further investigation of the negative feedback mutant and mutants breaking the polycistron will continue to elucidate how σE response kinetics is achieved.

ROLE OF RNA/DNA HYBRIDS IN INITIATING STRESS-INDUCED MUTAGENESIS AND NON-HOMOLOGOUS RECOMBINATION.

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Bacterial, yeast and human cells induce pathways of genomic instability under the control of stress responses, potentially enhancing their ability to evolve specifically when they are maladapted to their environment, i. e. are stressed. In an *Escherichia coli* model system, starvation-stressed cells induce two pathways of genomic instability controlled by the RpoS general stress response: stress-induced point mutagenesis, which creates -1 bp deletions and base substitutions via DNA polymerase errors, and gene amplification or copy-number variation, caused by non-homologous recombination. Both are instigated by DNA double-strand breaks (DSBs) and associated with DSB repair. We explored the possibility that both kinds of genomic instability might be targeted to actively transcribed genomic regions. This could create genetic diversity in genes the functions of which are required under a specific stress condition, and possibly accelerate adaptation. We provide evidence that RNA/DNA hybrids are intermediates in stress-induced amplification and point mutation. We show that overproduction of RNase HI, which removes RNA from RNA/DNA hybrids (R-loops), reduces amplification and point mutagenesis ~50%. Deletion of *rnhA*, encoding RNase HI, increases amplification and point mutagenesis ~5-fold. Most of the point mutagenesis and amplification in the *rnhA* cells, and about half of it in wild-type cells, required Mfd, a transcription-coupled DNA-repair factor, relating both events to transcription. Translation opposes R-loop formation by preventing nascent transcripts from reincorporation into their template DNA, and we find that pulse inhibition of translation with spectinomycin increased mutagenesis and amplification dependently on Mfd, implying transcriptional involvement. Finally, we show that RNaseH, and by implication R-loops, no longer affect amplification and mutagenesis when, rather than relying on spontaneous DSBs to initiate both events, we provide an I-SceI endonuclease-generated DSB in vivo. These data indicate that the role of R-loops in amplification and in point mutation is upstream of formation of DSBs. We suggest that R-loops prime adventitious replication forks that then collapse at single-strand DNA nicks, producing the DS-ends that instigate amplification and mutagenesis. The data suggest that genetic change might be targeted to regions under immediate selection, possibly accelerating adaptation. In the *E. coli* Lac assay, at least half the adaptive genetic changes might form by this mechanism.

A SUGAR TRANSFERASE REGULATING CELL SIZE HOMEOSTASIS IN *ESCHERICHIA COLI*

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Escherichia coli cell size is tightly coupled to nutrient availability and growth rate. During rapid growth in carbon-rich medium, *E. coli* cells are ~2-fold larger than when cultured in carbon-poor medium. While the mechanism regulating cell size is not known in *E. coli*, accumulation of UDP-glucose in the Gram positive model organism *Bacillus subtilis* serves as a proxy for coordinating cell size with carbon availability. Mutations in genes required for UDP-glucose biosynthesis uncouple *B. subtilis* cell size from growth rate and reduce cell size by ~35% under carbon rich conditions. In wild type cells, the UDP-glucose binding protein UgtP coordinates cell size with growth rate by interacting directly with the cell division protein FtsZ to delay division until cells reach the appropriate mass for a given growth rate. Although *E. coli* has no UgtP homolog, mutations in genes necessary for UDP-glucose synthesis cause a similar small-cell phenotype suggesting that UDP-glucose is an evolutionarily conserved sensor linking growth rate to division.

Our data for *E. coli* indicate that the pathway responsible for synthesizing osmoregulated periplasmic glucans also coordinates carbon availability with cell division maintaining cell-size homeostasis. Mutations in three genes in this pathway, *pgm*, *galU*, and *mdoH* reduced cell size by 25%, 18%, and 12% respectively during growth in carbon-rich medium. Consistent with a model in which this pathway coordinates division with carbon availability, a deletion in the first gene in this pathway, the phosphoglucomutase *pgm*, rescues cells from over-expression of the division inhibitor MinD. Moreover, a GFP fusion to the last enzyme in the pathway MdoH, localizes to midcell, suggesting it may interact with components of the division apparatus. MdoH is a glucosyltransferase, a function analogous to that of *B. subtilis* UgtP. Future studies will be aimed at validating MdoH as the *E. coli* size homeostasis effector protein both genetically and biochemically.

UNIFYING OUR KNOWLEDGE ABOUT *E. COLI* AS A MODEL ORGANISM

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E. coli is the best studied free-living organism and our knowledge about *E. coli* informs almost all aspects of basic and applied microbiology. Applying our understanding of *E. coli* is limited by the challenges of finding and comparing information from diverse sources. Our project seeks to unify access to information and tools about the biology of *E. coli*, its bacteriophages, plasmids, and mobile genetic elements. In the past year, we have focused on several areas: 1) Search: a new information-dense search system with provisions for adding new data sources. Our search currently returns links to gene and product information from EcoCyc, EcoGene, UniProt, and EcoliWiki. It finds expression profile data from the Stanford Microarray Database, gene families from the PANTHER database, and publications. 2) Expression data: we are curating and acquiring information from GEO and ArrayExpress to generate cross-study analyses of expression data from microarray experiments. New tools for mining available gene expression datasets for functional connections are being developed. 3) Genomes: Curating additional genomes for E. coli strains. 4) Evolution: large scale phylogenetic analysis of *E. coli* genes and gene families and 5) Community: resources to bring community members together 6) Tools for power users: EcoliHouse, a data warehouse to allow power users to perform complex queries across multiple data sources.

A MUTATION WITHIN THE B SUBUNIT OF E.COLI RNA POLYMERASE IMPAIRS TRANSCRIPTION FROM BACTERIOPHAGE T4 MIDDLE PROMOTERS.

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During infection of E.coli, bacteriophage T4 usurps the host transcriptional machinery, redirecting it to the expression of early, middle and late phage genes. Middle genes, whose expression begins about 1 minute post infection, are transcribed both from the extension of early RNA into middle genes and by the activation of T4 middle promoters. In this activation, the T4 co-activator AsiA structurally remodels a portion of sigma70, the specificity subunit of RNA polymerase. This remodeling allows the T4 transcriptional activator MotA to also interact with sigma70 and to bind to a middle promoter element at -30. Despite the fact that MotA and AsiA are required for middle promoter activation, T4 motA- or asiA- phage do grow, although poorly, in wild type E.coli. However, previous work has found that these mutants cannot plate on the E.coli mutant strain tabG. We show that the rpoB gene of tabG, which encodes the β subunit of RNA polymerase, contains two mutations, E835K and G1249D. We further show that it is the G1249D mutation that restricts the growth of either T4motA- or asiA-, and that this mutation impairs transcription from MotA/AsiA -activated middle promoters in vivo. Transcription from T4 early promoters is not harmed, and there is no significant growth phenotype for the rpoB E835K/G1249D strain in the absence of T4 infection. Using the reported structures of thermophilic RNAP, we find that G1249 is located along the RNA exit channel, immediately adjacent to a hydrophobic pocket (Switch III loop), which is thought to aid in the separation of the RNA from the DNA-RNA hybrid as RNA enters the exit channel. Our results suggest that the presence of MotA and AsiA may impair the function of this loop or that this portion of β may influence interactions among MotA, AsiA, and RNA polymerase.

HOMEOSTASIS OF INTRACELLULAR GTP AND ATP POOLS IN *LACTOCOCCUS LACTIS* STAINS WITH LOWERED GMP FLUX

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ATP and GTP serve as both energy donors and substrates for RNA and DNA synthesis. While ATP is used as the general household energy currency, GTP supplies energy to the most fundamental processes like protein synthesis and cell division. Synthesis of ATP and GTP follows a common pathway leading to the formation of IMP, after which the pathways branches out into an AMP and a GMP pathway, catalyzed by the PurA + PurB and GuaB + GuaA enzymes respectively. The pathways are symmetrically regulated at the enzymatic level by feed-back inhibition of the first reaction in each branch (PurA and GuaB) by AMP and GMP respectively, and the reciprocal use of ATP-bound energy in the GMP branch and GTP-bound energy in the AMP branch. At the genetic level, shortage of GTP is known to induce the synthesis of the *guaB* and *guaA* genes of the GMP branch by an unknown mechanism. We wanted to analyze whether the tight regulation was enough to secure a constant supply of GTP under conditions where the expression of the GuaB enzyme was limited by expression from weak promoters selected from an expression library. Strains containing single copies of the *guaB* gene transcribed from promoters with varying strength (approximately 10% to 500% of the wild type level under standard conditions) were analyzed with respect to growth rate and intracellular pools of intermediates in the ATP and GTP synthesis pathways. We found that while the growth rate was highly dependent upon a sufficient GuaB activity, and the intermediates XMP and GMP in the GMP branch varied more than 50-fold between the strains, the GDP and GTP pools varied less than 3-fold. The ATP/GTP ratio varied from 2 to 10 as the *guaB* level was changed from the highest to the lowest level.

PARTICIPATION OF A CHROMOSOME SEGREGATION PROTEIN PARAI OF *VIBRIO CHOLERAE* IN CHROMOSOME REPLICATION

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Vibrio cholerae carries homologs of *parA* and *parB* genes on both of its chromosomes. The genes contribute to segregation of many plasmids and chromosomes. Here, we have studied the par genes of chromosome I. Earlier studies suggest that ParBI binds to centromeric site *parSI* near the origin of replication (*oriI*), and *parSI*-ParBI complexes are placed at the cell poles by ParAI. Deletion of *parAI* and *parSI* caused the origin-proximal DNA to be less polar. Here, we found that deletion of *parBI* also resulted in a less polar localization of *oriI*. However, unlike the deletion of *parAI*, the deletion of *parBI* increased *oriI* number, indicating over-initiation of replication. Replication was normal when both *parAI* and *parBI* were deleted, suggesting that ParBI mediates its action through ParAI. Over expression of ParAI in a *parABI* deleted strain also increased the DNA content. The results are strikingly similar to those found in *Bacillus subtilis*, where ParA (Soj) stimulates replication and this activity is repressed by ParB (SpoOJ). As in *B. subtilis*, the stimulation of replication most likely involves the replication initiator DnaA. Our results thus establish that another conserved function of chromosomal par genes is likely to be control of chromosomal DNA replication.

ECOCYC AND ECOLIHUB – 43 *E. COLI* AND *SHIGELLA* GENOMES

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EcoCyc (EcoCyc.org) and EcoliHub (EcoliHub.org) together form a model organism database for *Escherichia coli* K-12 MG1655. Since 1994, EcoCyc curators have captured and summarized information found in the experimental literature, resulting in extensive coverage of the functions of individual *E. coli* gene products. A number of search and display tools allow users to learn about gene products and their organization into protein complexes, metabolic pathways, operons, and regulatory networks. The data can be analyzed computationally via built-in tools to manipulate, transform and analyze lists of objects and by programmatic access to the contents of the database.

Major recent enhancements to EcoCyc include extensions to the capabilities of the associated Pathway Tools software to allow the capture and representation of regulatory mechanisms that act after transcription initiation, such as regulation by attenuation, riboswitches, and regulation of translation initiation and mRNA stability by small RNAs. Individual regulatory interactions are displayed on gene and transcription unit pages within EcoCyc and are incorporated into the genetic regulation schematic. The entire transcriptional regulatory network of *E. coli* can be displayed and navigated via the Regulatory Overview, which is now available for both Web site and desktop software users.

The autumn release of the EcoCyc Web site will include a new global regulation diagram shown on the gene/protein pages. This diagram will provide a compact summary of all regulatory inputs to the expression and activity of a gene product, and is automatically generated from data in EcoCyc. The diagram shows the actions of all regulators that affect the final amount or activity of the product, including regulators of transcription, translation, and protein activity.

As part of the associated EcoliHub project, we have greatly expanded our collection of databases built from the genome sequences of other strains of *E. coli* and *Shigella*. Within the BioCyc collection at BioCyc.org, 43 *E. coli* genomes are represented, allowing comparative analyses between different pathogenic, non-pathogenic and laboratory strains of *E. coli*. Examples of comparative operations include displaying chromosomal regions around orthologous genes (see “Align in Multi-Genome Browser” button on gene/protein pages) and the ability to generate comparative reports on pathways, reactions, compounds, proteins, and transcription units (see “Comparative Analysis” under “Tools” menu). In addition, BioCyc tools such as the genome browser, zoomable metabolic map diagram, and omics viewers can be applied to these *E. coli* and *Shigella* genomes. We plan to add many additional genomes as they appear in GenBank.

NOVEL SMALL ENVELOPE PROTEINS REGULATED BY THE CPXAR TWO-COMPONENT ENVELOPE STRESS RESPONSE OF *ESCHERICHIA COLI*

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Escherichia coli is a Gram-negative inhabitant of the mammalian intestinal tract which encounters different environmental stresses while moving within and between hosts. Therefore, it has evolved several responses to sense and adapt to envelope-related stresses. The CpxAR two-component system is made up of a sensor histidine kinase, CpxA, its cognate cytoplasmic response regulator, CpxR, and a unique third component, the periplasmic protein CpxP. The Cpx pathway is thought to respond to misfolded periplasmic proteins and the classical members of its regulon include periplasmic chaperones and proteases that help to restore homeostasis by either refolding or degrading the misfolded proteins. Furthermore, in the enteropathogenic *E. coli* (EPEC) strain E2348/69, the Cpx pathway downregulates large virulence structures, presumably to clear the envelope of unnecessary protein traffic in times of stress. In an effort to further expand our knowledge of the Cpx regulon, microarray analyses were carried out under Cpx-inducing conditions in both the *E. coli* K-12 strain MC4100 and EPEC E2348/69. This analysis revealed that induction of the Cpx response is strain and media dependent, and several hundred genes are changed upon NlpE over-expresssion. Despite this, the genes that were changed in expression fell into similar functional clusters under all conditions. Some of these clusters are related to functions that were not previously linked to the Cpx response. For example, several novel genes encoding small, uncharacterized, predicted periplasmic (YnfD, YjfN, and YncJ) and inner membrane (YqaE) proteins were up-regulated. Since not all of these genes contain predicted CpxR consensus binding sites upstream of their promoters, we confirmed the Cpx regulation of these genes by investigating β-galactosidase expression from transcriptional *lacZ* reporters under several known Cpx-activating conditions. In an effort to determine what cellular processes these uncharacterized proteins might be involved with, we used microarrays to determine the downstream effects of the overproduction of these four proteins on global EPEC E3248/69 gene expression in defined media. The results of the microarray studies will be discussed.

IN VIVO TRANSCRIPTION AND TRANSLATION DYNAMICS OF THE GALACTOSE OPERON IN *ESCHERICHIA COLI*

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In order to understand dynamics of transcription and translation of the galactose operon of *Escherichia coli*, we have performed in vivo quantitative analyses of gal-specific transcripts, and proteins during times of growth when the operon is under full induction. When cells are actively dividing (exponential phase), 70 % of gal transcription initiated from the *P1* promoter. But when cells are not dividing (stationary phase), 70 % of gal transcription initiated from the *P2* promoter. Transition of the major promoter occurred due to different rate of transcription when cells enter stationary phase. The Gal proteins produced per unit amount of the corresponding gal mRNA is greater in stationary phase than exponential phase, suggesting that the *P2*-major status in stationary phase is to maintain a sufficient concentration of the Gal proteins when the transcripts are decreasing. The initial synthesis rate of the *P1* transcript is 0.24 min^{-1}, which is 1.5 times faster than that of the *P2* transcript. Considering that these analyses were done on the same numbers of cells at each time point, and that CRP is the activator of the *P1* promoter, it is likely that the transcription rate of *P1* depends on numbers galactose operon to which CRP is bound, instead of amounts of transcription from a single *P1* promoter.

SYNTHETIC PEPTIDE INHIBITORS OF DNA REPLICATION IN *STAPHYLOCOCCUS AUREUS*

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During the last decades bacteria have developed resistance towards many of the antibiotics on the marked. With this in mind it is important to continue the development of new antibacterial agents. A good target for development of antibiotics is one that has a conserved function in a wide spectrum of clinically important pathogens and is essential for bacterial proliferation. The bacterial replication apparatus fulfill the requirements for a good drug target. The replisome of *S. aureus* consists of 5 different subunits (β2, PolC2, tau4, δ and δ`) who's organization depends on multiple protein-protein interactions. Centrally in the replisome is the β-clamp where to multiple proteins binds through a conserved motif.
We have identified the protein-protein interactions in the replisome of *S. aureus* by use of a bacterial two-hybrid system. A reverse bacterial two-hybrid system (R-BTH) based on PyrF counterselection was developed to directly select for compounds able to disrupt selected interactions.
We have subsequently constructed a cyclic peptide library for intracellular synthesis of cyclic peptides using known technology. Several cyclic peptides were able to interfere with oligomerization of DnaN (β), DnaB and DnaX (tau). Three peptides identified as inhibitors of DnaN have been purified. Two of these peptides inhibited growth as well as DNA replication in *S. aureus*. The minimal inhibitory concentration (MIC) of the peptides was approximately 50 μg/ml. Overexpression of DnaN reduced the inhibitory effect of the peptides confirming the target of the peptides.

SPECIFIC DETECTION OF SALMONELLA AND ESCHERICHIA COLI STRAINS BY USING ELISA WITH BACTERIOPHAGES AS RECOGNITION AGENTS

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The use of bacteriophages, instead of antibodies, in ELISA-based detection of bacterial strains was tested. This procedure appeared to be efficient, and specific strains of Salmonella and Escherichia coli could be detected. The sensitivity of the assay was about 10^5 bacterial cells/well (10^6/ml), which is comparable with or outperform other ELISA tests detecting intact bacterial cells without an enrichment step. The specificity of the assay depends on the kind of bacteriophage used. Therefore, we conclude that the use of bacteriophages in detection and identification of bacteria by ELISA-based method can be an alternative to the use of specific antibodies. The advantages of the use of bacteriophages are their environmental abundance (and thus a possibility to isolate various phages of different specificities) and availability of methods for obtaining large amounts of phage lysates, which are simple, rapid, cheap and easy.

INFLUENCE OF THE BACTERIOPHAGE Λ *EXO-XIS* REGION ON EFFICIENCY OF PROPHAGE INDUCTION

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Although bacteriophage λ has served as a model organism in molecular biology for more than fifty years the functions of some of its genes are not yet clear. This concerns the region between *exo* and *xis* genes. This region is transcribed from the pL promoter, which is an early promoter, active during the lytic development of phage λ switched off by the *cI* gene product after lysogenization and during maintenance of the prophage. Induction of λ prophage in lysogenic *Escherichia coli* cells bearing either the whole λ *exo-xis* region (with truncated, thus nonfunctional, *exo* and *xis* genes) or particular genes from this region were investigated. We found that overexpression of this region from a plasmid increased efficiency of λ prophage induction by different agents and spontaneous induction at 37 ˚C. The highest increase efficiency of prophage λ induction occurred when the whole λ *exo-xis* region was present on a plasmid. Lower increase was also obtained when gene *ea22* in combination with open reading frames *orf60a*, *orf61* and *orf63* were present on a plasmid. However, *ea22* alone as well as *orf60a*, *orf61* and *orf63* did not reveal such a phenotype. This report presents that the λ *exo-xis* region may have influence on λ phage induction.

SPANIN-MEDIATED OUTER MEMBRANE DISRUPTION IS REQUIRED FOR LYSIS OF *E. COLI* BY Λ

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The λ "lysis cassette" is densely packed with coding information, consisting of three adjacent genes, *SRRz*, with a fourth gene, *Rz1*, entirely embedded in the +1 register of *Rz*. The *S* gene encodes two inner membrane (IM) proteins: the holin (S105) and the antiholin (S107). *R* encodes the muralytic endolysin, which accumulates in the cytosol. S105 and R have long been known essential for lysis: the former opens large, non-specific holes in the IM at an allele-specific time, allowing the latter to escape and attack the murein. Previously, it has been reported that *Rz/Rz1* have a conditional phenotype, required for lysis only if the OM is stabilized by millimolar concentrations of divalent cations. Under the non-permissive conditions, the absence of *Rz* or *Rz1* function leads to the formation of spherical cells, instead of lysis. *Rz* encodes a type II inner membrane (IM) protein, with a periplasmic domain predicted to be largely alpha-helical. *Rz1* encodes a 40 aa OM lipoprotein. Genetic and biochemical evidence indicates that Rz and Rz1 form a complex via C-terminal interactions. Because this complex would span the bilayer, Rz and Rz1 are called the IM and OM subunits of a spanin complex. Originally of interest mainly because of the bizarre gene structure, *Rz/Rz1* has recently become more of a focus because of the discovery that nearly all dsDNA phages have functionally equivalent genes. Remarkably, more than 40 unrelated gene families of two-component spanins have been found. This includes many others with the embedded gene arrangement like lambda but also three other distinct architectures, including overlapped genes, where the *Rz1* equivalent extends beyond the end of *Rz*, and completely separated genes. Moreover, some phages have a single gene, encoding a unimolecular spanin: a lipoprotein with a C-terminal TMD. The prototype, gp*11* of phage T1, which is embedded in the inner leaflet of the OM by the lipids on its N-terminal Cys and also traverses the IM with its TMD, complements Rz-Rz1 defects. We have shown that the purified periplasmic domains of Rz and Rz1 form a large rod-shaped complex of dimensions sufficient to span the periplasm. Here we show that in vivo, Rz and Rz1 are both dimers covalently linked by intermolecular disulfide bonds. Finally, videomicroscopic studies will be described that indicate the spanin function is, after all, required for lysis of the infected cell, a fact that we have missed because the spherical cells produced in an *Rz-Rz1* defective infection are sensitive to the shearing forces in aerated cultures. These results will be discussed in terms of three-step pathway for the lysis process.

LOCALIZATION OF THE BACTERIOCIN BINDING SITE ON THE RECEPTOR

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Bacteriocins are bacterially produced antimicrobial peptides that hold a great potential as second generation antibiotics in infection treatment and as preservatives in food production. To date, a large number of bacteriocins have been characterized; however, further progress in the application of these peptides relies on increased understanding of the molecular mechanisms involved in receptor recognition and mode of killing.

One of the major groups of bacteriocins is the so-called pediocin-like bacteriocins. These peptides are small in size (35-50 unmodified amino acids), cationic and they kill important pathogens such as *Listeria monocytogenes* and *Enterococcus faecalis* with high potencies. The mannose-phosphotransferase system (man-PTS) has been identified as target cell receptor for pediocin-like bacteriocins. This sugar importer, which is the major glucose transporter in many bacterial cells, is composed of two transmembrane proteins (IIC and IID) and a membrane-associated protein (IIAB), but only the transmembrane proteins IIC and IID are required for the receptor function.

By comparing man-PTSs from different origins, we recently found that the pediocin-like bacteriocins only target a phylogenetically defined subgroup of man-PTSs, and that homologous man-PTSs can have different receptor potencies, e. g., the listerial man-PTS is a highly potent bacteriocin receptor while the closely related lactococcal man-PTS cannot function as receptor. Thus, in order to localize the specific bacteriocin binding site on the receptor we have in the present study systematically constructed hybrid genes of the listerial and the lactococcal man-PTSs. Experiments with these hybrids revealed a region of 40 aa in the listerial IIC protein which is needed for this species-specific targeting. The 40 aa region contains a predicted extracellular loop and we demonstrate by site-directed mutations that some of the loop-residues were indeed important for the receptor function. Taken together, we believe that this 40 aa region is not only involved in the interaction with the peptides but that it also defines the species-specific targeting for these bacteriocins.

RSEB MODULATES THE Σ^E STRESS RESPONSE PATHWAY IN *ESCHERICHIA COLI*

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In *Escherichia coli* accumulation of unassembled outer membrane porins (OMPs) in the envelope leads to cytoplasmic activation of σ^E. A proteolytic cascade transmits OMP signal by regulating degradation of a membrane-spanning anti-sigma factor, RseA, whose cytoplasmic domain inhibits σ^E-dependent transcription. C-termini of OMPs signal RseA proteolysis by activating a membrane-anchored periplasmic protease, DegS. The RseA fragment generated by DegS cleavage is subsequently cleaved by a membrane-embedded protease, RseP, and finally degraded by cytoplasmic proteases. A periplasmic protein, RseB, binds RseA and inhibits its cleavage by DegS and RseP. Thus RseA/RseB interaction must be altered for the OMP signal to transduce. The mechanism of OMP-mediated DegS activation has been extensively studied and it is generally believed that σ^E induction is determined by the degree of DegS stimulation by OMPs. Considering the negative regulation of RseB on RseA degradation we sought to investigate whether OMPs also modulate RseB effects on σ^E activity. To this end, we used a library of OMP peptides and compared σ^E output in wildtype and Δ*rseB* strains. σ^E induction matched DegS activation parameters in Δ*rseB* but not in wildtype cells, and a portion of OMP peptide was uniquely required for σ^E activation in the presence of RseB. Our results clearly show that OMPs encode RseB modulatory sequences and that σ^E activation is a composite effect of DegS activation and RseB de-repression. It will be interesting to investigate whether OMPs directly mediate RseB inactivation or indirectly by leading to the buildup of another envelope signal that de-represses RseB. In either case RseB is the 'quality control sensor' of envelope integrity that collaborates with DegS in determining the magnitude of σ^E response. Our work additionally reveals that an important outcome of RseB inhibition on DegS activity is to desensitize σ^E pathway to low levels of OMP signal. Specifically, RseB raises the OMP peptide threshold for DegS activation. Our studies thus establish RseB as a 'gatekeeper' of σ^E pathway that conveys information regarding envelope integrity and ensures that the protease cascade is activated only when stress is sufficient to warrant response.

USING BLACK LIPID MEMBRANES TO PROBE TRANSPORT THROUGH TONB-DEPENDENT TRANSPORTERS.

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Micronutrients such as siderophore-bound iron and vitamin B12 cross the outer membrane of Gram-negative bacteria through a group of 22-stranded β-barrel proteins. They share the unusual feature that their N-terminal end inserts from the periplasmic side into the β-barrel and plugs the lumen. Transport results from energy-driven movement of TonB protein, which either pulls the plug out of the barrel or causes it to rearrange within the barrel.

Attempts to reconstitute native plugged channels in an ionconducting state in lipid bilayer membranes proved fruitful when we discovered that if the *cis* solution contained 4 M urea, then, with the periplasmic side of the channel facing that solution, macroscopic conductances and single channel events could be observed. These results were obtained with FhuA, Cir, and BtuB; for the former two, the channels were closed by removing the 4 M urea.

Channels generated by 4 M urea exposure were not a consequence of general protein denaturation, as their ligand-binding properties were preserved. With FhuA, addition of ferrichrome (its siderophore) to the trans, extracellular-facing side reversibly inhibited 4 M urea-induced channel opening and blocked the channels. With Cir, addition of colicin Ia (the microbial toxin that targets Cir) to the trans, extracellular-facing side prevented 4 M urea-induced channel opening.

Using this system, we probed the state of the plug domain of ion-conducting FhuA via the use of the endopeptidase trypsin. We found that if a FhuA-containing membrane is exposed to trypsin in the presence of 4 M urea, conductance increased faster compared to the membrane in the absence of trypsin. Furthermore, the removal of the urea resulted in little closing of the channels, suggesting that the plug domain of FhuA is accessible to and was cleaved by trypsin. Trypsin had no effect in the absence of urea indicating that urea facilitates the exposure of the plug domain of FhuA to Trypsin.

THE ROLE OF THE TYPE V AUTOTRANSPORTER XATA IN *XYLELLA FASTIDIOSA* VIRULENCE

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Xylella fastidiosa Temecula1 is the causative agent of Pierce's Disease of grapevine. This Gram-negative, endophytic bacterium is spread from infected plants to uninfected plants by xylem-feeding insects, such as sharpshooters and spittle bugs. An important feature of infection cycle is the ability of *X. fastidiosa* to colonize and interact with both the xylem tissue of susceptible plants and with the foregut of insects. One category of virulence determinants involved in colonization is the Type V AT-1 autotransporters. These proteins are dedicated to the secretion of a single specific polypeptide, the passenger domain, across the outer membrane. Based on genomic analysis, there are six AT-1 autotransporters in *X. fastidiosa* Temecula1. Our strategy has been to generate mutations in each gene and to examine their impact on cell physiology and virulence. Here, we describe our characterization of XatA, the *X. fastidiosa* autotransporter protein encoded by PD0528. Localization studies indicate that XatA is a major outer membrane protein and its passenger domain can be found in the supernatant. Moreover, mutational analysis indicates that XatA is important for autoaggregation and biofilm formation under laboratory conditions. We also found that introduction of the *xatA* gene into *Escherichia coli* strain UT5600 resulted in the presence of XatA in the outer membrane and in autoaggregation and biofilm formation, properties not normally observed for the UT5600 strain. These phenotypic properties allowed us to establish that XatA requires components of the Bam OMP assembly machinery for its translocation in *E. coli*. This dependency has been observed for other AT-1 proteins and suggests that a similar mechanism is operating in *X. fastidiosa*. Finally, to determine if XatA plays a role in *X. fastidiosa* virulence, we introduced a strain containing a *xatA* null mutation into Thompson seedless grapevines and compared its impact on PD symptom development to grapevines infected with wild type. Although wild-type infected grapevines exhibited symptoms sixteen weeks after inoculation, the *xatA* mutant infected grapevines did not. To confirm the presence of *X. fastidiosa* in the infected vines, petriole tissues were harvested one inch and six inches above the inoculation sites. Examination of the xylem tissue indicated the *xatA* mutant did not migrate as far from the inoculation site as wild type. In addition, the *xatA* mutant infected vines appeared to have approximately 10-fold fewer cells. Experiments are currently underway to determine the impact of the *xatA* mutation on the transmission of *X. fastidiosa* by the insect vector.

IDENTIFICATION AND CHARACTERIZATION OF A STABILIZER OF THE ESSENTIAL CELL DIVISION PROTEIN FTSZ IN *ESCHERICHIA COLI*

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The earliest identified event in bacterial cytokinesis is the formation of a ring structure by polymers of a tubulin-like GTPase, FtsZ at midcell, creating the framework for divisome assembly. The overall concentration of FtsZ is essentially unchanged through the cell-cycle, and its spatiotemporal control *in vivo* occurs through GTP-hydrolysis coupled assembly/disassembly processes. The assembly dynamics of FtsZ is influenced by a large group of regulatory proteins that ensure the spatial and temporal integrity of the FtsZ ring at midcell. These include stabilizers such as FtsA, ZapA, ZapB and ZipA (found only in γ-proteobacteria) and inhibitors like SulA, SlmA, MinC, ClpX, and EzrA (found only in low GC Gram-positive bacteria). In a localization screen in *E. coli*, we identified an as yet uncharacterized protein, YcbW, as being present at midcell. YcbW is dependent on FtsZ but not on FtsA or FtsI for midcell localization suggesting that YcbW is recruited early during divisome assembly. YcbW colocalizes with FtsZ and interacts with FtsZ in a protein interaction platform (PIP) assay in yeast - this indicates that YcbW and FtsZ are likely direct interacting proteins. Overexpression of YcbW leads to formation of aberrant FtsZ rings without affecting cellular FtsZ levels suggesting a role for YcbW in FtsZ assembly/stability. Removal of *ycbW* in the absence of FtsZ stabilizers, ZapA and ZapB, leads to a synergistic sick phenotype consistent with a role for YcbW in FtsZ stability *in vivo*. In FtsZ polymer pelleting assays with purified FtsZ, addition of purified YcbW-His increased the amount of FtsZ recovered in the pellet. Morphological characterization of the FtsZ polymer pelleting reactions by electron microscopy revealed enhanced bundling of FtsZ in the presence of YcbW. Based on our *in vivo* and *in vitro* data we propose that YcbW is a novel component of the *E. coli* divisome and likely belongs to the Zap (Z-associated protein) family of FtsZ stabilizers.

THE CHANGING OSCILLATION PATTERNS OF MIN PROTEINS BEFORE CYTOKINESIS

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During bacterial cytokinesis, FtsZ forms a ring structure at mid-cell which contracts and divides the cell into two daughter cells. The entire process must be tightly regulated spatially and temporally to ensure proliferation of the population. The Min proteins in Escherichia coli and Bacillus subtilis are responsible for keeping FtsZ rings from aberrantly assembling at cell poles and forming chromosome-free minicells. In E. coli, the oscillation of MinC-bound MinD sets up a gradient of the FtsZ inhibitor MinC, which is highest at the cell poles. To study how the Min proteins get equally partitioned into daughter cells, we examined GFP-MinD oscillation patterns in E. coli cells that were actively growing and dividing. Our results show that GFP-MinD oscillation patterns changed from a regular pole-pole oscillation pattern in newborn cells to an irregular oscillation pattern with frequent pauses at either side of the septum. These mid-cell pauses became more frequent as the cells approached division. The pattern then switched to double pole-septum oscillations on each side of the dividing cell. This double oscillation sometimes transiently reverted to the single irregular oscillation, but always switched back to double oscillation upon completion of cytokinesis. Because of the known interactions between MinC and FtsZ, we tested if mid-cell pausing was MinC-dependent and found that it was not. This suggests that MinD itself is targeted to a component of the developing new cell poles, which increasingly compete with the old poles for MinD binding. We hypothesize that the ability of MinD to bind to septal membranes is a precursor to the splitting of one MinD zone into two, which is required for the doubled oscillation and the equal distribution of MinD in daughter cells.

ROLES OF FTSN AND OTHER PROTEINS WITH A SPOR DOMAIN IN STARTING *E.COLI* CELL CONSTRICTION.

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In *Escherichia coli*, FtsN is the last known essential division protein to join the division apparatus before cell constriction ensues. FtsN is a bitopic inner-membrane (IM) protein with a small cytoplasmic portion and a large periplasmic one. The latter is composed of an α-helical juxtamembrane region, an unstructured Q-rich linker, and a C-terminal murein-binding SPOR domain (1). We recently showed that the essential function of FtsN can be performed by a surprisingly small essential domain (^{E}FtsN) of at most 35 residues that resides near the outer face of the IM, and that the C-terminal SPOR domain (^{S}FtsN) is a septal localization determinant. Interestingly, septal localization of ^{S}FtsN depends on the production and processing of septal murein and, thus, on the activity of ^{E}FtsN. This and other results indicate that FtsN joins the division apparatus in an unsusual self-enhancing fashion at the time of constriction initiation that involves both ^{E}FtsN and ^{S}FtsN (2).

SPOR domains are widely distributed in bacteria. The SPOR domains of three additional *E. coli* proteins of unknown function, DamX, DedD, and RlpA, as well as that of *Bacillus subtilis* CwlC, also accumulated sharply at constriction sites in *E. coli*, implying that septal targeting is a common property of SPORs. Further analyses showed that DamX and, especially, DedD are genuine division proteins that contribute significantly to the cell constriction process (2). These and additional results suggest that ^{E}FtsN controls cell constriction by allosterically modulating the activity of another essential septal ring component, and that DedD and DamX contribute to the triggering function of FtsN by enhancing ^{E}FtsN activity. We use several approaches to further define ^{E}FtsN, its predicted essential target(s), and the roles of the other SPOR domain proteins in the cell constriction process, and current results will be presented.

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CLPXP MODULATES CELL DIVISION IN *E. COLI* AFTER FORMATION OF THE FTSZ-RING BY A MECHANISM THAT INVOLVES ATP-DEPENDENT DEGRADATION

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Cell division in *Escherichia coli* begins with the recruitment and assembly of the cell division machinery at midcell. FtsZ, an essential cell division protein, assembles into a dynamic structure, the FtsZ-ring. One system the cell utilizes to ensure proper placement of the FtsZ-ring is the Min system, comprised of three proteins MinC, MinD and MinE. MinC is recruited to the membrane by MinD where it interacts directly with FtsZ to prevent FtsZ polymerization. MinE causes release of MinD from the membrane, thereby inhibiting the ability of MinC/MinD to prevent FtsZ polymerization. In *minCDE* deletion strains, division occurs at locations other than midcell, including locations near the cell poles. Since FtsZ is degraded in vivo and in vitro by ClpXP, a two-component ATP-dependent protease, we asked whether there is interplay between ClpXP and the Min system in regulating cell division.

In this study we demonstrate that deletion of *clpX* or *clpP* in combination with the *minC* deletion causes a delay in cell division that results in cellular filamentation. Plasmid-encoded *clpX*, but not *clpX(E185Q)*, which carries a mutation that renders ClpX deficient for ATP hydrolysis, complements the cell division delay in *clpX minC* deletion cells. Likewise, plasmid-encoded ClpP complements the defect in *clpP minC* deletion cells. The filamentous cells display several discrete FtsZ-rings by immunofluorescence microscopy suggesting that FtsZ-ring formation is not impaired. In wild type cells ClpX and ClpP localize to both the poles and septa of dividing cells consistent with a role in cell division. Additional experiments combining a filamentous temperature-sensitive mutant strain, *ftsZ84*, with deletions in clpX or clpP demonstrate that deletion of either *clpX* or *clpP* suppresses the filamentous temperature-sensitive phenotype caused by the mutation in *ftsZ*. These results suggest that ClpXP participates in the execution of cell division in *E. coli* after the establishment of the FtsZ-ring. This may occur through modulation of the equilibrium between free and polymeric FtsZ via degradation of FtsZ or other cell division proteins.

IN VIVO STRUCTURE OF THE *E. COLI* FTSZ-RING REVEALED BY PHOTOACTIVATED LOCALIZATION MICROSCOPY (PALM)

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During bacterial cell division, the FtsZ protein localizes to the midcell and assembles into a ring-like structure—the Z-ring. While it is known that the Z-ring serves as an essential scaffold to recruit all other division proteins and generates contractile force for cytokinesis, it is unknown what the structure of the Z-ring is. Electron microscopy (EM) has been unsuccessful in detecting the Z-ring due to the dense cytoplasm of bacterial cells, whereas conventional fluorescence light microscopy (FLM) has limited spatial resolution (200-300 nm) due to the diffraction of light. Here, we used photoactivated localization microscopy (PALM), a single molecule-based superresolution imaging technique, to characterize the in vivo structure of the Z-ring in E. coli with 30-nm spatial resolution. We discovered that in addition to the expected ring-like conformation, the Z-ring of E. coli may also adopt a novel compressed helical conformation with variable helical length and pitch. We measured the thickness and packing density of the Z-ring and discuss the potential arrangement of FtsZ protofilaments inside the Z-ring.

THE STAPHYLOCOCCAL SUPERANTIGEN PATHOGENICITY ISLANDS – A NEW PARADIGM FOR MOLECULAR PIRACY.

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The SaPI family of *Staphylococcus aureus* pathogenicity islands is a group of related genetic elements that carry staphylococcal enterotoxin genes and are mobilized at high frequency by specific helper phages. The interactions between SaPIs and their helper phages are reminiscent in several ways of the exploitation of temperate enterobacteriophage P2 by satellite phage P4. The most striking similarity is remodeling of the helper phage capsid to accommodate the smaller SaPI genome. In contrast to the external scaffold provided by the Sid protein of P4, however, SaPIs redirect capsid size via SaPI-encoded internal scaffolding proteins. A novel feature of SaPI mobilization is redirection of the phage packaging machinery for preferential encapsidation of the SaPI genome. This involves a SaPI-encoded small terminase subunit that replaces the phage-encoded small subunit in the terminase complex to allow specific recognition of SaPI DNA. The genes involved in hijacking the helper phage morphogenetic functions are highly conserved among different SaPIs, but each SaPI can be mobilized only by certain helper phages. A major determinant of helper phage specificity is SaPI derepression, the initial step required for SaPI mobilization. The master repressors encoded by different members of the SaPI family are nonhomologous. Different SaPIs commandeer different phage-encoded proteins as antirepressors, which block repressor action and allow the expression of SaPI genes leading to excision, replication and helper phage exploitation.

THE DEVELOPMENT OF BACTERIOPHAGE TAILS AS AN ANTI-STAPHYLOCOCCAL AGENT

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Given their specificity and the relative ease in countering developing phage-resistance, bacteriophages (phages) and bacteriophage products are promising alternatives to traditional chemical antibiotic treatment. Although phages have been used previously to treat infections, there are serious disadvantages to this method: phages can transfer pathogenicity genes across bacterial species; lysogeny can provide resistance to future phage infection; and the cell lysis at the end of infection can release toxins from within the cell to the surrounding environment.

Our aim is to explore the use the tails of phages to kill *Staphylococcus aureus* as a potential alternative to traditional chemical antibiotics. Phage tails and tail-like particles (pyocins) have been shown to limit the growth of *Pseudomonas* and *Bacillus sp*. Growth inhibition is thought to occur because potassium ions leak through a pore created by the phage tail. During infection with intact phage this pore is repaired, but it persists when created by a defective phage and the cell is unable to recover from the ion leakage.

We have screened a 13 staphylococcal phages against 32 strains of *S. aureus* to identify suitable candidates for creating tails with anti-staphylococcal activity. We have identified a group of phages that display broad host range for both adsorption and plaque formation. We have successfully created phage tails from several of these by both chemical disruption of intact phage particles as well as through the construction of mutant phage lysogens. We observe that these phage-tail particles can adsorb to staphylococcal cells and we are currently developing methods to assess potassium flux and effects on bacterial growth.

USING WHAT PHAGE HAVE LEARNED TO CONTROL GRAM-POSITIVE BACTERIA

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Bacteriophages have evolved with bacteria for ~1 billion years. In this primordial soup, phages have evolved at least two systems that may be used to control bacteria. The first is the lytic system used to release progeny phage from infected bacteria. The second is the finding that the binding domains of lysins bind essential cell wall components that may be used as targets for antibiotic development. Lysins are highly evolved molecules used by the phage to destroy the bacterial cell wall to release phage progeny. We have developed lysins to nearly all gram-positive pathogens that kill these pathogens on mucous membranes, in blood and infected tissues. Our results show that in vitro 10^7 bacteria can be reduced to sterility seconds to minutes after enzyme contact. In animal model experiments, we colonize mice with either streptococcal or pneumococcal species (orally or nasally) and remove them to undetectable levels with lysins delivered to these sites using a single lysin dose. A model of murine MRSA sepsis was developed demonstrating widespread infiltration of all organs within 3h. When mice were treated intraperitoneally with 1 mg of a staphylococcal-specific lysin every 12 hrs for 2 days, bacteremia was eliminated and 85% of the animals survived; all controls died. So lysins may be a new strategy to control pathogens on mucous membranes and infected tissues.

The selection of targets for antibiotic development is critical for the identification of antibiotics that are difficult to become resistant against. However, there is no established method to identify these targets. Unlike enzymes from gram-negative phage, lysins from gram-positive phage have evolved binding domains that bind at high affinity to the bacterial cell wall. In the cell wall they target essential structures (usually carbohydrates) that the bacteria are unable to easily change. In a proof-of-principle study we used the binding domain from the gamma phage lysin to identify its carbohydrate receptor in the *B. anthracis* cell wall. We identified the genes in the pathway responsible for the carbohydrate's synthesis and identified an essential enzyme (N-acetylglucosamine 2-epimerase) in that pathway. The crystal structure of the 2-epimerase was determined and an inhibitor (RU04) for the enzyme that blocked the growth of *B. anthracis* both in vitro and in vivo down to ~3uM was modeled *in silico*. When we searched for resistant *B. anthracis* to this compound, it was found to be $<10^{-10}$ frequency, below our detectable limit. Thus, phage lytic enzymes may be used to identify critical pathways for the development of new anti-infectives for resistant gram-positive pathogens.

FUNCTIONAL AND STRUCTURAL STUDIES OF THE BIFUNCTIONAL *TOXIN* PHAGE ABORTIVE INFECTION AND TOXIN-ANTITOXIN SYSTEM

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The 10^{30} bacteriophages on Earth relentlessly drive the adaptive evolution of protective mechanisms in their bacterial hosts. Abortive infection, during which an infected bacterial cell altruistically commits suicide to protect the clonal population, can be mediated by the toxin-antitoxin pair, ToxIN. This system was found encoded on a cryptic plasmid, pECA1039, from the phytopathogen *Erwinia carotovora* subspecies *atroseptica*. This two-component abortive infection system is comprised of toxic ToxN protein and antitoxic ToxI non-coding RNA. ToxIN is the defining member of a new (Type III) class of protein-RNA toxin-antitoxin modules, of which there are multiple homologues, cross-genera.
The ToxI gene sequence comprises five and a half repeats of thirty-six nucleotides. We showed that one of these repeats is sufficient to inhibit the toxicity of ToxN. This knowledge allowed co-over-expression of ToxN protein with ToxI RNA, for use in X-ray crystallographic studies. The solved macromolecular structure showed an aesthetically beautiful complex formed of multiple ToxN monomers and ToxI RNAs. The structure highlights the diverse potential of non-coding RNAs, specifically indicating a role as inhibitors (or suppressors) of cognate processing enzymes. This is the first reported structure of a hybrid protein-RNA toxin-antitoxin system, and represents a platform for further investigation of ToxN homologues and their modes of binding diverse RNAs. In addition to the structure, we are also investigating how some, but not all, phages activate ToxIN to instigate suicidal abortive infection.

THE *ESCHERICHIA COLI RNLA-RNLB* IS A NEW TOXIN-ANTITOXIN SYSTEM THAT IS ACTIVATED BY BACTERIOPHAGE T4 INFECTION

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The *Escherichia coli* endoribonuclease LS was originally identified as a potential antagonist of bacteriophage T4. When the T4 *dmd* gene is defective, RNase LS cleaves T4 mRNAs and antagonizes T4 reproduction. This RNase also appears to play an important role in RNA metabolisms in *E. coli*. The *rnlA* is essential for RNase LS activity and encodes an endonuclease activity. In this meeting, we report that the *rnlA-rnlB* operon is a new chromosomal toxin-antitoxin locus. RnlA expression caused rapid degradation of *E.coli* mRNAs and impaired cell growth. However, the presence of *rnlB* (formerly *yfjO*), which is located downstream of *rnlA*, counteracted this nuclease activity and growth defect. Over-expression of RnlB could also suppress the growth defect of T4 *dmd* phage. Immunoprecipitation assays showed the interaction of RnlA with RnlB or Dmd, and both of which specifically blocked an RnlA nuclease activity *in vitro*. From these data, an RnlA endonuclease is a toxin, and RnlB and Dmd function as an antitoxin for RnlA. While *E.coli* would have the *rnlA-rnlB* operon as a defense mechanism that inhibits the spread of phage T4, T4 phage might evolve the *dmd* gene for its survival.

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DIRECT ACTIVATOR/CO-ACTIVATOR INTERACTION IS ESSENTIAL FOR BACTERIOPHAGE T4 MIDDLE GENE EXPRESSION

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The bacteriophage T4 AsiA protein is a bifunctional regulator that inhibits transcription from the major class of bacterial promoters and also serves as an essential co-activator of transcription from T4 middle promoters. AsiA binds the primary σ factor in *Escherichia coli*, σ^{70}, and modifies the promoter recognition properties of the σ^{70}-containing RNA polymerase (RNAP) holoenzyme. In its role as co-activator, AsiA directs RNAP to T4 middle promoters in the presence of the T4-encoded activator MotA. According to the current model for T4 middle promoter activation, AsiA plays an indirect role in stabilizing the activation complex by facilitating interaction between DNA-bound MotA and σ^{70}. Here we show that AsiA also plays a *direct* role in T4 middle promoter activation by contacting the MotA activation domain. Furthermore, we show that the recently characterized interaction between AsiA and the β-flap domain of RNAP is important for co-activation. Based on our findings, we propose a revised model for T4 middle promoter activation, with AsiA organizing the activation complex via three distinct protein–protein interactions.

POLYMORPHIC REGULATION OF P_{RM} BY Λ CI

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Bacteriophage λ can grow either lytically or as a lysogen. The λ repressor, CI, is transcribed from the promoter, P_{RM}, and is responsible for the maintenance of the lysogenic state. In a lysogen, the lytic promoters, P_L and P_R, are turned off by CI to prevent lytic growth and at the same time the P_{RM} is turned on by CI to maintain lysogeny. The operator regions for CI binding consist of a left region (operator O_L) and a right region (operator O_R). O_L and O_R each contain a subset of three CI binding sites, (O_L1, O_L2 and O_L3) and (O_R1, O_R2 and O_R3), respectively. In a lysogen, one pair of CI dimers binds cooperatively to O_R1~O_R2 and a second pair binds cooperatively to O_L1~O_L2, repressing P_R and P_L, and activating cI gene. Two pairs of dimers at O_R1~O_R2 and O_L1~O_L2 bind cooperatively to each other forming a DNA loop held together by an octamer of CI. At higher CI concentrations, two CI dimers also bind cooperatively to O_L3 and O_R3, forming a tetramer and repressing P_{RM}. Recent studies show that O_L3 located 2.3 kbp from O_R3, plays a role in efficient repression of P_{RM}. The object of our work was to investigate in detail the role of each of the six operators in the activation and repression of P_{RM}. We analyzed various operator mutations by *in vitro* transcription assays. We found that mutation in O_L1 or O_L2 reduced the repression of P_{RM}. Mutation in O_R1 resulted in the repression of P_{RM}, while mutation in O_R3 or O_L3 failed to repress P_{RM}. Mutation in O_R2 resulted in both activation and repression of P_{RM}. Our results would be put in the context of the current model of the regulation of CI in lysogenic maintenace.

MECHANISM OF TRANSCRIPTION AUTOREGULATION OF THE Λ REPRESSOR REVEALED BY SINGLE-MOLECULE IMAGING IN LIVE E. COLI CELLS

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We developed a single-molecule reporter to monitor the autoregulation of a transcription factor in single E. coli cells. Using the regulatory motif controlled by the bacteriophage λ repressor CI as a model system, we used a cotranslational fusion and cleavage strategy to count the production of CI molecules one by one in real time. This strategy circumvents the problem that a fused fluorescent protein moiety disturbs CI function. We discovered that positive autoregulation of CI results in an elevated frequency of transcriptional events, or bursts, and the number of mRNA molecules produced during each burst, or burst size. Negative autoregulation by CI reduces the frequency, but not the size, of transcriptional bursts. These observations are consistent with a model in which CI activates its own transcription by enhancing the recruitment of RNA polymerase and the cooperativity of the transcription process. We investigate possible molecular mechanisms by which λ repressor activates its own transcription

PHAGE GIL01 GOES SOS: THE UNCONVENTIONAL ROLE OF HOST LEXA AT A LYTIC SWITCH

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The transition from a latent state to the lytic cycle is a finely regulated process in temperate phages. The switch is classically initiated when a lytic gene repressor, usually phage-encoded, is inactivated. In the lambda model, the protein repressor CI is cleaved as a consequence of the SOS response to DNA damage in a same fashion as the global SOS repressor LexA.
Phage GIL01 has a linear genome with a terminal protein bound to the ends and does not integrate the host chromosome upon infection. Instead, it remains inside the cell as a plasmid and is passed down through generations as cells divide. GIL01 occasionally forms clear plaque mutants that can be subdivided into two categories: mutants in genes predicted to code for DNA-binding proteins and operator mutants. The operator is a conserved LexA box that is effectively bound by host LexA *in vitro*. Disruption of either the LexA box or one of the phage-coded regulators abolishes the ability to enter the lysogenic state. Intriguingly, LexA does not repress transcription from the phage promoter but rather, it is part of a repressosome composed of multiple protein actors in which each actor appears to be critical for the integrity of the system. The present study describes a new function for LexA in the control of mobile DNA.

ALTERNATIVE SIGNALING PATHWAYS FOR THE RCS REGULON

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The capsule regulatory system of *E. coli* (Rcs) is composed of RcsF, an outer-membrane lipoprotein, RcsC a transmembrane sensor-kinase, RcsD a transmembrane phospho-transfer protein, RcsB, the DNA-binding response regulator and RcsA, an auxiliary protein. The nature of the physiological signals activating this pathway and, in particular, the nature of the signal going from RcsF to RcsC remains elusive. Work by Kadokura *et al*. (Science (303):534-537) indicated that RcsF interacts directly with the disulfide bond forming protein (DsbA). The presence of 4 highly conserved cysteine residues in the periplasmic domain of RcsF suggested the possibility that RcsF may be monitoring the redox potential of the periplasm as a physiological signal and transmitting this signal to RcsC. However, DsbA signaling is dependent on RcsC and RcsD but not RcsF, indicating that the disulfide bonds in RcsF are required for structure, not signaling. We also find that DsbA signaling to RcsC is independent of the conserved cysteines in the periplasmic loop of RcsC, although these residues do play a role in RcsF-dependent signaling. Our results suggest the presence of two signaling pathways to RcsC.

PROBING THE DEVELOPMENT AND EVOLUTION OF HOST SPECIFICITY IN SYMBIOTIC *VIBRIO FISCHERI*

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Animal biology is characterized by sophisticated relationships with symbiotic microorganisms. In many instances symbionts are acquired from the environment each generation, and filtering of the relevant symbiotic partner from a milieu of unwanted relationships is a formidable obstacle. Despite many examples of specificity in both beneficial and harmful relationships, integrated systems to study the genetics, development, ecology, and evolution of specificity in animal-bacterial partnerships have been lacking.

The bioluminescent Gram-negative bacterium *Vibrio fischeri* forms natural monospecific light-organ associations with squid and fish hosts, and specific colonization of *Euprymna scolopes* squid by *V. fischeri* is due to the RscS-Syp bacterial signal transduction pathway. The RscS-Syp pathway controls symbiotic biofilm formation and had been shown to be important for normal squid colonization. My work has pointed to the role of a single regulatory gene, *rscS*, in allowing Pacific *V. fischeri* to form specific relationships with *E. scolopes*. Comparative genomics, functional studies, population studies, and phylogenetic analyses together have suggested that *rscS* is necessary and sufficient for host determination in squid populations of the North Pacific Ocean.

In my own laboratory I am expanding on these approaches to take advantage of the natural diversity of *V. fischeri* isolated from squid and fish hosts in the Pacific Ocean, from squid hosts in the Mediterranean Sea, and from non-symbiotic sources. I have performed targeted molecular analysis of these isolates and found significant natural variation in the canonical RscS-Syp pathway. Mutant analysis has revealed isolates that can colonize the squid host without a functional RscS. Approaches underway to dissect the pathway include whole genome sequencing and forward genetic analysis. As such, the natural diversity provides a rich starting point for fine-scale dissection of the evolutionary processes that underlie the development of host specificity and identification of novel mechanisms of host association.

SENSITIVITY OF *E. COLI* TO BLEOMYCIN IS MEDIUM DEPENDENT

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Bleomycin (BLM) is a glycopeptide antibiotic and anti-tumor agent that targets primarily the furanose rings of DNA. Degradation by BLM is initiated by generating a free radical in deoxyribose resulting in two different types of DNA damage: oxidized apurinic/apyridiminic (AP) sites and single-and double-strand breaks. Consistent with these damages, we found that recombination-deficient strains of *E. coli* K-12 are very sensitive to the cytotoxic action of BLM at low doses when cells were grown in rich medium. As expected, the *recBCD* pathway strains were sensitive but the *recF* pathway strains were not. Surprisingly, *recG* and *recN* mutant strains were sensitive to the action of BLM. An *xthA nfo* strain deficient in AP endonucleases was also sensitive to BLM consistent with damage to DNA bases. Surprisingly, all the above strains, and the wildtype, were resistant to BLM at the same concentrations when grown and exposed to BLM in minimal medium. The number of BLM-induced double-strand DNA breaks, however, was the same in cells grown in both media as determined by pulse field gel electrophoesis. Only mutants deficient in DNA ligase (*lig-4* and *lig-7*) were sensitive in both media.

BACTERIAL CELL WALL POLYMERASES REQUIRE PROTEIN COFACTORS TO BUILD PEPTIDOGLYCAN

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Most bacteria fortify their cytoplasmic membrane with a crosslinked polysaccharide meshwork called peptidoglycan (PG) to protect themselves from osmotic lysis. The major cellular PG synthases are the bifunctional penicillin-binding proteins (PBPs), which possess both transglycosylase activity for synthesizing the glycan strands of PG and transpeptidase activity for crosslinking them. When supplied with lipid-II substrate, these purified PBPs can polymerize and crosslink glycan strands *in vitro*. However, the PBPs alone are insufficient for the proper assembly PG assmbly *in vivo*. Their activity must be spatially and temporally regulated. Cytoskeletal polymers of FtsZ and MreB are thought to accomplish this by organizing the assembly of distinct multi-protein complexes containing the PBPs and other PG assembly factors and directing their activity to appropriate subcellular locations. Many questions regarding the function and composition of these PBP-containing, multi-enzyme complexes remain to be addressed. One of the most fundamental is whether such complexes promote PG synthesis simply by providing the PBPs with access to substrate, or whether they also contain critical accessory factors that facilitate and/or regulate PBP activity by affecting lipid-II utilization or the incorporation of nascent PG into the existing network. We therefore sought to identify factors essential for the *in vivo* activity of the bifunctional PBPs using a synthetic lethal screen we developed for the model gram-negative rod *Escherichia coli*. Our approach revealed two outer membrane lipoproteins of previously unknown function as new PG assembly factors. We have designated them LpoA and LpoB for lipoprotein activators of PBP1 function from the outer membrane. LpoA was found to be specifically required for PBP1a function while LpoB was found to be specifically required for PBP1b function. Results will be presented indicating that these factors directly modulate the activity of their cognate PBPs through the formation of specific trans-envelope lipoprotein-PBP complexes.

ROLE AND REGULATION OF *CAULOBACTER CRESCENTUS CSP* PARALOGUES IN RESPONSE TO STRESS

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Bacteria are organisms that can live in very different habitats and therefore must be able to cope with challenges that may somehow affect their growth. One critical environmental factor is temperature, which requires quick physiological responses. The most characteristic response to temperature downshift is the induction of small proteins with a conserved domain called "Cold Shock Domain" (CSD).
Caulobacter crescentus has four genes encoding proteins with CSD (*cspA, cspB, cspC* and *cspD*) being *cspA* and *cspB* cold-induced while *cspC* and *cspD* are induced upon entry into stationary phase. Mutant strains deleted of each one of these genes were constructed and mutations were combined generating double and triple mutants. Cold sensitivity assays suggested that there is a hierarchy of importance in cold shock response, being CspA and CspC the most important ones followed consecutively by CspB and CspD. Studies of gene expression made possible to determine that in ΔCD mutant strain *cspA* expression is increased at early stages of cold shock, while *cspB* pattern showed no alteration of cold induction but has decreased levels of expression even at 30ºC. The upstream region of *cspA* and *cspB* were aligned in order to determine common regions that could mediate the cold shock induction of these two genes. Gene expression assays using translational fusions, showed that *cspB* inductions is not dependent of a downstream box but there is a very important putative upstream box that apparently contributes for the major induction by cold shock.
Freezing survival involves the ability of coping with cold and osmotic stresses, since differential compartment freezing may create osmotic flux of water to the outside of cells. CSP mutants in *C. crescentus* showed decreased freezing resistance, which raised the possibility of their involvement in osmotic stress response. Survival tests under high osmolarity showed that CspC and CspB are the most relevant CSPs in this stress response, but only *cspA* and *cspC* show transcriptional induction in this condition.
All results taken together allowed to conclude that *csp* paralogues in *C. crescentus* respond to a wide range of stresses like osmolarity, cold and stationary phase, and are very important for viability under these conditions.

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COMMUNITY ASSESSMENT OF COMMUNITY ANNOTATION USING ONTOLOGIES (CACAO): FUNCTIONAL ANNOTATION WITH UNDERGRADUATES

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The rapid advances in DNA sequencing technology mean that we are discovering genes faster than we can figure out what they do. For most new sequences, gene function will be inferred by comparison with well-studied model systems such as *Escherichia coli*. This means that the quality of functional annotation for these models is very important for the future of biology. With the growing demand for inexpensive, high quality curation, we developed an activity called CACAO, or Community Assessment of Community Annotation with Ontologies. During a pilot course run in April 2010, we trained teams of undergraduates at Texas A&M University on how to generate literature-based functional annotation of proteins using Gene Ontology (GO). The teams, totaling 16 students, submitted 153 annotations to EcoliWiki (http://ecoliwiki.net) and the competition concluded with evaluation and challenging of annotations contributed by other teams and declaration of a "winning" team. Experienced curators reviewed the students' annotations and accepted 117. These annotations will be submitted back to the GO consortium where they will be publicly available for databases and bioinformatics groups to use. There was a very positive response from the students and feedback showed support for learning about bioinformatics and functional annotation. In the future, annotations will be added to GONUTS, a database that is not restricted to a single model organism. We will be repeating CACAO on a larger scale in the Fall of 2010 with a multi-institutional set of teams after positive feedback and interest from other undergraduate educators.

PREDICTING PHAGE PREFERENCES: LYTIC VS LYSOGENIC LIFESTYLE FROM GENOMES

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There are two distinct phage lifestyles: lytic and lysogenic. The lysogenic lifestyle has many implications for phage therapy, genomics, and microbiology, however it is often very difficult to determine whether a newly sequenced phage isolate grows lytically or lysogenically just from the genome. Using the ~200 known phage genomes, a supervised random forest classifier was built to determine which proteins of phage are important for determining lytic and lysogenic traits. A similarity vector is created for each phage by comparing each protein from a random sampling of all known phage proteins to each phage genome. Each value in the similarity vector represents the protein with the highest similarity score for that phage genome. This vector is used to train a random forest to classify phage according to their lifestyle. To test the classifier each phage is removed from the data set one at a time and treated as a single unknown. The classifier was able to successfully group 188 of the 196 phages for whom the lifestyle is known, giving my algorithm an estimated 4% error rate. The classifier also identifies the most important genes for determining lifestyle; in addition to integrases, expected to be important, the composition of the phage (capsid and tail) also determines the lifestyle. A large number of hypothetical proteins are also involved in determining whether a phage is lytic or lysogenic.

FUNCTIONAL CHARACTERIZATION OF LER, THE POSITIVE REGULATOR OF VIRULENCE GENES IN ENTEROPATHOGENIC *ESCHERICHIA COLI* (EPEC).

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The Locus of Enterocyte Effacement (LEE) is essential for virulence in EPEC and other attaching and effacing bacteria such as EHEC and *Citrobacter rodentium*. The LEE-encoded regulator (Ler) is the key transcriptional regulator of EPEC virulence. Ler is a 15-kDa protein that exhibits amino acid sequence similarity with the C-terminus of H-NS, a nucleoid-structuring protein that affects, mainly negatively, bacterial gene expression. As for H-NS, the Ler N- and C-terminal domains are predicted to participate in oligomerization and DNA binding, respectively. Ler acts as an antirepressor protein that overcomes the H-NS-mediated silencing on the LEE promoter regions. In this study, characterization of randomly selected Ler mutants incapable of activating the expression of a Ler-dependent promoter, led to the identification of critical amino acids and functional motifs. Using a bacterial two-hybrid system and pull-down assays, we found that all mutants, including those located at the predicted "coiled-coil" domain, were still able to dimerize. By performing DNA binding assays using the purified proteins, the Ler mutants were separated in different functional groups: mutants that interact with the Ler DNA targets as well as the wild type; mutants that do not bind to DNA; and mutants that form aberrant DNA-protein complexes. Furthermore, analysis of the oligomeric state of wild type Ler in native acrylamide gels and by gel filtration, suggested that the functional DNA-binding form of this protein is an octamer, and that mutants forming aberrant complexes with DNA also generate different oligomeric forms in solution even at low protein concentrations.

Mutations predicted to disrupt the putative "coiled-coil" domain located at the N-terminal region of Ler, did not affect its ability to interact with itself or with DNA, but affected its activity, as well as its capacity to form octamers, suggesting that this domain mediates high order oligomerization, while the important residues for dimerization are located at the end of the N-terminal domain. According to these observations, the dimerization-oligomerization domain of Ler has an inverted organization in comparison with H-NS. Overall, these results further our understanding of the modular nature of Ler and identified critical functional residues involved in oligomerization and DNA binding.

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STRUCTURE-FUNCTION ANALYSIS OF A BACTERIOPHAGE-ENCODED REGULATOR OF Σ^A-DEPENDENT TRANSCRIPTION IN *S. AUREUS*

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RNA polymerase in bacteria consists of a catalytically proficient core enzyme (subunit composition $\alpha_2\beta\beta'\omega$) that must associate with one or another σ factor to recognize specific promoter sequences. Anti-σ factors comprise a large and diverse family of proteins that regulates σ factor function. Typically, anti-σ factors target alternative σ factors and inhibit promoter utilization by preventing their association with the RNAP core enzyme. However, the bacteriophage T4-encoded AsiA protein provides a well-characterized example of an anti-σ factor that interacts with a primary σ factor (*E. coli* σ^{70}) in the context of the RNAP holoenzyme, altering its promoter-recognition properties. Here we examine another bacteriophage-encoded anti-σ factor that targets a primary σ factor: the ORF67 protein of *S. aureus* phage G1. First identified as an inhibitor of *S. aureus* growth (1), ORF67 forms a tight complex with conserved region 4 of *S. aureus* σ^A and inhibits σ^A-dependent transcription. Biochemical evidence indicates that ORF67 associates stably with the σ^A-containing RNAP holoenzyme, suggesting that the ORF67-σ^A region 4 interaction is not incompatible with holoenzyme formation. Here we use a bacterial two-hybrid assay to further characterize the protein-protein interactions of ORF67. Taking advantage of the fact that ORF67 does not interact detectably with σ^{70} region 4, we use σ region 4 chimeras to identify the specificity-determining residues. A high-resolution crystal structure of the ORF67-σ^A region 4 complex locates these residues at the ORF67-σ^A region 4 interface. By adapting our two-hybrid assay for the detection of bridging interactions, we show further that the ORF67-σ^A region 4 complex can interact with the flap domain of the RNAP β subunit, which is the attachment point for σ region 4 in the context of the RNAP holoenzyme. Current efforts are directed towards understanding precisely how ORF67 modulates the promoter-recognition properties of the σ^A-containing RNAP holoenzyme.

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INTERROGATING GENES OF UNKNOWN FUNCTION IN *E. COLI.*

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We are addressing the roles of bacterial proteins encoded by genes for which no known function has been assigned with an emphasis on those genes that have been deemed essential or those that are highly conserved. In *E. coli*, there are many genes that do not tolerate disruption and it has been concluded that these genes most likely encode important or essential gene products. Surprisingly, a notable fraction of these genes have no assigned function despite the fact *E. coli* has been studied at a genetic and biochemical level for decades. A significant hurdle in identifying the roles these genes play stems from the fact that many are essential, so standard genetic techniques that reduce the amount of gene product end up killing the cells and precluding any meaningful physiological measurement. For those genes that are non-essential, genetic manipulations designed to remove the gene can result in a re-balancing of cellular process that compensate for the absence of the encoded gene product or second-site mutations can suppress overt phenotypes, a bane of classical genetics.

One of our approaches to address the roles of essential genes of unknown function, we are implementing a targeted degradation system that allows for normal cell growth using a modified target gene encoding a conditional degradation peptide tag that is specifically recognized by a processive cellular protease. Induction of proteolysis leads to the rapid elimination of the target protein from the cell, leaving the other proteins intact. This approach allows the characterization of cellular networks depleted of an essential target soon after its removal.

GENETIC DISSECTION OF THE RCS TWO-COMPONENT SIGNALING PATHWAY AND ITS ROLE IN SWARMING MOTILITY IN *PROTEUS MIRABILIS*

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Proteus mirabilis is a gram-negative, rod-shaped bacterium when grown in liquid media. During growth on surfaces, *P. mirabilis* undergoes a distinct physical and biochemical change that culminates in the formation of a swarmer cell. Swarmer cells are elongated, polyploid, and hyper-flagellated cells that up-regulate virulence factors. How *P. mirabilis* senses a surface is not fully understood, however, the inhibition of flagella rotation has been proposed to be a sensory mechanism. Our lab has isolated a transposon insertion in *waaL*, encoding O-antigen ligase, that results in loss of swarming, but not swimming motility. Upon further examination, it was shown that these cells fail to activate *flhDC*, the class 1 activator of the flagellar cascade, when grown on solid surfaces. A mutation in the *wzz* gene, which results in short O-antigen side chains, also prevented activation of *flhDC* on solid surfaces. We propose that surface sensing is relayed by O-antigen to the RcsCDB phosphorelay, a known repressor of *flhDC*. In order to test this hypothesis, mutations were made in *rcsC, rcsB, rcsF*, and *umoB* (*igaA*) in wild-type and *waaL* backgrounds. By comparing the swarming phenotypes of the single and double mutants, we have begun to establish a working model for the role of O-antigen in surface sensing and the Rcs pathway in *P. mirabilis*. The data demonstrates that along with RcsF, there is another input acting on RcsC, and that at least one of these inputs appears to be activated by surface sensing.

DIFFERENCES IN OXIDATIVE STRESS-RESPONSE MECHANISMS OF *E. COLI* AND *B. SUBTILIS* CORRELATE WITH THEIR ENVIRONMENTAL DEMANDS

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Several processes that restrict bacterial growth are known to induce the accumulation of Reactive Oxygen Species (ROS) inside the cell. High levels of ROS such as peroxide can lead to an increased rate of mutagenesis and cell death by damaging cellular proteins, lipids and nucleic acids. As a defense, prokaryotes have evolved sensitive and specific inducible response mechanisms to detect peroxide. Two well-studied peroxide stress-response mechanisms are the OxyR and PerR regulons of *E. coli* and *B. subtilis* respectively. Both OxyR and PerR are transcription factors that react with peroxide, change their activity and thereby modulate the expression of target enzymes that are likely to protect the cell from peroxide. However there is an important difference between the designs of these two inducible stress-detection mechanisms. The Fe-mediated oxidation of metallo-protein PerR in the presence of peroxide is irreversible but the oxidation of OxyR by peroxide is reversible. We have constructed mathematical models of the OxyR and PerR regulatory circuits and used them to perform a mathematically controlled comparison. The results indicate that even though the steady state response sensitivities to changes in peroxide concentrations are very similar, structural disparities in the two circuits result in important differences in their response dynamics. Our models show that OxyR responds to changes in peroxide concentrations faster than PerR. On the other hand, the PerR circuit results in less noise in target gene expression as compared to the OxyR circuit. These trade-offs can be interpreted in the context of *E. coli* and *B. subtilis* natural environments to explain the differences in response strategies.

TRANSLATIONAL BIASES IN THE GENES OF MYCOBACTERIOPHAGE

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Codons in prokaryotes are used in a biased fashion, adapted to the cellular levels of tRNAs.[1,2] Highly expressed genes often exhibit a marked degree of bias towards the most abundant tRNAs,[3] although there is considerable variation over the lengths of such genes, with low abundance tRNAs more represented at the 5' end.[4] Bacteriophages might be expected to share the biases of their hosts, whose translational apparatus they exploit. This is not always the case, and it has been suggested that tRNAs encoded by phage genomes may be responsible for altered biases in phage genes.[5] The relationship between phage translational biases, phage-encoded tRNAs, and the biases of phage hosts remains incompletely understood.

We have used the large number of sequenced mycobacteriophage genomes,[6] along with those of seven additional mycobacteriophages we have sequenced, to explore codon and tRNA usage biases and their relationship to phage hosts and phage-encoded tRNA. A comparison of codon biases between mycobacteriophages and a collection of 13 sequenced mycobacterium genomes produced the original host, *Mycobacterium smegmatis*, as the top match in all tested cases but one. Such an analysis may therefore be useful in predicting the host of phages identified from environmental samples whose host is unknown. The exceptional case was the mycobacteriophage Cornelius, which did not match any mycobacterium particularly well. Cornelius is unusual in that it carries 10 tRNAs, the significance of which will be discussed.

Codon biases seen in *M. smegmatis* are sometimes amplified within the mycobacteriophages that infect it. Codons that are highly used in the bacterium tended to be even more highly used in the phage, while those that are underutilized in the bacterium were even less used in the phage. This codon amplification of bias was seen as well in genes of the bacterium expected to be highly expressed (e.g. those encoding ribosomal proteins).

This work was done within the context of annotating the new phage genomes, and we will discuss the efficacy of using tRNA usage biases along the length of annotated genes to refine the identification of the genes' 5' ends.

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TARGET SPECIALIZATION OF *E. COLI* TRANSCRIPTION FACTOR DKSA

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A group of bacterial transcription factors including GreA, GreB and DksA of *E. coli* regulate transcription by acting through the secondary channel of RNA polymerase (RNAP). Transcript cleavage factors GreA and GreB stimulate RNAP intrinsic nucleolytic activity and thereby suppress transcription pause and arrest, enhance transcription fidelity and promoter escape. Unlike Gre factors, DksA does not stimulate transcript cleavage. Instead, depending on the promoter, it can destabilize the RNAP-DNA open promoter complexes. DksA alone or together with alarmone ppGpp activates and/or inhibits transcription both *in vivo* and *in vitro*. That these proteins act through RNAP's secondary channel, raises the question as to how they regulate transcription. Do these factors compete with each other? If they don't, what is the mechanism of their target specificity? To address these questions and to better understand the molecular mechanism of action of these factors, we (i) characterized the molecular interfaces of GreA/DksA-RNAP complexes using site-directed mutagenesis of Gre/DksA and RNAP, and by site-specific protein crosslinking; (ii) determined the binding affinities of Gre/DksA towards three functionally distinct complexes: open promoter complex, backtracked and active elongation complexes; (iii) analyzed functional activities of Gre/DksA *in vitro* transcript cleavage, run-off transcription and DNA-footprinting assays. We identified the key residues in Gre, DksA and RNAP secondary channel required for specific Gre/DksA-RNAP interactions. Based on our data, we created plausible models of Gre/DksA-RNAP complexes. In the models, Gre and DksA bind to RNAP at the β'-loop connecting two α-helices of the β' coiled-coil (β'-CC) - the rim of the secondary channel. However, the contact residues for Gre and DksA on β' are distinct. The high-affinity binding of DksA to RNAP is determined by its Zn-binding domain which anchors to the β'-CC, and by the C-terminal α-helix which binds to the nonconserved element SI1 of β. The N-terminal coiled-coil domain of DksA is essential for its functional activity. It interacts with the β' conserved trigger loop (G-loop), β' F-loop and the α-helix of the β-subunit substrate-binding element. We show that Gre and DksA possess different binding affinities to different RNAP complexes. In spite of the fact that Gre and DksA compete for binding to RNAP core and holoenzyme, DksA does not bind to elongation complexes and does not compete with Gre during transcription. We propose that conformational changes in the β SI1 element and, possibly in the β' G-loop, define the targeting of DksA to different classes of transcription complexes.

TRANSPOSON TN7 CONTROLS TARGETING INTO REPLICATING DNA USING INTERACTIONS WITH HOST- AND TRANSPOSON-ENCODED PROTEINS.

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The bacterial transposon Tn7 has two pathways of transposition that are catalyzed by a combination of five proteins, TnsA, TnsB, TnsC, TnsD, and TnsE (TnsABCDE). TnsABC form the core machinery that is required, but not sufficient, for transposition. The TnsABC + TnsD proteins can direct transposition into a single chromosomal site found in all bacteria (called *attTn7*). The TnsABC + TnsE proteins direct transposition into actively replicating DNA. Replication associated with the conjugal transfer of plasmids is preferentially targeted in a process that facilitates horizontal transmission of the element. We have made important advances on how Tn7 recognizes active DNA replication and how it communicates the molecular decision to target certain DNAs.

We find that TnsE physically and functionally interacts with the processivity factor (β-clamp) of the DNA replication machinery. The ability of TnsE to recognize the highly conserved β-clamp protein helps explain the wide distribution of Tn7-like elements in a variety of environments. Using a reconstituted *in vitro* system we have been able to discern the minimal requirements for targeting. Preliminary work suggests that other unrelated transposons may use a similar mechanism to recognize aspects of DNA replication. In more recent work we have found additional host proteins that associate with TnsE that have been identified by Mass Spectrometry. These proteins are being pursued as additional host factors that allow transposon targeting.

Continued work with this system is establishing the molecular mechanism that allows Tn7 to regulate target site selection. We find that we can use gain-of-activity mutations in TnsE and TnsC to engineer vast changes in Tn7 targeting *in vivo*. *In vitro* analysis of these mutant proteins has revealed biochemical activities that are crucial for the targeting decision. Our findings have implications for how Tn7-like elements may naturally alter targeting to suit their selection environment and how elements can be reengineered as tools for genomics.

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RECOMBINATION PHENOTYPES OF A *GREA* MUTANT

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Wild type phage λ behaves as if it were a *red* mutant in an *E.coli* strain bearing the *greA* D41N mutation (but not a *greA* deletion). It forms small plaques at high efficiency, and is defective for recombination via the Red pathway. In the absence of the rest of λ, with Red expressed from the Ptac promoter, the D41N strain produces fewer recombinants with electroporated DNA species than the wild type. This defect in Red-mediated recombination is not due to reduced Red expression; the D41N mutation has no apparent effect on expression of Redβ or λExo, as visualized by Western blot. In contrast to its defect in Red-mediated recombination, the D41N strain is apparently hyper-rec in the RecA-RecBCD pathway: it exhibits elevated frequencies of transduction of chromosomal markers by phage P1.

RE-EVALUATION OF GROWTH RATE CONTROL BY PPGPP

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It is widely accepted that the DNA, RNA and protein content of *Enterobacteriaceae* is regulated as a function of exponential growth rates, which was demonstrated more than 50 years ago in the classic works of Kjeldgaard, Maaloe and Schaechter. This phenomenon, called growth rate control, primarily involves regulation of ribosomal RNA synthesis. However, it was uncertain whether the global regulator ppGpp is the major determinant for growth rate control or whether there are robust redundant mechanisms contributing to the same degree. Here, we re-evaluate the effect of ppGpp on macromolecular content at different balanced growth rates in defined media. We determined the specific amino acid requirements of strains lacking ppGpp ($ppGpp^0$), which we exploit to vary unrestricted growth.

We find that growth rate control does not occur in the absence of ppGpp. Instead, RNA/protein and RNA/DNA ratios are equivalent in fast and slow growing ppGpp0 cells and are maintained at the levels of fast growing wild type cells. We have verified that under these conditions the excessive RNA content of slow growing $ppGpp^0$ cells is ribosomal RNA, detectable in sucrose gradients. Furthermore, ribosomal subunit composition is unchanged indicating ribosomal maturation is unaffected. On the other hand, polysome content of $ppGpp^0$ cells is reduced when compared to wild type cells, while the monosome fraction is increased as if excess mRNA is present as well.

Artificial elevation of ppGpp or introduction of stringent RNA polymerase mutants in $ppGpp^0$ cells restores growth rate control. We believe these findings strongly argue in favor of ppGpp and against redundant regulation of ribosomal content by other factors in enteric bacteria.

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PRODUCTION OF ENGINEERED AND FUNCTIONAL T4 BACTERIOPHAGES BANK THAT TARGET PATHOGENIC GRAM NEGATIVE BACTERIA

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Infectious threats due to microbes of high pathogenic potential remain a steady but unpredictable risk for human and animal health. The emergence and/or reoccurrence of pathogenic bacteria together with ever increasing antibiotic multi-resistances constitute significant therapeutic problems. Bacteriophages do provide some solutions but their use is limited by a rapid development of resistance in bacterial target populations and the ineffectiveness of selecting new phage strains from nature. Therefore, we decided to genetically engineered bacteriophages in order to change their host range, while maintaining their capacity to replicate. In our study, we generated millions of variants of three T4 bacteriophage genes involved in Escherichia coli host recognition (cf. Poster, Genetical engineering of the gp37, gp38 and gp12 T4 bacteriophage genes involved in the phage-host recognition with Escherichia coli). We have created a method to efficiently introduce such variant genes in T4 phage genome: the lytic cycle of the obligate phage T4 was reversibly interrupted within its host and the exchange of these genes was carried out by homologous recombination. Then, the lytic cycle was reactivated to produce a very large, genetically engineered infective recombinant progeny (engineered T4 phage bank). The screening of this bank allowed a rapid isolation of recombinant T4 particles capable of infecting and destroying hosts belonging to bacterial species far removed from the original host: Yersinia ruckeri and Pseudomonas aeruginosa. We are currently testing our bank on a wide range of Escherichia coli which are wild-type T4 phage resistant and antibiotic multiresistant.

THE LUXR-FAMILY TRANSCRIPTIONAL REGULATOR $CARR_{39006}$ FROM *SERRATIA* SP. ATCC 39006 IS NATURALLY LIGAND-INDEPENDENT

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Strains of *Erwinia carotovora* are important plant pathogens, causing tuber soft-rot and blackleg disease in potato. Some strains of *Erwinia carotovora* subsp. *carotovora* (*Ecc*) synthesise a carbapenem antibiotic (1-carbapen-2-em-3-carboxylic acid) under quorum sensing (QS) control. In *Ecc* strain 39048, the LuxR-type transcriptional regulator $CarR_{Ecc}$ activates transcription of the carbapenem biosynthetic operon, and requires an *N*-acyl-homoserine lactone (AHL) ligand to function. *Serratia* sp. ATCC 39006 produces the same carbapenem antibiotic, controlled by the corresponding $CarR_{39006}$ transcriptional activator. These CarR activators are closely related to a sub-clade of variant LuxR-family repressor proteins, which fold and function in the absence of AHLs and are antagonised by AHL ligands.

This study presents evidence that, like other LuxR-family activators, specific binding to its cognate AHL ligand increases the affinity of $CarR_{Ecc}$ for its DNA target, and may prevent proteolytic turnover of $CarR_{Ecc}$. These AHL-mediated effects were abolished when a key residue in the ligand-binding pocket of $CarR_{Ecc}$ was changed by site-directed mutagenesis.

Previous genetic evidence suggested that, uniquely, the *Serratia* $CarR_{39006}$ protein is naturally able to function in the absence of AHL ligands. In contrast to its close homologue $CarR_{Ecc}$, this study presents direct evidence that, at physiological protein concentrations, $CarR_{39006}$ binds with high affinity to the *Serratia* 39006 carbapenem operon promoter, resists proteolytic turnover and activates transcription in the absence of AHLs. These biochemical data are consistent with our previous genetic evidence that this LuxR-family activator is naturally ligand-independent.

GRLR AND GRLA, THE REGULATORY SENTINELS OF THE LOCUS OF ENTEROCYTE EFFACEMENT IN ATTACHING AND EFFACING *ESCHERICHIA COLI*.

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EPEC, EHEC and *Citrobacter rodentium* belong to a group of pathogens that share the ability to form ‘‘attaching and effacing’’ (A/E) lesions on intestinal epithelia. The A/E lesion is characterized by the localized destruction of the apical microvilli of enterocytes and important cytoskeleton rearrangements beneath the adherent bacteria, leading to the formation of actin-rich cup-like structures and intimate bacterium-host cell interactions. The genes required for the formation of the A/E lesion are located within a pathogenicity island known as the locus of enterocyte effacement (LEE). The specific regulation of LEE genes relies on three LEE-encoded regulators, Ler, which activates the expression of the LEE operons by disrupting the repression mediated by the global regulator H-NS; GrlA, which is required for the specific activation of ler; and GrlR, which represses the expression of LEE operons. Here, we show that in the absence of GrlR the transcriptional activity of LEE operons increases significantly under repressing growth conditions. Overexpression of GrlR exerts a strong repression over LEE genes in wild type EPEC and, unexpectedly, even in the absence of H-NS, suggesting that GrlR acts directly as a repressor. In contrast to the notion that GrlR acted as a repressor by inactivating GrlA through this interaction, here we show that an inactive GrlA mutant that still interacts with GrlR prevents GrlR-mediated repression and that a GrlR mutant that still interacts with GrlA does not longer repress LEE gene expression when over expressed. Under inducing growth conditions GrlA traps GrlR to keep it from forming an active dimer and repressing LEE gene expression; while under repressing growth conditions the predominant functional GrlR dimer favors negative control. Overall, our data unveiled that LEE gene expression is negatively regulated at two levels, which are mediated by both a global regulator (H-NS) and an EPEC specific regulator (GrlR) and that GrlA has a dual positive regulatory role as a specific regulator of *ler* expression by acting as a DNA binding protein and as an antagonist of GrlR through protein-protein interactions. Our work also illustrates that the LEE probably facilitated its horizontal transmission by down regulating its own expression through GrlR, thus preventing potential detrimental effects on bacterial fitness and ensuring assimilation by its current bacterial hosts, but at the same time providing the positive regulatory proteins (GrlA and Ler) that counteract its self- and host-encoded repressors (GrlR and H-NS, respectively).

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RHO-DEPENDENT TRANSCRIPTION TERMINATION IN *E. COLI*: ROLES FOR NUSA AND THE H-NS FAMILY OF NUCLEOID PROTEINS

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In eubacteria and archaebacteria, translation is a cotranscriptional process which requires both the binding of a pioneer ribosome to the nascent transcript as well as dynamic inter-regulation of the rates of transcript elongation and polypeptide chain elongation. In *E. coli*, the synthesis of nascent transcripts which fail to be simultaneously translated is terminated by an active mechanism (termed as polarity) involving the protein Rho. The factors NusA and NusG have generally been described to function antagonistically in modulating both transcription elongation rate and polarity (increased by NusG and decreased by NusA, in both instances); on the other hand, a recent report (Cardinale et. al., 2008) has suggested that both NusA and NusG act to promote polarity. The Rho, NusA, and NusG proteins are all essential for viability in "wild-type" *E. coli* MG1655.

Earlier work in this laboratory had shown that the transcription termination-defective (ie. polarity-relief) phenotypes conferred by recessive missense mutations in *rho* and *nusG* are suppressed by expression of a dominant-negative variant of the nucleoid protein H-NS that represents the N-terminal 64 amino acids of the 136 amino acid-long protein (H-NSΔ64) (Harinarayanan and Gowrishankar, 2003). In this study, we have identified for the first time a recessive missense mutation in *nusA* (R258C) that (i) restores polarity relief in *rho* or *nusG* strains with H-NSΔ64, (ii) by itself confers a polarity-relief phenotype but is unaffected for the rate of transcription elongation, and (iii) is synthetically lethal with the *rho* or *nusG* missense mutations in the absence of H-NSΔ64. We have also found that suppression of polarity relief in *rho* or *nusG* mutants by H-NSΔ64 is dependent on the presence of the H-NS-related genes *ydgT* or *hha* on the chromosome, and that multicopy-*ydgT* by itself can substitute for H-NSΔ64 in eliciting the suppression phenotype.

These results indicate (i) that NusA function is indeed needed for efficient Rho-dependent transcription termination, (ii) that the structure of the nucleoid is a determinant in the efficiency of the process, and (iii) that an entire spectrum of efficiencies of Rho-dependent termination can be generated by various combinations of perturbations in the genes encoding Rho, NusA, NusG, and the H-NS family of proteins, with the corresponding phenotypes extending from polarity through polarity relief to lethality.

References:
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IDENTIFYING CELLULAR FUNCTIONS FOR DNA GLYCOSYLASES BY MEASURING REPAIR OF OXIDATIVE DNA DAMAGE *IN VIVO*

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To deal with oxidative DNA damage, organisms have a suite of highly conserved DNA glycosylases that often have partially overlapping or redundant substrate specificities *in vitro*. A remaining challenge has been to determine whether these enzymes are truly redundant *in vivo*, or have more specialized roles inside the cell. To begin to address this possibility, we measured the rate at which Endonuclease III (Endo III) and formamidopyrimidine N-glycosylase (Fpg) repaired their respective substrates *in vivo*. *In vitro*, these are the primary enzymes that remove two common forms of oxidized base damage, 8-oxoguanine (8-oxoG) and thymine glycol (Tg), respectively. In contrast to biochemical studies, we observe that Fpg is responsible for the rapid removal of both lesions *in vivo*. By comparison, the absence of Endo III did not significantly alter the rate by which either lesion was removed. The results imply that *in vivo*, Fpg is the predominant enzyme that removes both 8-oxoG and Tg lesions from the bulk genomic DNA, whereas the functional role of Endo III and Endo VIII may be more specialized *in vivo*. Surprisingly, the recovery of replication was not prevented in any of the mutants examined, even when lesions persisted in the DNA, suggesting that cells contain a uniquely efficient mechanism to repair or tolerate oxidative lesions encountered during replication.

A STUDY OF COVALENTLY LINKED HFQ DIMERS AND THE REQUIREMENTS FOR RNA BINDING

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Hfq, an RNA chaperone necessary for the stability and activity of many sRNAs in *E. coli*, belongs to the Sm and Lsm family of proteins. Involved in splicing in eukaryotes, Hfq in its active form associates into a homohexameric ring. This structure has been shown to bind both sRNAs and mRNAs and facilitate their pairing. However, the mechanism by which hexameric Hfq facilitates these RNA-RNA interactions remains elusive. Mutations that disrupt binding to specific substrates have been identified, but it is still unclear the role that each subunit plays in these interactions, or if more than one ring of Hfq is necessary to promote pairing. In some alpha-proteobacteria, we found that *hfq* is encoded as a covalent dimer. Using a linker from these Hfq homologues, we have created covalently linked *E. coli* dimers, which are active for the Hfq-dependent positive regulation of RpoS. Mutations in single subunits of the dimer, as well as the construction of larger covalently linked multimers, are allowing us to define the contributions of individual Hfq subunits to sRNA-dependent regulation.

GLOBAL REGULATION BY CSRA IN *E. COLI*

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Global regulators allow rapid and coordinated responses to environmental changes and/or modifications inside the bacterial cell. Global regulators act at each level of gene expression control (transcription, translation and protein stability). In *E. coli*, the global regulator CsrA (carbon storage regulator A) regulates central carbon fluxes, mobility and biofilm formation and acts at the post-transcriptional level either positively or negatively by affecting the stability of various mRNAs. It has recently been shown in our lab that the *csrA* gene is essential for growth on glycolytic carbon sources due to an imbalance of carbon fluxes.

We have shown that a *csrA* deletion mutant is affected in growth and viability, especially during stationary phase, and also strongly affected in central carbon metabolism. Indeed, expression of the small RNA *sgrS* as well as the universal stress protein A UspA is induced in this mutant. This shows that the *csrA* deletion mutant suffers from the so-called 'hexose-phosphate stress' (accumulation of glucose-6-phosphate and/or fructose-6-phosphate). This is in accordance with reduced glycolysis and enhanced gluconeogenesis presented by the *csrA* deletion mutant.

Moreover, cAMP accumulates in the *csrA* deletion mutant (4-fold increase as compared to the wild-type strain). cAMP accumulation is independent of the hexose-phosphate stress, since cAMP also accumulates in a *sgrR csrA* double mutant. Molecular mechanism of cAMP overproduction is currently under investigation in our lab. It is known that CsrA negatively regulates the production of PGA (poly-β-1-6-N-acetylglucosamine), an exopolysaccharide involved in biofilm formation. Our preliminary data indicate that CsrA might regulate expression of another type of exopolysaccharide.

DEVELOPMENT OF AN ONTOLOGY FOR MICROBIAL PHENOTYPES (OMP)

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The rapid generation of microbial genome and metagenome sequences is producing a growing demand for functional analysis of microbes. Phenotypes are the observable manifestation of genotypic variation. Phenotype analysis has been used in many biological systems to elucidate the evolution of functional modules, to establish correlations between particular gene sets and strain characteristics, and to predict how genome engineering can produce new strains for basic research or biotechnology. However, the systematic analysis of phenotypes in bacteria and other microbes is hindered by the lack of a controlled terminology to describe and classify them. Our project aims to develop an annotation system to improve the ability of microbiologists and bioinformaticians to use both existing and new phenotype information and to capture it in a consistent and standardized manner. We have begun the development of an Ontology for Microbial Phenotypes (OMP), which is a controlled vocabulary to describe and classify phenotypes and provide connections between related phenotypes. Phenotypes will also be connected to a specific assay, e.g. "growth rate at 37°C in LB medium (Optical Density)" or "motility (swarming assay)," and supplemented by references to detailed descriptions of the assay.

MISMATCH REPAIR COLLAPSES FOCI OF THE REPLICASE DNAE IN *BACILLUS SUBTILIS*

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Mismatch repair (MMR) corrects DNA polymerase errors that occur during genome replication. One model to explain how mismatches are recognized in the Gram-positive bacterium *Bacillus subtilis* is that MMR proteins MutS and MutL associate with the DNA replication machinery to detect mismatches immediately following their incorporation. In support of this model, MutS and MutL fused to Green Fluorescent Protein (GFP) require ongoing DNA replication to localize as foci following the addition of 2-aminopurine (2-AP), a DNA base analog used to generate mismatches *in vivo*. Furthermore, MutS-GFP and MutL-GFP localize to midcell in a manner similar to the replication machinery in live cells. In an effort to understand how the MMR pathway is coupled to the DNA replication machinery, we monitored the subcellular localization of the MMR and replication proteins fused to GFP upon challenge of cells with 2-AP. We found that foci of the DNA polymerase DnaE-GFP collapsed when cells were in the presence of 2-AP. We show that loss of DnaE-GFP foci following 2-AP depends exclusively on the MMR pathway, and that loss of DnaE-GFP foci does not occur when cells are challenged with a damaging agent that alkylates DNA. In addition, we show a strong and specific interaction between DnaE and the MMR proteins MutS and MutL, and we find that cells challenged with 2-AP exhibit a MutS-dependent growth defect. We propose that MutS directly contacts the replisome to pause DNA synthesis, providing MMR proteins with access to the 3' or 5' termini of the mismatch-containing DNA strand to direct repair. These data also suggest that DnaE may be recruited by MutL to re-synthesize the DNA following removal of the mismatch.

GENOMIC REARRANGEMENTS AND MUTATIONS IDENTIFIED BY NEXT GENERATION SEQUENCING.

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The control of chromosomal DNA replication is tightly coupled to cell growth in *E. coli*. The key element is the DnaA protein that associates with ADP or ATP, the ATP form being more active in some reactions. The Hda protein converts the ATP form of DnaA to the ADP form in a process termed RIDA. RIDA depends further on active DNA replication sensed by active beta-subunits of the DNA Polymerase III holoenzyme (termed PCNA in eukaryotes).
We have earlier inactivated the *hda* gene and observed that only cells concurrently acquiring a suppressor mutation survived. Eight strains carrying a *hda* suppressing mutation (*hsm* mutants) were characterized further and one strain carried a point mutation in the *dnaA* gene that is likely to affect ATP hydrolysis[1)]. Thus these suppressor mutations may reveal aspects of the control of DNA replication in bacteria.
We have sequenced the genomes of each of these eight *hda* suppressing mutants and the *hda*(wt) mother strain using the Illumina GAII platform and we analyzed the sequence with our short read analysis program R2R (from Reads to Results).
We identified seven mutations common to most or all strains and we further identified one strain specific mutation in each of the eight *hsm* strains. Some of these mutations have been shown to cause the *hsm* phenotype and further genetic characterization is in progress. The types of mutations detected include simple point mutations, small indels, sequential deletions and insertions, transpositions and major chromosomal rearrangements. Several of these mutations would have been very difficult to map using classical genetic techniques.
We conclude that Illumina sequencing is superior to classical genetic techniques for identification of genome wide mutations since it is faster, cheaper and much more sensitive.

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A SMALL-MOLECULE INHIBITOR OF THE *E. COLI* ARAC/XYLS FAMILY TRANSCRIPTIONAL ACTIVATOR RHAS.

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RhaS is a member of the very large AraC/XylS family of transcriptional activators. In the presence of L-rhamnose, RhaS activates expression of the *E. coli* L-rhamnose catabolic enzymes. Here we present the results of a high-throughput screen to identify small-molecule AraC/XylS inhibitors. We assayed 110,000 compounds for inhibition of a RhaS-activated *lacZ* reporter fusion relative to a non-inhibited control, and identified ~300 potential inhibitors. To identify specific RhaS inhibitors, the impacts of the potential inhibitors on the RhaS-activated *lacZ* fusion were compared with an isogenic control, LacI-repressed, *lacZ* fusion. We identified one compound that strongly inhibited expression of the RhaS-activated *lacZ* fusion, but had little effect on the LacI-repressed control fusion, with a concentration dependence expected for a specific inhibitor. We also determined that the compound inhibited activation by the RhaS DNA-binding domain (RhaS-DBD) and full-length RhaS to similar degrees. This suggests that the compound likely inhibits DNA binding or contacts between RhaS-DBD and RNA polymerase. Our lead compound appears to have a different mode of action from, and is not structurally related to, previously identified AraC/XylS inhibitors. We plan to use purified RhaS-DBD to further identify the mechanism of action of the inhibitor; however, an obstacle has been obtaining soluble, active protein. Previously, we have had some success purifying RhaS-DBD, but this required that we refold the protein from inclusion bodies or use detergent to maintain solubility. The refolded protein bound specifically to RhaS-binding site DNA but formed small aggregates, while the detergent complicated functional assays due to its interaction with DNA. Here we report the expression and purification of RhaS-DBD fused with the 56-amino acid solubility-enhancement tag GB1. GB1 has been found to dramatically enhance the solubility of many proteins without impacting their structure. RhaS-DBD-GB1 exhibited >10-fold increased solubility relative to RhaS-DBD without GB1. We have purified RhaS-DBD-GB1 to near homogeneity. Dynamic light scattering indicated that the protein was monodisperse. We have also obtained an ^{15}N-^{1}H-HSQC spectrum of RhaS-DBD-GB1 at 1.0 mM (22.5 mg/mL) with well-separated peaks, indicating that the protein was well-folded, stable, and monodisperse at high concentration. We plan to determine the structure of RhaS-DBD by NMR and further characterize the mechanism of action of our lead inhibitor using in vitro studies.

THE GENETIC AND FUNCTIONAL RELATIONSHIP BETWEEN DNA REPLICATION AND CENTRAL CARBON METABOLISM IN *ESCHERICHIA COLI*

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DNA replication is a tightly regulated process. Particularly, unicellular organisms (like bacteria) have to control DNA replication precisely in order to obtain optimal adaptation to environmental conditions. The regulation of initiation of DNA replication is relatively well-studied, although little is known about the control of replication elongation process in accordance to alterations of nutrient limitations and availability.
Central carbon metabolism (CCM), a group of reactions that convert nutrients into compounds for macromolecules' synthesis, is involved in the regulation of DNA replication elongation in *Bacillus subtilis*. The suppression of the replication defects by mutants in enzymes involved in glycolysis was recently reported by Janniere and colleagues. Our recent studies involving the Gram-negative model bacteria *Escherichia coli* indicate that this phenomenon is more general – the temperature-sensitive phenotype of certain mutations in genes coding for replication enzymes (DnaB, DnaC, DnaE) can be suppressed by mutations in genes encoding carbon metabolism enzymes. Interestingly, the mutator phenotype of mutations in dnaQ and dnaX genes could be also suppressed by mutations in CCM. The suppressor mutants involved not only glycolysis, but also other pathways of central carbon metabolism. The observed suppression strongly suggests an existence of the link of two major cellular processes - DNA replication, leading to cell reproduction, and fundamental metabolic pathways involving carbon metabolism. The presence of the system integrating replication and metabolism in genetically distant bacterial species, such as *E. coli* and *B. subtilis*, indicates that this network of genetic and functional interactions could be universal, and serves as a basis for maintaining genome stability and adaptation of cell propagation to environmental requirements.

INDEPENDENT AND ANTAGONISTIC ACTIONS OF PPGPP AND DKSA AT THE BACTERIOPHAGE LAMBDA PR PROMOTER

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Transcription control in bacteria is often achieved by employing global regulatory networks. Such systems are designated for precise and suitable adaptations of unicellular organisms to alterations in environment and the availability of nutrient sources. The effector of the stringent response, a specific nucleotide, guanosine tetraphosphate (ppGpp) is rapidly produced in response to variety of physico-chemical and nutritional stresses. ppGpp affects the activity of various bacterial promoters by binding to the RNA polymerase and modulating its activity. Guanosine tetraphophate also inhibits bacteriophage lambda plasmid replication during the stringent response due to direct down-regulation of lambda pR promoter activity. Recent reports show that the DksA protein plays an important role in regulation by ppGpp. It was proposed that DksA works as a co-factor for ppGpp, affecting RNA polymerase transcription capacity. This results in synergistical amplification of the positive or negative effect of ppGpp depending on specificity of the given promoter. In order to investigate the role of DksA in pR transcription regulation, we employed the set of strains carrying ppGpp/DksA deficiency for the i*n vivo* analysis. Our results demonstrated that pR promoter activity decreases dramatically in DksA-deficient strain and in the triple mutant devoid of ppGpp and DksA (*dksA relA spoT*). This line of evidence suggesting DksA stimulatory role in pR transcription was also confirmed by *in vitro* experiments, showing that DksA significantly activates pR-initiated transcription even in the presence of ppGpp. The main mechanism of DksA stimulation of pR transcription involves enhancing of RNA polymerase binding to the promoter region, which results in more productive transcription initiation. Our study provides thus the evidence supporting the hypothesis that DksA can play not only synergistic role to stringent response alarmone in transcription regulation but also can act independently and moreover, antagonistically, to ppGpp, and that DksA-mediated regulation could be predominant over ppGpp effects.

ESCHERICHIA COLI DNAG PRIMASE IS DIRECTLY INHIBITED BY STRINGENT RESPONSE ALARMONES, (P)PPGPP

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DNA primase is an enzyme required for replication of both chromosomes and vast majority of plasmids. Guanosine tetra- and penta-phosphate (ppGpp and pppGpp, respectively) are alarmones of the bacterial stringent response to starvation and stress conditions, and act by modulation of the RNA polymerase activity. Recent studies indicated that the primase-catalyzed reaction is also inhibited by (p)ppGpp in *Bacillus subtilis*, where a specific regulation of DNA replication elongation, the replication fork arrest, was discovered. Although in *Escherichia coli* such a replication regulation was not reported to date, we found that *E. coli* DnaG primase is directly inhibited by ppGpp and pppGpp. This was demonstrated in *in vitro* experiments with purified compounds. Experimental systems with and without DnaB helicase indicated ppGpp-mediated inhibition of DnaG activity. Contrary to the *B. subtilis* primase response to the stringent control alarmons, the *E. coli* DnaG was inhibited more efficiently by ppGpp than by pppGpp. A lack of reports demonstrating effects of ppGpp-mediated inhibition of *E. coli* DnaG activity *in vivo*, which might be expected in the light of the results presented in this paper, in intriguing. One possible explanation is that since ppGpp appears to inhibit the initiation of DNA replication relatively strongly, any effects of this nucleotide on the elongation phase of DNA replication could be either masked by predominant effects at the initiation stage or overlooked by investigators.

A SITE-SPECIFIC INSULATION SYSTEM LIMITS A MATP-DEPENDENT CONSTRAINING PROCESS TO THE TER REGION OF THE E. COLI CHROMOSOME

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In E. coli, cytological and genetic analyses based on long distance DNA interactions have revealed a structuring process of the chromosome that differentiates large structured regions called Macrodomains (MDs) from less-structured regions called Non-Structured (NS) regions. The four different MDs (Ori, Ter, Left and Right) are spatially isolated from each other whereas NS regions can interact with both flanking MDs. The Ter MD is flanked by the Left and Right MDs, whereas the Ori MD is flanked by the two NS (NSRight and NSLeft) regions. Analysing the positioning, the segregation pattern and the motility of fluorescent tags has shown that the dynamic behaviour of loci belonging to various MDs and NS regions is radically different; loci in MDs showed a much lower mobility than those in NS regions. The organization of the Ter macrodomain relies on the interaction of the MatP protein with a 13 bp motif called matS repeated 23 times in the 800-kb-long domain. MatP accumulates in the cell as a discrete focus that colocalizes with the Ter macrodomain. The effects of MatP inactivation reveal its role as the main organizer of the Ter macrodomain: in the absence of MatP, DNA is less compacted and the mobility of markers is increased.
To understand how NS regions and MDs are maintained in the E. coli chromosome, we have developed a new method to transpose large chromosomal segments at defined positions. By swapping the Right MD and the NSRight region, we have observed that the properties of the NSRight region were similar to those of a MD when the NSRight region is adjacent to the Ter MD. This effect is dependent on MatP as these new properties are reversed in a matP mutant. The same MatP-dependent effect is detected on the left arm of the chromosome when the NSLeft region is adjacent to the Ter MD. These results suggested that determinants are present in the Right and Left MDs to prevent MatP from affecting the properties of the NS regions in the wt chromosome configuration. By using chromosomal rearrangements in which various segments of the Right MD separate the NSRight from the Ter MD, the insulation determinants of the Right MD were mapped in a 2 kb region. Deletion and insertion analyses allowed the identification of a 12 bp palindromic sequence necessary and sufficient to insulate the NSRight region from a MatP effect; this sequence has been called ridS for right insulator determinant Sequence. The MatP-dependent structuring process of NS regions and the insulation mechanism involving ridS will be discussed.

INTRA- AND INTER-SPECIES QUORUM SENSING IN *ERWINIA CAROTOVORA*

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Quorum sensing is a process in which bacteria sense their cell-population density by measuring the concentration of small secreted signal molecules, called autoinducers, and respond with changes in gene expression. Thus, cell-cell communication allows a group of organisms to coordinate its behavior and synchronize activities that are productive only at a high population density, such as antibiotic production, biofilm formation, virulence and other developmental programs.
Erwinia carotovora spp *carotovora* (also known as *Pectobacterium carotovora*) is a Gram-negative bacterium and a plant pathogen found in the soil. This bacterium causes soft-rot diseases in various commercially important plants, including potatoes, celeries and Chinese cabbage. The plant cell wall-degrading enzymes (PCWDE) secreted by the bacteria are the major factors contributing to the overall virulence and are composed mainly by pectinases. Previous studies identified three major pathways involved in the regulation of PCWDE production, specifically the acyl-homoserine lactone (AHL) intra-species system, the autoinducer-2 (AI-2) inter-species system and the GacS/GacA system, whose signal is still unknown.
Towards the understanding of how these quorum sensing systems regulate virulence in *E. carotovora* we want to determine the factors involved in the cross-talk between the different systems and the molecular mechanism involved in integrating these three signaling pathways.
We have shown that in this bacterium the intra-species signals, AHLs, induce the production of the inter-species signal, AI-2. Next we will determine if this regulation occurs at transcription or post-transcription level. Because the receptor for AI-2 recognition in this bacterium has not been identified we are combining genetic as well as chemical approaches to identify it.
Additionally, the signal controlling the GacS/GacA pathway is still unknown and we are performing a genetic screen to identify the genes involved in signal production as well as in regulation. We have constructed a library of 15,000 transposan mutants in *E. carotovora* and we are screening this library for mutants impaired in PCWDE production using the pectin lyase assay. Several candidates were selected and those will be subject to a second screen for complementation with cell-free supernatants from wild type. We expect selected mutants to have a transposan insertion in genes responsible for signal production or regulation. Finally we want to understand the role of such signals in vivo by doing mixed infections of potatoes with bacteria that produce and sense those signals.

LOSS OF THE RNA CHAPERONE PROTEIN HFQ ACTIVATES THE CPX ENVELOPE STRESS RESPONSE IN ENTEROPATHOGENIC, BUT NOT NON-PATHOGENIC, *ESCHERICHIA COLI*

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The RNA-binding protein Hfq is conserved among many species of bacteria, and is best known for its role in facilitating the annealing of small, non-coding regulatory RNAs (sRNAs) to their target mRNAs. Many of the mRNAs that are known to be regulated by sRNAs encode outer membrane proteins (OMPs). In mutants lacking Hfq, these sRNAs are no longer able to regulate expression of their target OMPs. For this reason, mutation of *hfq* in several Gram-negative species leads to increased accumulation of OMPs and therefore activation of the σ^E envelope stress response. In this study, we investigated whether mutating *hfq* in *Escherichia coli* would activate an additional, distinct envelope stress response - the CpxAR two-component system. While the σ^E response is thought to mainly detect misfolding of OMPs, many of the known activating cues of the Cpx response involve the aggregation of periplasmic proteins such as pilins. Using *cpxP* expression as a reporter for activation of the Cpx pathway, we found that mutating *hfq* strongly activated the Cpx response in enteropathogenic *E. coli* (EPEC), but not in *E. coli* K-12 strains. The activation of the Cpx response in the EPEC *hfq* mutant could be partially attributed to increased expression of the bundle-forming pilus. In contrast, we found that the σ^E envelope stress response was activated in both pathogenic and non-pathogenic strains of *E. coli*. These results suggest that regulating OMP expression, and therefore preventing induction of the σ^E envelope stress response, is a conserved function of Hfq in several strains of *E. coli*. On the other hand, EPEC appears to have co-opted the ancestral regulator Hfq to control expression of its horizontally acquired virulence traits, possibly in order to prevent envelope stress that would activate the Cpx response.

REDUNDANT REGULATION BY TRANSCRIPTION FACTORS IN *ESCHERICHIA COLI*.

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We have mapped all binding sites for the *Escherichia coli* transcription factor, GalR. In addition to the 5 known targets, we identified 7 putative GalR-regulated genes. These include several genes with no obvious connection to the well-described function of GalR in regulating galactose metabolism. Furthermore, none of the novel target genes show a detectable change in mRNA level following addition of galactose (inactivates GalR). We present evidence that these novel target genes are regulated redundantly by GalR and additional transcription factors. This suggests that bacterial transcription factors often function redundantly. Hence, traditional phenotypic analyses fail to identify many regulatory targets. Furthermore, our data suggest that even well-studied transcription factors may have unexpected regulatory roles.

BYPASS OF THE ESSENTIAL PCSB CELL DIVISION PROTEIN BY A CARBOXYL-TERMINAL FRAME-SHIFT MUTATION IN DIVIVA REQUIRES THE CARBOXYPEPTIDASES DACA AND NEWLY IDENTIFIED DACB IN SEROTYPE 2 D39 *STREPTOCOCCUS PNEUMONIAE*.

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The interplay between cell division and cell wall biosynthesis in *S. pneumoniae* (pneumococcus) is poorly understood. PcsB is an essential cell division protein that contains a CHAP domain found in peptidoglycan (PG) amidases. Expression of PcsB is positively regulated by the $WalRK_{Spn}$ two-component system (TCS), and PcsB has been implicated as a protective vaccine candidate against pneumococcal infections. We report here the isolation and characterization of a pcsB bypass suppressor which contains a frame-shift mutation in the well characterized cell division protein, DivIVA. The *divIVA* carboxyl frame-shift (*divIVA-C43*) is most likely a gain-of-function mutation, because its presence alone did not lead to defects in cell growth, morphology, or global transcription patterns. In addition, *divIVA-C43* bypass suppression is allele specific, since deletion of divIVA or translation termination at the site of the frame-shift mutation were insufficient to bypass the function of PcsB. Repeated attempts to complement $divIVA_{Spn}$ mutants were not successful and have not been reported in the literature, implying that DivIVA may require co-translation of one or more upstream *ylm* gene(s) for proper function.

To understand the basis for this bypass suppression of pcsB function, we constructed and characterized knockout mutations in all genes predicted to encode PG hydrolases in *S. pneumoniae*. Only three genes or operons of this set were required for *divIVA-C43* bypass of Δ*pcsB* mutations: *dacA* (D,D-carboxypeptidase), *dacB* (D,L-carboxypeptidase), and the *spd_1874* operon (encoding a putative N-acetyl-muramidase) which is also positively regulated by the $WalRK_{Spn}$ TCS. We confirmed biochemically that *dacB* encodes a new D,L-carboxypeptidase involved in PG maturation. Notably, *dacB* mutants, similar to *dacA* mutants, showed severe defects in cell shape and septation, consistent with the idea that the availability of precursors is important for proper PG biosynthesis. Epistasis analysis indicated that DacB hydrolyzes the peptide bond to the penultimate D-Ala in stem peptides sequentially after removal of the ultimate D-Ala by DacA. Together, these results suggest that the carboxyl-terminal extension of the DivIVA-C43 protein changes cell division to bypass the requirement for PcsB function, and this bypass is dependent on the activities of carboxypeptidases and possibly a lysozyme that adjust the precursor pools required for PG biosynthesis.

A MUTATION IN THE INHIBITORY PERIPLASMIC PROTEIN CPXP FACILITATES THE DEGRADATION OF THE BUNDLE-FORMING PILUS BY THE CHAPERONE-PROTEASE DEGP IN ENTEROPATHOGENIC *ESCHERICHIA COLI*

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Bacteria need to adapt to changing environmental conditions in order to survive. The Cpx two-component system senses stress to the bacterial envelope through the sensor kinase CpxA and transduces the signal to the cytoplasm through a conserved phosphotransfer to the response regulator, CpxR, which upregulates a number of protein-folding and degrading factors to restore envelope homeostasis[4]. One of these factors, CpxP, inhibits CpxA activation in the absence of stress[2,5] and has been suggested as an adaptor for DegP, a dual chaperone-protease, to degrade misfolded P-pilin subunits from uropathogenic *Escherichia coli* expressed heterologously in *E. coli* K-12[3]. We have also previously identified mutations that cause single amino acid changes in CpxP and negate its ability to inhibit the Cpx response in *E. coli* K-12[1]. Here, we show that CpxP over-expression inhibits the Cpx response in enteropathogenic *Escherichia coli* (EPEC), as in *E. coli* K-12, and that the previously isolated CpxP mutants also have a loss-of-function phenotype in EPEC with respect to Cpx pathway inhibition. Recently, our lab has shown that CpxP is required for normal elaboration of the bundle-forming pilus (BFP) in EPEC[6]. The BFP mediates autoaggregation of EPEC and loose attachment to the surface of host cells[7]. We found that expression of one CpxP mutant, CpxPR60Q, can facilitate the degradation of the BFP in a DegP-dependent manner in EPEC. In agreement with this observation, an EPEC strain expressing CpxPR60Q is defective in BFP-mediated autoaggregation and localized adherence to Hep-2 cells, confirming that cells expressing CpxPR60Q cannot elaborate a functional BFP. Interestingly, CpxPR60Q forms high molecular weight complexes with periplasmic proteins that are resistant to SDS and boiling. These results describe the first gain-of-function mutation in CpxP with respect to its ability to facilitate DegP proteolysis of pilin subunits and suggest that further study of CpxPR60Q may elucidate the mechanism by which CpxP performs this function.

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IT'S A FEAST OR A FAMINE – CAN *ESCHERICHIA COLI* O157:H7 SURVIVE THE RIGORS OF STARVATION AND GO ON TO LIVE ANOTHER DAY?

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Escherichia coli O157:H7 is a food-borne pathogen which causes serious, often life-threatening illnesses. Infections by *E. coli* O157:H7 have been linked to the consumption of undercooked ground beef, contaminated meat products and salad vegetables. Ruminants such as cattle are asymptomatic carriers of *E. coli* O157:H7 and act as important reservoirs. *E. coli* O157:H7 can also survive outside the ruminant in both soil and water, increasing the range of potential sources for transmission to humans. *E. coli* O157:H7 is the most prevalent disease-causing serotype of Shiga-toxin producing bacteria isolated in New Zealand. However, unlike most other countries, human infection by *E. coli* O157:H7 in New Zealand are sporadic, commonly associated with environmental contact rather than through contaminated food sources. While infections linked to food sources can be prevented by improved hygiene practices, controlling *E. coli* O157:H7 in the environment is far more complex. This is in part due to the lack of knowledge of the mechanisms used by *E. coli* O157:H7 to survive in the environment and how they respond to the transition to favourable growth conditions. In this study, we have used a combination of shifts in temperature and starvation to trigger bacterial survival responses, followed by recovery on solid-media with different nutrient availability to emulate the cycling of *E. coli* O157:H7 between its natural host and the environment.

In this research project, survivability has been measured as the maintenance of individual cell integrity, the metabolic activity of the population upon nutrient up-shift and the ability of individual cells to form colonies. Data from prolonged starvation experiments of up to 98 days, carried out at a range of environmentally significant temperatures will be presented.

NANORNAS PRIME TRANSCRIPTION INITIATION IN VIVO

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Initiation of DNA synthesis by DNA polymerase requires use of a primer whereas it is presumed that, in vivo, the initiation of RNA synthesis by DNA-dependent RNA polymerases occurs de novo. Here, using the model Gram-negative bacterium Pseudomonas aeruginosa, we demonstrate that depletion of the small RNA-specific exonuclease, oligoribonuclease, causes the accumulation of ~2-4 nt RNA transcripts, “nanoRNAs”, which serve as primers for transcription initiation at a significant fraction of promoters. Widespread use of nanoRNAs to prime transcription is coupled with global alterations in gene expression. We propose that nanoRNAs represent a distinct class of functional small RNAs that can affect gene expression through direct incorporation into a target RNA transcript rather than through a traditional antisense-based mechanism.

A BACKTRACK-INDUCING SEQUENCE IS AN ESSENTIAL COMPONENT OF Σ^{70}-DEPENDENT PROMOTER-PROXIMAL PAUSING

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RNA polymerase (RNAP) of both bacteria and eukaryotes can stall or pause within tens of base pairs of its initiation site at the promoter, a state that may reflect important regulatory events in early transcription. In the bacterial model system, the σ^{70} initiation factor stabilizes such pauses by binding a downstream repeat of the promoter segment, especially the promoter -10 sequence. σ^{70} region 2 retains contacts with this -10-like element while the active site of RNAP translocates downstream and elongates the RNA, a process akin to DNA 'scrunching' for initial transcribing complexes. This downstream, scrunched elongation complex is believed to be the substrate required for efficient RNAP modification by the lambdoid phage late antiterminator Q protein. In addition to σ^{70} region 2-dependent promoter-proximal pauses, we have identified a σ^{70} region 4-dependent pause in the late gene promoter of the lambdoid phage 82. In this pause variant, σ^{70} region 4 interacts with a -35-like element displaced 9nt downstream of the promoter -35 element and scrunches 5-6nts.

We show that an essential element of either type of pause is a sequence that stabilizes RNAP backtracking. Although the scrunched pause is not intrinsically backtracked, we suggest that the same sequence element is required both to stabilize the paused state and to potentiate backtracking.

SIGMA FACTOR SPECIFIC TRANSCRIPTIONAL CONTROL BY A SEQUENCE IN THE PROMOTER SPACER REGION

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Transcription of *cbpA* in *Escherichia coli* is driven by overlapping promoters specific for RNA polymerase associated with either sigma70 or sigma38. The sigma38 dependent promoter is the most active and is responsible for the bulk of *cbpA* transcription. We randomly mutated the *cbpA* regulatory region and selected derivatives with increased transcriptional activity. One of the mutations that we identified falls in the overlapping spacer regions of the sigma70 and sigma38 dependent promoters. Further genetic and biochemical analysis of this mutant showed that it specifically stimulated transcription by sigma70 whilst not affecting sigma38, resulting in a “flip” in the overall sigma factor preference for *cbpA* transcription. We introduced the same mutation into other promoters shared by sigma70 and sigma38 and saw similar effects.

We conclude that, at overlapping promoters, or single promoters shared by more than one sigma factor, the sequence of the promoter spacer region can play an important role by influencing sigma factor preference.

SIGMA-MEDIATED PAUSING AT PROMOTER PROXIMAL POSITIONS ENHANCES THE SIGMA CONTENT OF DOWNSTREAM TRANSCRIPTION ELONGATION COMPLEXES

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Promoter recognition depends on the sigma subunit of RNA polymerase (RNAP), which forms an extended interface with the catalytically proficient core enzyme. During the transition from initiation to elongation, the stability of sigma's interaction with core decreases, but the complete release of sigma is not obligatory and sigma factors can be detected as components of transcription elongation complexes. Work from Jeff Roberts' lab first established a functional role for σ^{70} (the primary sigma factor in *E. coli*) during early elongation, namely to engage a promoter-like pause element associated with the bacteriophage lambda late promoter. The recognition of this early elongation pause element is required for the expression of the phage late genes under the control of the Q antiterminator protein, which gains access specifically to the paused elongation complex. Here we show that this engagement of promoter-proximal pause elements by the σ^{70}-containing RNAP enhances the σ^{70}-content of downstream transcription elongation complexes. Furthermore, we demonstrate that the presence of a promoter proximal pause element enables the recognition of downstream σ^{70}-dependent pause elements, presumably because of the enhanced probability that σ^{70} will be retained in elongation complexes that have paused at promoter proximal positions. Our findings suggest that sigma-dependent promoter-proximal pause elements may function broadly to regulate the behavior of the transcription elongation complex.

ROLE OF THE ELEMENTS OF RNA POLYMERASE SECONDARY CHANNEL IN THE FUNCTION OF *E.COLI* TRANSCRIPT CLEAVAGE FACTORS GREA AND GREB

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Bacterial transcription elongation factors GreA and GreB stimulate the intrinsic nucleolytic activity of RNA polymerase (RNAP) thus helping the enzyme to read through pausing and arresting sites on DNA, accelerating RNAP transition from initiation to elongation, and enhancing transcription fidelity. Previous studies showed that Gre factors act by binding to the rim of RNAP secondary channel, protruding their extended coiled-coil domain into the channel, and placing two conserved acidic residues near the enzyme catalytic center where they coordinate one of the two essential Mg^{2+}-ions required for nucleolytic reaction. Despite extensive studies of Gre structure-function in the past, the details of their interactions with RNAP and their molecular mechanism of action and regulation remain poorly understood. Here, we characterize the interface between RNAP and GreA/GreB and describe the roles of different elements of RNAP secondary channel in the function of Gre factors. We used a combination of side-directed mutagenesis of Gre and RNAP β' subunits, suppressor mutation analysis of β' resistant to dominant lethal GreA mutant, and site-specific proximity probing of Gre-RNAP complexes by Fe^{2+}-BABE and protein-protein crosslinking. Incorporating our new genetic and biochemical data, and available structural data, we constructed a refined 3D-model of Gre-RNAP complex that includes the 188-residue insertion into a conserved G-loop (trigger loop) element of β' of *E. coli* (β'GNCD) in proper spatial orientation within RNAP. The model shows how the elements of the secondary channel participate in the binding of Gre C-terminal domain (CTD) and in adjusting position of the N-terminal domain (NTD) towards RNAP catalytic center. Gre anchors to RNAP through β' coiled-coil element (the "fulcrum") that forms the rim of the secondary channel. The binding occurs mostly through specific "steric" recognition by β' G671 assisted by hydrophobic interactions from A657, I664 and F668. G1055 and L1056 of the "shaft", the mobile β-sheet loop of β'GNCD, abut Gre-CTD from the side, and β' residues G732D/S733Y and G1245D of the two "pincer"-like loops emerging from the secondary channel wall clasp the distal portion of Gre. A small push by the shaft causes Gre-NTD, which acts as a lever, to swing. The swinging motion is restricted by residues of the "pincers" to insure proper positioning of Gre-NTD tip with respect to the catalytic center and RNA 3' terminus. This mechanistic view is consistent with most of the observed functional activities of Gre factors.

TRANSLATION-UNCOUPLED TRANSCRIPTION CAN MEDIATE THE BYPASS OF RNASE E ESSENTIALITY IN *ESCHERICHIA COLI*

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RNase E (encoded by *rne*) in *E. coli* is an essential endonuclease that has been implicated in processing and maturation of tRNAs, rRNAs and tmRNA, as well as in global degradation of mRNA and non-coding small RNA; however, the C-terminal half (CTH) of the protein (which has been implicated as scaffold for assembly of the "degradosome") is dispensable for cell viability. RNase E catalytic activity is modulated by the status of the 5'-end of its RNA substrate, with maximal efficiency on 5'-monophosphorylated (5'-monoP) RNA. RppH is an RNA 5'-pyrophosphohydrolase (that generates 5'-monoP RNA) which has been reported to participate in global mRNA degradation mediated by RNase E, but Δ*rppH* mutants are viable.

In this work, we have found that whereas the single mutants Δ*rppH* and *rne*-ΔCTH are viable, the combination is synthetically lethal. Δ*rppH* was not synthetic lethal with Δ*rhlB* Δ*pnp* (that encode other major components of the degradosome). The inviability associated with Δ*rppH rne*-ΔCTH was rescued by mutations in *rho* or *nusG* that reduce the efficiency of termination of translation-uncoupled transcripts. Lethality suppression of Δ*rppH rne*-ΔCTH by *rho* or *nusG* was independent of RNase G, but it required either RNase H1 or the ectopic expression of phage T4-encoded UvsW, two enzymes with the ability to remove RNA-DNA hybrids (R-loops) by hydrolysis and helicase action, respectively. Likewise, the *rne* allele encoding an RNase E variant with a combined deletion of its CTH and an R169Q substitution in its "5'-sensor-domain" was also inviable, suppressible by *rho* or *nusG*. Finally, the *rho* and *nusG* mutations could also restore viability in strains with complete deficiency of both RNase E (that is, Δ*rne*) and RppH.

Our results provide support to the following models, namely (i) that the essential function of RNase E is in catalyzing mRNA turnover; (ii) that the enzyme has two pathways for endonucleolytic cleavage, one of which is rendered defective by C-terminal truncation and the other by either the R169Q alteration in its 5'-sensor-domain or by RppH deficiency; and (iii) that the *rho* and *nusG* mutations restore viability in RNase E- and RppH deficient bacteria by providing a bypass route for mRNA turnover, through the formation of R-loops from nascent untranslated transcripts that fail to be terminated in these strains.

AN INHIBITION-ANTITERMINATION HYBRID MECHANISM FOR OVERCOMING THE RHO-DEPENDENT TRANSCRIPTION TERMINATION BY AN ANTITERMINATOR.

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Lamdoid phages have evolved an antitermination system to overcome the Rho-dependent transcription termination in bacteria. N protein from these phages modifies the host-transcription machinery to overcome both the Rho-dependent and the -independent termination. N binds to a specific site called *nut* site on the mRNA, which also overlaps with the Rho-loading site (the *rut* site of the *tR1*terminator), on the same RNA, using its N-terminal ARM motif and interacts with the elongating RNA polymerase (RNAP) through its C-terminal domain. We hypothesized that the mechanism of overcoming the Rho-dependent termination by N should at least involve two steps; inhibition of Rho by blocking its entry at the *nut*-site and an antitermination mechanism by modifying the RNAP most-likely at the RNA exit channel which could be the access point for Rho. Here we tested these hypotheses by using the N protein from a lambdoid phage H-19B. We used two types of lacZ reporter cassettes fused with either a single terminator, H-19B *nutR/tR1* (having both N and Rho-binding sites; H-19B *nutR/tR1-lacZYA*), or with two terminators, H-19B *nutR/tR1* and *trpt'* (H-19B *nutR/tR1-trpt'-lacZYA*). Expressions of β-galactosidase enzyme from the single terminator construct will report the N-Rho competition for the same site, whereas due to the presence of an extra Rho-dependent terminator, *trpt'* (a Rho-entry site that does not bind to N), in the double terminator construct, will report the existence of an antitermination mechanism. On the *nutR/tR1*-lacZYA construct, mutations in the ARM motifs of N were defective for overcoming Rho-dependent termination, whereas deletions in the C-terminal RNAP binding did not show significant defect. Similarly, RNAP β'-mutants defective for N-function also did not have significant defect on this construct. In vitro transcription assays on this construct also followed the same trend. The rate of RNA-dependent ATPase activity on the RNA with a *nutR/tR1* site by Rho was reduced in the presence of N. These results strongly indicate that N inhibits the Rho-entry at the *nut site*. Interestingly, to overcome the Rho function on the double terminator construct, functional N-CTD–RNAP interactions were found to be essential which also suggests the existence of a true antitermination mechanism. Ability of an N-modified stalled elongation complex (EC) to overcome the Rho-mediated RNA release in vitro, also indicated that this antitermination step is a result of a specific modification of RNAP and not by uncoupling the "kinetic coupling" between Rho and the EC, which may arise from the increase in the elongation rate of the latter by N. We propose that the antiterminator N uses an inhibition-antitermination hybrid mechanism to overcome the Rho-dependent termination.

RHO FACTOR RESOLVES R-LOOPS IN VIVO.

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R-loops are RNA-DNA hybrids formed when transcribed ssRNA hybridizes with DNA, forming a stable structure. In *E. coli*, R-loops prime DNA replication, stimulate SOS, and blockade transcription. Most R-loops are degraded by RNaseHI, the product of the non-essential rnhA gene. Transcription termination factor Rho is an RNA-DNA helicase which unwinds RNA-DNA hybrids in vitro. In this work we present evidence that Rho factor resolves R-loops in vivo, including R-loops that are not resolved by RNaseHI.

We developed a physical assay for detecting chromosomal R-loops. Intact chromosomes were treated with a combination of mung bean ssDNA nuclease and RNaseH, which cleaves DNA at the sites of R-loops. The cleaved DNA is detected using pulsed field gel electrophoresis. We found that inhibiting Rho factor with the antibiotic bicyclomycin (BCM) increased R-loop formation.

R-loops generate new origins of replication (oriK) that can replace oriC. Deletion of rnhA suppresses a dnaAts mutation by allowing oriC independent replication. We found that the RNA-DNA helicase defective rho115 mutation also suppressed a dnaAts mutation. Interestingly, overexpression of RnhA did not reverse rho115 suppression. We suggest that Rho RNA-DNA helicase removes R-loops that cannot be degraded by RNaseHI. The role of Rho in resolving R-loops may be an important contributor to the fitness of cells.

FUNCTIONAL ANALYSIS OF DUAL TOXIN-ANTITOXIN INTERACTIONS

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The plasmid prophage of bacteriophage P1 is stabilized, in part, by a toxin-antitoxin system that acts to arrest plasmid-free segregants. Transcription of this toxin-antitoxin operon is negatively regulated by the protein products. The antitoxin, Phd, binds as a dimer to adjacent palindromic sequences in the operator. The toxin, Doc, appears to enhance repression primarily by bridging adjacent dimers of Phd. Here, we used site directed mutagenesis and physiological analyses to assess the functional roles of two structurally defined Phd-Doc interactions. Since the two interactions involve overlapping parts of Phd, but disjoint parts of Doc, we made a number of structurally guided mutations in Doc intended to specifically disrupt one or the other of the two interactions. The results indicate that the first interaction is both necessary and sufficient to neutralize the toxin. In vivo competition experiments indicate that it is also the higher affinity interaction. Interestingly, within this interface, the part of Phd that is most important for binding to Doc (helix 3) is distinct from the part of Phd (helix 4) that actually occludes residues in Doc that are important for toxicity. The second, smaller and lower affinity interaction is required to enhance repression of the addiction promoter, as expected by the bridging hypothesis. Although the second interaction is neither required nor sufficient for neutralization under the conditions tested, it does occlude at least one residue of Doc that is critical for toxicity, and so may contribute in some minor way to neutralization.

PROTEIN ACETYLATION IMPACTS SIGNAL TRANSDUCTION AND TRANSCRIPTION

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Three global proteomic studies have revealed the existence of many acetylated proteins associated with diverse functions in Escherichia coli and Salmonella enterica (Yu et al., 2008, Wang et al., 2010, Zhang et al., 2009). Of particular interest to us was the report of an acetylated lysine (K291) in the C-terminal domain of the alpha subunit (alpha-CTD) of RNA polymerase (RNAP). Since the alpha-CTD impacts transcription through its ability to interact with both DNA and transcription factors, we hypothesized that acetylation of the alpha-CTD could influence transcription. We tested this hypothesis on the cpxP promoter because its transcription is reported to depend only on RNAP and the two-component response regulator, CpxR. Here, we report that acetyl phosphate-dependent cpxP transcription can be inhibited by the action of K291 and a GCN5-like acetyltransferase (GNAT). We propose that this GNAT acetylates K291 using acetyl-CoA as its acetyl donor. We further propose that this acetylation event interferes with the ability of phospho-CpxR to activate transcription. Since K291 sits on a surface that is not involved in DNA binding, we speculate that its acetylation might influence the ability of the alpha-CTD to interact with CpxR and/or other transcription factors.

BOTH SIDES: DISULFIDE BOND FORMATION IN CRENARCHAEA.

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Many thermophiles make disulfide bonds in cytoplasmic proteins. This is thought to contribute to thermostability. Protein Disulfide Oxidase, PDO, a cytoplasmic thioredoxin-like protein is found in most thermophiles with cytoplasmic disulfide bonds and is thought to be the oxidant responsible for their formation. There is nothing in the literature about how PDO is reoxidized after it is reduced during disulfide bond formation in its substrate proteins.
We have used a bioinformatic approach to answer the question "Do all Bacteria make disulfide bonds using the same, well characterized pathway found in E. coli?" (Dutton et al 2008). The answer to the question is a resounding "No!"

Many anaerobic bacteria do not make disulfide bonds and most bacteria that do make them do not use the E. coli pathway. The E. coli pathway involves a periplasmic thioredoxin-like protein, DsbA, as the direct oxidant and a transmembrane protein DsbB to reoxidize DsbA. All bacteria have exported thioredoxins that could concievably serve as DsbA-like oxidants but only Proteobacterial relatives of E. coli have DsbB. Most Bacteria that make disulfide bonds have instead a homolog of the Metazoan transmembrane protein vitamin K epoxide reductase, VKORC1, which in Mammals is involved in blood clotting. We think bacterial vitamin K epoxide reductase, VKOR, reoxidizes exported thioredoxin-like proteins that have DsbA-like function in most aerobic bacteria.

Many sequenced thermophilic Crenarchaea, some of which have been shown to have cytoplasmic disulfide bonds, have two VKORs. Could it be that one of these is responsible for reoxidizing a thioredoxin-like protein that oxidizes exported proteins and the other oxidizes PDO and is responsible for the cytoplasmic disulfide bonds? The answer is a qualified "Yes!"

Dutton RJ, Boyd D, Berkmen M, Beckwith J. 2008. Bacterial species exhibit diversity in their mechanisms and capacity for protein disulfide bond formation. PNAS 105:11933.

PROBING THE FUNCTIONS OF O-LINKED PROTEIN GLYCOSYLATION IN NEISSERIA GONORRHOEAE

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Protein glycosylation based on both *N*- and *O*-linked modifications is now well established in bacterial pathogens and symbionts of man. The prevalence of bacterial protein glycosylation strongly suggests that these systems are advantageous and impact on fitness. In fact, mutants in the general glycosylation systems of *C. jejuni* and *B. fragilis* are defective in mucosal colonization although the fundamental basis for the observations remains unclear. In some cases, no overt phenotypes are seen in mutants while in others, defects in protein stability and trafficking have been documented.

We have shown that the human pathogen *Neisseria gonorrhoeae* has a broad spectrum *O*-linked glycosylation system that targets at least 12 different proteins. The majority of these glycoproteins are predicted to be localized to the periplasm, although the most abundant *N. gonorrhoeae* glycoprotein is PilE, the protein subunit of type IV pili. In the strain we have used, the glycoproteins are modified with the disaccharide Gal-DATDH (2,4-diamino-2,4,6-trideoxyhexose), but there is considerable intra- and interstrain glycan heterogeneity among Neisserial isolates.

We have made many efforts to identify phenotypes associated with different *pgl* backgrounds in gonococci with little success. With regard to pilus-associated phenotypes such as adherence to human cells, competence, and autoagglutination, we have seen no compelling phenotypes associated with different *pgl* backgrounds. Here, it is relevant to note that this system has been shaped by selection *in vivo* while we can only examine the system *in vitro*.

Recognizing that simply altering pgl status may be insufficient to see *in vitro* effects, we devised an approach to analyze synthetic interactions based on the observation that expression of certain pilins carrying polyhistidine extensions at their C-termini inhibited bacterial growth but only when co-expressed with wild-type pilin. Realizing that this system might provide a sensitive means to assessing the efficacy of pilin subunit interactions, we examined the effects of altering glycosylation status and disrupting pilus dynamics (assembly and retraction) and found that all of these affected the growth arrest phenotype. As both pilin-pilin and pilin-assembly/retraction component interactions takes place in the cytoplasmic membrane, protein glycosylation appears to play a role in intracellular processes occuring at the inner membrane. This hypothesis is consistent with the glycosylation status of other inner membrane-localized proteins that are unlikely to be trafficked to the cell surface.

GLOBAL APPROACHES TO STUDY THE IMPACT OF CCPA ON SUGAR METABOLISM IN *STREPTOCOCCUS PNEUMONIAE* D39

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Streptococcus pneumoniae is a strictly fermentative organism that relies on sugar metabolism for energy production. In the host, migration between different niches requires adaptation of *S. pneumoniae* to changing sugar availability. In Gram-positive bacteria glucose is generally the preferred sugar and represses the utilization of other sugars via the global regulator catabolite control protein A (CcpA). In *S. pneumoniae* previous research showed that inactivation of *ccpA* results in decreased virulence, but its role as a global regulator of gene expression has not been, however, established. To address the role of *ccpA* on the physiology of this pathogen we performed whole-transcriptome and metabolic analysis of *S. pneumoniae* D39 (WT) and its *ccpA* mutant at mid-exponential and transition to stationary phases of growth. Cells were grown in a defined medium (CDM) containing glucose or galactose, and this choice was based on their specific host niche occurrence; glucose is present in the blood (infection) and galactose in the nasopharynx (colonization). In this work we show that *ccpA* inactivation results in altered expression of more genes involved in central metabolism than previously anticipated. Unexpectedly, inactivation of *ccpA* affected the expression of more genes during growth on galactose than on glucose. This finding supports the current view that galactose plays a crucial role on pneumococcal physiology and virulence (Yesilkaya *et al*, 2009). Furthermore, the data show that sugar-specific CcpA-independent regulatory mechanisms are also in effect in *S. pneumoniae*. In the *ccpA* mutant, end-product analysis showed a slight shift from homolactic to mixed acid fermentation during growth on glucose. In galactose, mixed acid fermentation was observed for both strains, though at a less extent in the *ccpA* mutant. Despite this observation, in the *ccpA* mutant mRNA levels of genes involved in mixed-acid fermentation were higher than in the WT strain. This suggests regulation at the translational or post-translational levels. ^{31}P-NMR analysis of cell extracts obtained for strain D39 and its *ccpA* mutant indicates that glycolytic metabolite pools differ considerably from those in the model and closely related organism *Lactococcus lactis* (Neves *et al*, 2006). This finding supports different regulatory mechanisms at the level of pyruvate kinase and/or sugar transport in these organisms. The impact of our results on pneumococcal virulence will be discussed.

TIME-COURSE EXPERIMENTS ANALYZING THE CELLULAR RESPONSE TO PURINE AVAILABILITY IN THE PARTIALLY PURINE-STARVED LACTIC ACID BACTERIUM *LACTOCOCCUS LACTIS*

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Lactococcus lactis is an industrially important Gram-positive lactic acid bacterium, generally believed to be domesticated from a plant environment. By adaptation to growth in milk the bacterium has become auxotrophic for a range of amino acids. When fermenting milk or chemically defined medium, its growth rate can be increased by supplying exogenous purines, showing that it is naturally starved for purines. Purine biosynthesis genes are strongly regulated in response to the presence or absence of purines, and we have previously shown genetic evidence that PurR is the responsible regulator. PurR in *L. lactis* is an activator and contains a LysR type DNA binding domain and a phosphoribosyltransferase domain binding 5-phosphoribosyl-1-pyrophosphate (PRPP). *Bacillus subtilis* contains a close homolog of PurR, but functions as a repressor. PRPP is needed as a precursor for both biosynthesis and salvage of purines, and has been found to be a feed-forward inducer of PurR regulated genes in *B. subtilis*. The PRPP synthetase is inhibited by adenine nucleotides, resulting in lowering of PRPP synthesis. We have examined the PurR-DNA interaction and found that the PurR regulon comprises a wider range of genes, suggesting a more global role for the regulator in environmental adaptation. In time-course experiments during purine run-out we have simultaneously analyzed the transcriptomic responses and nucleotide pool changes. The analysis revealed a sharp increase in the PRPP pool upon run-out resulting in a new homeostasis at a higher level. We saw abrupt changes in the expression of genes both related and unrelated to purine-nucleotide metabolism, as well as clear responses in the intracellular nucleotide pools.

CONTROLLING D-AMINO ACIDS PRODUCTION AND BIOFILM DISASSEMBLY.

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Bacteria form structured communities known as biofilms, which have a finite life span. As the biofilm ages, nutrients become limiting and waste products accumulate, forcing the bacteria to return to a planktonic existence. We have shown that prior to biofilm disassembly, Bacillus subtilis produces a factor that prevents biofilm formation and triggers the destruction of existing biofilms (Kolodkin-Gal *et al*., Science 328, 629). The factor is a mixture of D-leucine, D-methionine, D-tyrosine and D-tryptophan. Waldor and co-workers (Lam *et al*., Science 325, 1552) have shown that D-amino acids are produced by a wide variety of bacteria and are incorporated into the cell wall, and we have found that they also prevent biofilm formation by the pathogens *Staphyloccus aureus* and *Pseudomonas aeruginosa*. In B. subtilis, incorporation of D-amino acids into the cell walls triggers the release of amyloid-like fibers in the matrix that hold cells in the biofilm together. We are now focusing on the question of how D-amino acid production is controlled. We have identified two putative racemase-like genes *racX* and *ylmE* that are needed for the production of D-tyrosine and D-leucine. Experiments in progress and to be discussed indicate that transcription of *racX* is induced at a late stage of biofilm formation and is directly or indirectly under the control of the regulatory circuit governing the expression of genes involved in matrix production.

TRANSCRIPTIONAL REGULATION OF RESD-ACTIVATED NITRITE REDUCTASE GENES BY NO-SENSITIVE NSRR IN BACILLUS SUBTILIS.

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Nitric oxide (NO) plays an important role in bacterial physiology as it does in mammalian physiology. Bacteria encounter NO through many different sources. In order to protect from the lethal effects of NO, bacteria have evolved a wide range of NO sensors. NsrR which is recognized as a NO-sensitive transcription regulator plays a vital role in controlling NO metabolism in many bacteria. In *Bacillus subtilis*, NsrR represses genes involved in nitrate respiration (*nasDEF*: nitrite reductase genes) and NO detoxification (*hmp*: flavohemoglobin) that are under a positive control of the ResD-ResE two-component signal transduction system. The NsrR-dependent repression is relieved through NO which is generated during nitrate respiration or by exogenous NO source. How NO modifies the NsrR activity is the subject of our research. Here we show that NO targets the [4Fe-4S] cluster of NsrR, thereby decreasing the binding affinity of NsrR to the *nasD* promoter. We also show evidence that NsrR represses *nasD* transcription by blocking the formation of a ternary complex formed of ResD, RNA polymerase (RNAP) and the *nasD* promoter DNA, by competing with RNAP for the binding site. NO restores ternary complex formation through inactivation of NsrR by reacting with the [4Fe-4S] cluster. How NsrR inhibits RNAP binding to the promoter region of *nasD* is currently under investigation. By identifying and examining the *cis*-regulatory regions of NsrR in the *nasD* promoter, we will be able to illustrate how NsrR abrogates RNAP binding to the promoter DNA. These results will lead to a better understanding of how the interaction of NsrR and DNA affects ResD-dependent activation of *nasD*.

REGULATION OF *VIBRIO CHOLERAE* VIRULENCE GENE EXPRESSION AND PATHOGENESIS IN RESPONSE TO MICROAEROPHILIC GROWTH CONDITIONS

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Vibrio cholerae, a Gram-negative bacterium causes the human diarrheal disease cholera. As a model organism, it is a genetically tractable system for understanding bacterial pathogenesis, as evidenced by the successful identification of some of its virulence factors. Yet, much remains unknown with respect to the organism's mechanisms to sense and respond to virulence activating stimuli within the host microenvironment. Anaerobic growth has been shown to increase virulence gene expression in Gram-negative enteric and non-enteric bacteria. *V. cholerae* is subjected to an oxygen-gradient during colonization of the host intestine leading to disease, suggesting a link between hypoxia and virulence gene expression. A non-redundant and arrayed transposon library was screened to identify two-component system (TCS) mutants showing significant reduction in cholera toxin (CT) production under microaerobic conditions compared to the wild-type parent. Four unique TCS that potentially sense and respond to oxygen, osmolarity or host metabolites were identified. In-frame unmarked deletion strains lacking the identified TCS sensor proteins were constructed and showed reduction in CT production only under microaerobic conditions and were significantly attenuated in an infant mouse model of Vibrio colonization in competition with the wild-type parent (P less than 0.05). Furthermore, these TCS were found to regulate CT production in response to oxygen levels in two different *V. cholerae* biotypes suggesting an important link between the host-imposed oxygen-gradient and disease outcome.

HEXAVALENT CHROMIUM INDUCED MUTAGENESIS AND OXIDATIVE STRESS REGULATION IN THE MULTICELLULAR CYANOBACTERIUM *NOSTOC CALCICOLA*

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Chromium(VI) and its compounds are known mutagen and carcinogen. Cr(VI) and its intracellularly reduced form Cr(III) together with reactive oxygen species (ROS) induce oxidative DNA damage, DNA strand breaks, DNA-DNA and DNA-protein cross-links and mutations. Here we present, *Nostoc calcicola*, a filamentous multicellular cyanobacterium which display multiple phenotypic characteristics, adapt to different environmental stimuli and amenable to genetic manipulation as a model organism for Cr(VI) induced mutagenesis. *N. calcicola* exposed to Cr(VI) was investigated on ultrastructural changes (transmission electron microscope, TEM), protein, DNA profiles and Cr speciation. The analysis of fine structures obtained from TEM studies of Cr(VI) exposed cells exhibited discernible intracellular granular macromolecular structures throughout the cell which were compartmentalized to the intrathylakoidal spaces, and changes to the membranous systems and the cell wall architecture. The spherical structures of unknown functions, intracellular protein aggregates and localized necrosis were observed. The overexpression of cytosolic superoxide dismutase (Fe-SOD) and malate dehydrogenase (MDH) which are key indicators of cellular oxidative stress have shown a concentration dependent increase and sharp decline in the enzyme activity. Cr(VI) induced stress proteins were identified by MALDI-TOF MS/MS as Methyl-Accepting Chemotaxis Protein (MCP) and Malate dehydrogenase (MDH). The cellular responses of *N. calcicola* to Cr(VI) was found to be distinct when tested with the cells exposed to Cadmium(II) and Zinc(II). Cr(VI) was converted into Cr(III) and bound to the proteins as electron dense intracellular protein-Cr aggregates. The genomic DNA isolated from the cells treated with Cr(VI) indicated protein bound DNA and Cr(VI) induced DNA strand breaks. The investigation has led to the identification of the formation of intracellular granular macromolecular structures as the primary cellular defense mechanisms against Cr(VI) and Cr(III). Supported by the Council of Scientific and Industrial Research (CSIR), New Delhi.

ROLE OF THE ESSENTIAL GTPASE RBGA IN RIBOSOME ASSEMBLY

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Ribosomes are complex macromolecular machines comprised of three RNA molecules and over fifty proteins. While the structural and biochemical analyses of ribosome function have yielded important insights into the mechanism of translation, how ribosomes are formed in the cell is not well understood. Recent evidence in bacteria and eukaryotes strongly implicate GTPases in the process of ribosome formation. My laboratory investigates GTPases involved in the formation of the 50S subunit in Bacillus subtilis, with most of our attention focused on RbgA (ribosomal biogenesis GTPase A). Cells depleted of RbgA fail to form mature 50S subunits and accumulate a ribosomal intermediate that migrates at 45S in sucrose gradients. Our primary objectives are to 1) understand the structural differences that exist between the 45S ribosomal subunit intermediate and mature 50S subunits and 2) understand how RbgA participates in the maturation of the 45S intermediate into a functional 50S subunit. Recent data from genetic and biochemical experiments aimed at elucidating the role of RbgA in ribosome assembly will be presented.

PROCESSING OF THE 5’ TERMINUS OF SOME TRNA PRECURSORS BY RNASE P IN *ESCHERICHIA COLI* REQUIRES THE PRIOR CONVERSION OF THE 5’ TRIPHOSPHATE TO A 5' MONOPHOSPHATE BY RPPH.

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The processing of tRNA precursors in *E. coli* involves either endonucleolytic cleavages by RNase E or RNase P to separate pre-tRNAs contained in polycistronic transcripts (1-2) or removal of Rho-independent transcription terminators by either RNase E, RNase G or PNPase (2-3). Subsequently, endonucleolytic cleavages by RNase P generate the mature 5’ termini. Since it has been shown that RNase E activity is stimulated by the presence of a 5’ monophosphate at the 5’ terminus of a target substrate, we were interested in determining if inactivation of the enzyme involved in the conversion of a 5’ triphosphate to a 5’ monophosphate (RppH, formerly NudH) altered the processing of polycistronic tRNA operons that have been shown to be dependent on RNase E for their initial processing. To our surprise, we saw no differences in the processing of the *glyW cysT leuZ* polycistronic operon in a *ΔrppH* strain compared to a wild type control. Subsequently, we examined a number of monocistronic tRNA transcripts (*leuX*, *pheU*, *pheV*, *asnT*, *asnU*, *asnV*, and *asnW*). While we did not expect to see an RppH affect on *leuX* (3), the other tRNAs have been shown to be dependent on RNase E for the removal of their Rho-independent transcription terminators. However, the inactivation of RppH did not affect the initial processing of any of these seven tRNAs. In contrast, in the case of *pheU* and *pheV*, a prominent processing intermediate was present that was identical in size to one observed in an RNase P mutant. Detailed Northern blot and primer extension analysis showed that inactivation of RppH dramatically inhibited the processing of the 5’ terminus by RNase P. Interestingly, the 5’ processing of the *leuX* and *asn* transcripts was not dependent on RppH. Our data suggest that RNase P is inhibited by a 5’ triphosphate, if the leader region is less than 10 nt in length. We are currently working to elucidate the nature of the inhibition of RNase P by the proximity of a 5’ triphosphate. This work was supported in part by grants (GM81554 and GM57220) to S.R.K.

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HEAT SHOCK INDUCES YOEB-MEDIATED A-SITE MRNA CLEAVAGE IN *ESCHERICHIA COLI*

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Prolonged translational arrest leads to mRNA degradation into the A-site of paused ribosomes. This process appears to require multiple nucleases, including RNase II, which is the primary 3′-to-5′ exonuclease responsible for mRNA turnover in *E. coli*. Here, we demonstrate that growth at elevated termperatures (42 – 44 °C) restores A-site cleavage activity to cells lacking RNase II. Examination of the known E. coli toxin-antitoxin modules led to the identification of YoeB as the heat-induced A-site nuclease. Thermal activation of YoeB requires the Lon protease, consistent with its role in degradation of the cognate YefM antitoxin. YoeB activity could be induced at lower temperatures (30 °C) through overexpression of either σ32 or Lon. These latter results indicate that increased Lon expression is responsible for YoeB activation, rather than thermal dissociation of the YefM-YoeB complex. Taken together, these results suggest that YoeB plays a role in molecular quality control during heat shock. However, both Δ*yefM-yoeB* and Δ*lon* mutants grow at the same rate as wild-type cells at elevated temperatures. Although the physiological role of heat-induced YoeB activity is still unclear, these observations are consistent with the stress-response hypothesis of toxin-antitoxin function formulated by Gerdes and colleagues.

THREE DIFFERENT TRNA MIMICS THAT ALL CONTRIBUTE TO ANTIBIOTIC RESISTANCE

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Transfer RNAs (tRNAs) are primarily responsible for translation of the genetic code, and have also been shown to regulate gene expression by acting as sensors of the translation status of the cell. These highly stable molecules can also provide a structural framework for small RNAs and proteins that regulate other cellular functions such as viral replication, amino acid biosynthesis and cell wall remodeling. Here we describe three divergent examples of molecular mimicry that utilize the $tRNA^{Lys}$ scaffold, all of which contribute to antibiotic resistance in bacteria. Multiple peptide resistance factor (MprF) is a membrane protein that diverts amino acids from protein synthesis by binding aminoacylated tRNA in competition with translation elongation factors. MprF uses lysine, alanine and arginine to neutralize the negative charge of phosphatidylglycerol in the cell membrane, providing resistance to cationic antimicrobial peptides and other antibiotics. The second example is $tRNA^{Other}$, which mimics the $tRNA^{Lys}$ secondary structure but does not participate in translation. $tRNA^{Other}$ instead functions as an iron-regulated small RNA that coordinates the expression of a number of genes involved in antibiotic resistance. The most divergent example of a tRNA mimic that contributes to antibiotic resistance is elongation factor P (EF-P). EF-P is a protein that mimics both the structure and function of tRNA, to the extent that it can even be modified with lysine by a paralog of lysyl-tRNA synthetase. Lysine modification of EF-P is required for a novel mechanism of post-transcriptional control of gene expression, and contributes to a number of virulence phenotypes including antibiotic resistance. Taken together these three examples illustrate how tRNA provides a versatile molecular scaffold that can control a wide range of fundamental cellular processes.

A UNIQUE POST-TRANSLATIONAL MODIFICATION ON ELONGATION FACTOR P (EF-P) IS CRITICAL FOR VIRULENCE AND STRESS RESISTANCE IN *SALMONELLA*.

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We have identified an interaction between *poxA*, encoding a paralog of lysyl tRNA-synthetase, the closely linked *yjeK* gene, encoding a putative 2,3-β-lysine aminomutase, and *efp* encoding elongation factor P, that is critical for virulence and stress resistance in *Salmonella enterica*. *Salmonella poxA* and *yjeK* mutants share extensive phenotypic pleiotropy including attenuated virulence in mice, an increased ability to respire under nutrient limiting conditions, hypersusceptibility to a variety of diverse growth inhibitors, poor growth under conditions of low osmolarity, and altered expression of approximately 60 proteins including several encoded on the SPI-1 pathogenicity island. PoxA mediates post-translational lysylation of bacterial elongation factor P (EF-P) at a highly conserved lysyl residue to generate a unique lysyl-lysine amino acid. This modification is analogous to the modification of the eukaryotic EF-P homologue, eIF5A, with hypusine. Unlike eIF5A, however, EF-P is not essential and appears to selectively control the translation of a relatively limited number of proteins. The phenotypes of a *Salmonella efp* mutant are nearly identical to, but more severe than those of *poxA* and *yjeK* mutants. The modification of EF-P is a novel mechanism of regulation whereby PoxA acts as an aminoacyl-tRNA synthetase that attaches an amino acid to a protein resembling tRNA rather than to a tRNA.

COMPETENCE IN *STREPTOCOCCUS PNEUMONIAE* IS REGULATED BY THE RATE OF DECODING ERRORS DURING PROTEIN SYNTHESIS

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Competence for genetic transformation in *Streptococcus pneumoniae* is activated in response to a secreted peptide pheromone but is also repressed by factors including the pneumococcal HtrA protease. Because the homologous DegP protease of *Escherichia coli* displays a broad specificity for digesting unfolded proteins, we hypothesized that HtrA might modulate competence activity in response to changes in the accuracy of protein synthesis. In particular, production of miscoded proteins with intrinsic folding defects might interfere with the ability of HtrA to repress competence. Induction of competence in response to the antibiotics streptomycin and kanamycin has previously been shown. We tested whether the effect of these agents on competence was due to induction of decoding errors during protein translation. Using an assay for the suppression of nonsense codons in a *lacZ* reporter, we demonstrated that translational errors are promoted by these antibiotics at the concentrations required to induce competence. Another antibiotic—kasugamycin—had an opposing effect, reducing the basal rate of translational errors and counteracting the induction of errors by streptomycin. Addition of kasugamycin to pneumococcal cultures blocked the development of spontaneous competence and prevented the induction of competence by streptomycin. Other antibiotics that reduced translational errors (tetracycline and spectinomycin) were also found to repress competence, while conversely paromomycin promoted translational errors and induced competence. To test further the relationship between translational fidelity and competence, we examined the effect of a point mutation in the gene encoding the S12 ribosomal protein. This mutation, which confers streptomycin resistance, increases the accuracy of decoding and was found to impair the development of competence even in the absence of antibiotics. Consistent with the proposed role of the HtrA protease in this modulation of competence, the presence of a wild-type *htrA* allele repressed competence when translational accuracy was high but did not affect competence when the rate of translational errors was raised using streptomycin. Together these data suggest that as mistranslated proteins accumulate and provide alternative substrates for degradation, HtrA is less able to repress competence signaling, allowing HtrA to act indirectly as a sensor for the accuracy of protein synthesis. Although our studies have manipulated ribosomal decoding to test this model, similar protein miscoding may result from mutational damage to the genome.

COMPUTATIONAL AND EXPERIMENTAL DISCOVERY OF SMALL RNAS IN *STAPHYLOCOCCUS AUREUS* REVEALS RSAE, A RIBOREGULATOR OF CENTRAL METABOLISM

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Using a biocomputing strategy that exploits the entirety of available bacterial genomes to classify all noncoding elements of a selected reference species as well as an experimental approach, we identified twenty-two new small RNAs (sRNAs) of the pathogen *Staphylococcus aureus*. These sRNAs are either encoded in intergenic regions generated by premature transcription termination associated with riboswitch activities, or expressed from the complementary strands of mRNAs. The detailed analysis of sRNA expression reveals that some sRNAs accumulate in exponential, pre-stationary, or stationary phases. We observed abrupt changes in the expression levels of several sRNAs between OD600 = 6.0 and the stationary phase. We focused our study on RsaE, an sRNA that is highly conserved in the bacillales order and is deleterious when over-expressed. As many sRNAs act via base-pairing to target mRNAs and thereby affect their stability and/or translation, we hypothesized that RNA targets of RsaE would vary upon RsaE accumulation, and could thus be identified by transcriptome analysis. RsaE accumulation led to the downregulation of twenty-five genes. Strikingly, mRNAs that are downregulated by RsaE can be clustered around two essential pathways: i) the folate one-carbon shuffle metabolism, and (ii) the Krebs cycle. Computer predictions indicate that RsaE-dependent downregulation is likely mediated via pairing of RsaE with mRNA 5' UTRs of these genes. As we observed that RsaE accumulates transiently in late exponential growth, a possible RsaE function is to ensure coordinate downregulation of the central metabolism when carbon sources become scarce. Our results indicate that the concept of coordinated control of metabolic enzymes by sRNAs should be extended to the Gram-positive pathogen, *S. aureus*.

POLYNUCLEOTIDE PHOSPHORYLASE IS ESSENTIAL FOR THE POSTTRANSCRIPTIONAL REGULATION OF GENE EXPRESSION BY SMALL NONCODING RNAS IN *ESCHERICHIA COLI*

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In *Escherichia coli* and other bacteria, small noncoding RNAs (sRNAs) posttranscriptionally regulate gene expression by annealing to mRNAs in an Hfq dependent manner and altering their stability and/or translation. In a combined genetic selection and screen for mutants defective in the posttranscriptional regulation of gene expression by sRNAs, we isolated *hfq* mutants and null mutants in *pnp*, encoding polynucleotide phosphorylase. Deletion of *pnp* decreases the stability of at least two sRNAs, RyhB and SgrS, decreases the negative regulation of *sodB* by RyhB and *ompX* by CyaR, another sRNA. We have found that deletion of *pnp* increases the levels of the essential endonuclease RNase E. The C-terminal scaffold domain of RNase E binds a number of proteins, including Pnp. Unexpectedly, introduction of *rne* alleles that encode RNase E lacking the entire C-terminal scaffold domain or the RhlB/enolase binding domain into a *pnp* deletion mutant suppresses the cold sensitivity of the *pnp* mutant and restores the negative regulation of *ompX* by CyaR. These alleles of *rne* were previously shown to encode a less active RNase E. This results suggests the increased degradation of specific substrates by RNase E is responsible for the cold sensitivity of a *pnp* null mutant. Moreover, the lack of sRNA-mediated regulation in the absence of an active form of Pnp may be due to the rapid turnover of the sRNAs resulting from an increase in RNase E activity, and possibly an increase in access of nucleases to the sRNAs.

SMALL RNA REGULATION IN A PATHOGEN THAT NATURALLY LACKS HFQ: ENHANCED STREPTOKINASE ACTIVITY BY MODULATION OF MRNA STABILITY

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Small RNA molecules play key regulatory roles in bacteria. Commonly, these small regulatory RNAs (sRNAs) require the RNA-binding protein Hfq for activity. However, half of all genome-sequenced bacteria lack Hfq, including pathogens of the genera *Streptococcus*, *Enterococcus*, *Helicobacter*, and *Mycobacterium*. Moreover, there is little mechanistic information for sRNAs in these species. Here, we analyzed the relationship between a putative group A *Streptococcus* (GAS) sRNA and production of streptokinase (SKA), a secreted virulence factor exploited by the medical community for over six decades due to its ability to induce blood clot degradation. Homologues of the putative sRNA-encoding gene fibronectin/fibrinogen-binding/hemolytic-activity/streptokinase-regulator-X (*fasX*) were identified in four different pyogenic streptococcal species. However, despite 79% *fasX* nucleotide identity, a *fasX* allele from the animal pathogen *Streptococcus zooepidemicus* failed to complement a GAS *fasX* mutant. Using a series of precisely-constructed *fasX* alleles we discovered that FASX is a bona-fide sRNA that post-transcriptionally regulates SKA production in GAS. By base-pairing to the 5' end of *ska* mRNA, FASX enhances *ska* transcript stability, resulting in a ~10-fold increase in SKA activity. Mutation of complementary *fasX* or *ska* nucleotides reduced *ska* transcript stability, while stability was restored upon introduction of compensatory mutations. Our data provides the first nucleotide-level characterization of a molecular mechanism governing a sRNA regulatory pathway in a bacterium that naturally lacks Hfq. In addition, we enhance our understanding of the regulation of a key virulence factor from a medically important Gram-positive pathogen.

BEYOND TRANSCRIPTION: COMPLEX REGULATORY NETWORKS FOR RESPONDING TO CHANGING ENVIRONMENTS

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This year marks the 100th anniversary of Monod's birth, a useful moment to reflect on what we have learned about regulatory networks since the pioneering publications of Jacob and Monod on the lac and lambda regulatory systems 50 years ago. The basic ideas are the same – the cell responds to changing environments by optimizing expression of the needed and unneeded proteins. The early work on these systems aimed at defining how this change in expression worked, and concluded (Pardee, Jacob, and Monod, J. Mol. Biol. 1:165-178, 1959) ".. the repressor model may lead to a generalizable picture of the regulation of protein synthesis; according to this scheme, the basic mechanism common to all protein-synthesizing systems would be inhibition of specific repressors formed under the control of particular genes, and antagonized, in some cases, by inducers". The nature of the repressor was not yet known; it was suggested that it might be RNA. Now we know that regulatory networks include repressors (both protein and RNA), activators, and molecules that play both roles. Regulation takes place not only at the level of synthesis, but at the level of degradation of proteins and RNAs, and for many genes, at every possible level. I will discuss some of our recent work on post-transcriptional regulatory mechanisms – how small RNAs and regulated proteolysis changes how we think about regulatory circuits, adaptation, and feedback control.

THE NUCLEOID OCCLUSION FACTOR SLMA IS A DNA-ACTIVATED FTSZ ANTAGONIST

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Two negative regulatory systems, nucleoid occlusion (NO) and the Min system, control assembly of the cytokinetic FtsZ-ring in *E. coli* and *B. subtilis*. NO in *E. coli* is mediated by a TetR-family protein, SlmA. Previous work demonstrated a direct interaction between SlmA and FtsZ, but the mechanism by which SlmA blocks FtsZ polymerization over the nucleoid has remained mysterious. Here we show that SlmA blocks FtsZ polymerization *in vitro*. In addition, we used a ChIP-on-chip approach to identify 24 SlmA-binding sites (SBS) on the chromosome. The sites were enriched around the origin of replication and under represented in the terminus region. This suggests that, like the Noc protein from *B. subtilis*, SlmA segregation with the origin region can be used as a timing mechanism to coordinate chromosome replication and Z-ring assembly. We further show that SlmA specifically binds SBS *in vitro*. Remarkably, SlmA binding to SBS dramatically enhanced its ability to interfere with FtsZ polymerization. Thus, SlmA is a DNA-activated FtsZ antagonist. Because particular regions of the chromosome have distinct cellular addresses, coupling SlmA activation to specific DNA binding provides a mechanism for the precise localization of its anti-FtsZ activity within the cell.

MATP, THE SEPTAL RING AND REPLICATION CONTROL THE SEGREGATION OF THE TER MACRODOMAIN OF THE *E. COLI* CHROMOSOME.

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The large size of bacterial genomes compared to cell dimensions imposes an extensive compaction of chromosomes compatible with the DNA transactions occurring during transcription, replication, recombination and segregation. Two different levels of chromosome organization have been identified. At a small scale, DNA supercoiling partitions the chromosome into topological micro-domains averaging 10 kb. At higher scale, in *E. coli*, cytological and genetic analyses based on long distance DNA interactions revealed a structuring process that spatially insulates four large regions of the chromosome called macrodomains. The dynamic behavior of loci belonging to various macrodomains and less constrained regions is radically different. In macrodomains, constraints on mobility are apparent, whereas in Non-Structured regions, markers exhibit a greater mobility. We have recently identified a protein, MatP, and its binding sites that constrain the mobility of the DNA inside the macrodomain containing the terminus region of the chromosome (Ter MD). The absence of MatP as dramatic effects on chromosome segregation. A cell biology analysis of the behavior of *E. coli* linearized chromosomes during the cell cycle has allowed us to characterized the property of MatP to anchor the terminus region of the chromosome at mid-cell. This step, mediated by an interaction between MatP and the septal ring, delays segregation of sister Ter MDs and contribute to the condensation of the macrodomain. Finally, we have demonstrated that Ter MD segregation from the pole to mid-cell is controlled by the replication machinery but not by MatP.

TEMPORAL AND SPATIAL RESTRICTION OF MUTATION IN THE *ESCHERICHIA COLI* CHROMOSOME

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Mutation pathways controlled by stress responses can limit mutation to times of stress, when cells are maladapted to their environment, and possibly to local genomic regions, changing basic ideas about the randomness of mutation that drives evolution. Stress-induced mutation may provide superior models for mutations that drive pathogen-host interactions, antibiotic resistance and many other problems of evolution under stress. The most well documented stress-induced-mutation mechanism occurs in starving *Escherichia coli* and requires the RpoS general/starvation- and SOS DNA-damage-stress responses, SOS- and RpoS-upregulated DinB error-prone DNA polymerase, and double-strand breaks (DSBs) and their repair, but was thought to be peculiar to an F' conjugative plasmid, not a mechanism that affects cells generally. We show that this DSB-repair-coupled stress-induced-mutation mechanism occurs in chromosomes of starving F- *E. coli*. Endonuclease-induced DSBs increase mutation 50-100-fold, SOS-, RpoS-, DinB- and DSB-repair-protein-dependently. When added back, the F' enhanced this only 2-fold via an extra dinB gene. Moreover, we demonstrate that the mutations are localized near DSBs regardless of DSB position in the chromosome and decrease exponentially up to 30-60kb away. Restriction of mutation in genomic space could allow rare cells in a population with an adaptive mutation to survive, and promote local concerted evolution within genes. Furthermore, fully half of spontaneous mutation during starvation requires SOS, RpoS, DinB and DSB-repair proteins indicating that this is a mutation pathway that stressed cells usually employ. Mechanisms that temporally regulate and spatially restrict mutation could enhance the ability of cells to evolve.

A DNA DAMAGE CHECKPOINT IN *CAULOBACTER CRESCENTUS* USES A NOVEL MECHANISM TO REGULATE CELL DIVISION

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Following DNA damage, cells typically delay cell cycle progression and inhibit cell division until their chromosomes have been repaired. The bacterial checkpoint systems responsible for these DNA damage responses are incompletely understood. Here, we show that *Caulobacter crescentus* responds to DNA damage by coordinately inducing an SOS regulon and inhibiting the master regulator CtrA. Included in the SOS regulon is *sidA*, a small membrane protein that helps to delay cell division following DNA damage, but is dispensable in undamaged cells. SidA is sufficient, when overproduced, to block cell division. However, unlike other regulators of bacterial cell division, SidA does not disrupt the assembly or stability of the cytokinetic ring protein FtsZ, nor does it affect assembly of other components of the cell division machinery. Instead, we provide evidence that SidA inhibits division by targeting FtsW, an essential protein that may coordinate septal peptidoglycan synthesis with final constriction of the FtsZ ring.

E. COLI PRE-RC ASSEMBLY IS DIRECTED BY HELICALLY-PHASED ARRAYS OF CLOSELY SPACED, LOW AFFINITY DNAA RECOGNITION SITES IN ORIC

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All cells must duplicate their genomes before dividing into two daughter cells. During the E. coli cell cycle, new rounds of chromosome replication are triggered when sufficient amounts of the initiator protein, DnaA, accumulate and bind to multiple high and low affinity recognition sites in the unique origin of replication, oriC. DnaA occupation of oriC results in formation of a pre-replication complex (pre-RC) that unwinds the DNA duplex and recruits replisome proteins. Pre-RC assembly is tightly coordinated with cell growth to ensure that genome duplication begins at a precise mass, and is limited to once per cell division cycle. The pre-RC assembles in stages, and begins with occupation of three high affinity DnaA binding sites in oriC immediately after the previous initiation event is completed. Just prior to the onset of DNA replication, the remaining lower affinity sites in oriC are filled with DnaA, forming the pre-RC, but the mechanisms controlling this transition are not yet fully understood. In this study, we have investigated how the spatial arrangement of DnaA binding sites in oriC facilitates low affinity site loading and ordered pre-RC assembly. We have found that oligomeric-proficient DnaA bound at high affinity sites is necessary for low affinity DnaA/oriC interactions. Cooperative binding is optimal when weak DnaA recognition sites are arrayed, in the same orientation, and closely spaced in helical phase. OriC contains two such arrays, one in each half of the origin, although some of the sites in the arrays do not resemble canonical DnaA binding sites. Mutations that altered orientation, sequence, or spacing of arrayed sites also perturbed formation of higher-order complexes in vitro, and reduced oriC function in vivo. These data are consistent with a model in which pre-RC assembly requires strong sites to anchor DnaA oligomers whose extension is stabilized by binding to closely spaced weak sites. We suggest that in bacteria, the oriC nucleotide sequence carries the information needed to ensure ordered pre-RC assembly.

THE INITIATOR OF *VIBRIO CHOLERAE* CHROMOSOME II USES INTERACTIONS BETWEEN TWO KINDS OF DNA BINDING SITES TO CONTROL REPLICATION

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The origin of replication (*oriII*) of *Vibrio cholerae* chromosome II (chrII) is similar to commonly found plasmid origins that have repeated initiator-binding sites (iterons). Extra iterons can be found outside of plasmid origins and their deletion increases the plasmid copy number, indicating that they serve as inhibitors of replication. We show that in addition to extra iterons, ChrII has another conserved sequence that we designate as 39-mer. 39-mer binds the chrII-specific initiator and lowers the copy number of *oriII*-based plasmids, as do the iterons. Copy number decreased significantly when the extra iterons were deleted, suggesting that the iterons dampen the 39-mer activity to help promote chrII replication. This is opposite to the inhibitory role that they play in plasmids. The presence of 39-mer improved coupling of iteron-carrying DNA, an activity believed to inhibit initiation. The two kinds of sites thus can influence each others activities. The damping role of the extra iterons can be understood if they serve as decoys to engage the 39-mer, reducing its effect on the *oriII* iterons. The presence of many counterbalanced inhibitions and activations is desirable for homeostasis of the chromosomal copy number.

CO-DIRECTIONAL COLLISIONS BETWEEN THE REPLICATION AND TRANSCRIPTION MACHINERIES LEAD TO REPLICATION FORK STALLING AND RESTART AT rRNA OPERONS IN *B. SUBTILIS*

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Highly expressed bacterial genes are generally encoded on the leading strand of DNA such that there is co-directional replication and transcription. There is likely strong selective pressure to maintain this co-orientation and to avoid head-on collisions between the replication and transcription machineries that result in slowing of the replication fork. While this co-orientation avoids head-on collisions, there is still the potential for co-directional collisions because the rate of replication is approximately 10-fold greater than the rate of transcription.
Our results indicate that in *B. subtilis* during rapid growth, there are co-directional collisions in vivo between the replication and the transcription machineries and that these collisions result in replication fork pausing and restart. Primosomal proteins DnaD and DnaB are required for loading of the replicative helicase (DnaC) both at oriC and at regions of replication restart in *B. subtilis* and other low G+C content gram positive bacteria. We performed chromatin immunoprecipitations (ChIPs) of both DnaD and DnaB followed by genomic microarrays and qPCR and found that DnaD and DnaB associated significantly with ribosomal RNA genes (rrn) during rapid growth when rRNA genes are most highly expressed, but not during slow growth. Association of these replication restart proteins with highly transcribed regions was confirmed by testing a strong constitutively expressed promoter (P*xis*) transcribed in the head-on direction. The association of DnaD and DnaB with rRNA genes was dependent on active transcription; blocking RNA polymerase function with rifampicin alleviated the association. In agreement with the idea that DnaD and DnaB were present at the rrn loci due to replication fork stalling and restart, we found that the replicative helicase, DnaC, was also associated with these regions in a transcription dependent manner. Our results indicate that during rapid growth, there is replication fork stalling and restart at highly transcribed rRNA genes, and that this is likely due to co-directional collisions between the replication and transcription machineries.

PREVENTION OF THE CONFLICT BETWEEN TRANSCRIPTION AND REPLICATION

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The fundamental processes of replication and transcription take place on the same DNA template and must occur both efficiently and accurately. This requires these processes to be coordinated with each other to avoid potential conflicts. We study the bacteria *Bacillus subtilis* and *Escherichia coli* and found they have developed diverse mechanisms to prevent transcription from interfering with replication. 1) In *B. subtilis*, there is a strong genome-wide bias to co-orient transcription with replication, with essential and/or highly-expressed genes further enriched co-directionally. This feature helps to avoid conflicts between head-on replication and transcription. We defined the consequences of this conflict by engineering genomic inversions. We found that while reversing transcription bias in an extended genomic segment results in decreased replication speed and competitive disadvantage relative to wild-type cells in minimal medium, inversion of rDNA disrupts replication in rich medium, resulting in activation of DNA damage responses and cell death. Our work supports the hypothesis that the impact of transcription on replication is an important driving force in the evolution of genome organization, and suggests that the precise cost might vary depending on both the gene identity and the growth environment. 2) In *E. coli*, the transcription factor DksA ensures replication completion by removing transcription roadblocks. In the absence of DksA, replication is frequently interrupted by transcription and is rapidly arrested by amino acid starvation. We found evidence suggesting that this function of DksA depends on its less-studied transcription elongation activity. GreA/B elongation factors also prevent replication arrest during nutrient stress. We conclude that in E. coli, transcription elongation factors alleviate fundamental conflicts between replication and transcription. In summary, our work highlights the unified theme of challenges to replication, and the diverse strategies used by organisms to overcome these challenges and maintain genome integrity.

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VISITOR INFORMATION

EMERGENCY	CSHL	BANBURY
Fire	**(9) 742-3300**	**(9) 692-4747**
Ambulance	**(9) 742-3300**	**(9) 692-4747**
Poison	**(9) 542-2323**	**(9) 542-2323**
Police	**(9) 911**	**(9) 549-8800**
Safety-Security	**Extension 8870**	

Emergency Room **Huntington Hospital** 270 Park Avenue, Huntington	**631-351-2300** **(1037)**
Dentists Dr. William Berg Dr. Robert Zeman	 **631-271-2310** **631-271-8090**
Doctor MediCenter 234 W. Jericho Tpke., Huntington Station	**631-423-5400** (**1034**)
Drugs - 24 hours, 7 days Rite-Aid 391 W. Main Street, Huntington	**631-549-9400** (**1039**)

Free Speed Dial
Dial the four numbers (********) from any **tan house phone** to place a free call.

GENERAL INFORMATION

Books, Gifts, Snacks, Clothing, Newspapers
BOOKSTORE 367-8837 (hours posted on door)
Located in Grace Auditorium, lower level.

Photocopiers, Journals, Periodicals, Books, Newspapers
Photocopying – Main Library
Hours: 8:00 a.m. – 9:00 p.m. Mon-Fri
10:00 a.m. – 6:00 p.m. Saturday
Helpful tips - Obtain PIN from Meetings & Courses Office to enter Library after hours. See Library staff for photocopier code.

Computers, E-mail, Internet access
Grace Auditorium
Upper level: E-mail only
Lower level: Word processing and printing.
STMP server address: mail.optonline.net
To access your E-mail, you must know the name of your home server.

Dining, Bar
Blackford Hall
Breakfast 7:30–9:00, Lunch 11:30–1:30, Dinner 5:30–7:00
Bar 5:00 p.m. until late
Helpful tip - If there is a line at the upper dining area, try the lower dining room

Messages, Mail, Faxes

Message Board, Grace, lower level

Swimming, Tennis, Jogging, Hiking

June–Sept. Lifeguard on duty at the beach. 12:00 noon–6:00 p.m. Two tennis courts open daily.

Russell Fitness Center

Dolan Hall, west wing, lower level
PIN#: **Press 64480 (then enter #)**

Concierge

On duty daily at Meetings & Courses Office.
After hours – From tan house phones, dial x8870 for assistance

Pay Phones, House Phones

Grace, lower level; Cabin Complex; Blackford Hall; Dolan Hall, foyer

CSHL's Green Campus

Cold Spring Harbor Laboratory is pledged to operate in an environmentally responsible fashion wherever possible. In the past, we have removed underground oil tanks, remediated asbestos in historic buildings, and taken substantial measures to ensure the pristine quality of the waters of the harbor. Water used for irrigation comes from natural springs and wells on the property itself. Lawns, trees, and planting beds are managed organically whenever possible. And trees are planted to replace those felled for construction projects.

Two areas in which the Laboratory has focused recent efforts have been those of waste management and energy conservation. The Laboratory currently recycles most waste. Scrap metal, electronics, construction debris, batteries, fluorescent light bulbs, toner cartridges, and waste oil are all recycled. For general waste, the Laboratory uses a "single stream waste management" system, removing recyclable materials and sending the remaining combustible trash to a cogeneration plant where it is burned to provide electricity, an approach considered among the most energy efficient, while providing a high yield of recyclable materials.

Equal attention has been paid to energy conservation. Most lighting fixtures have been replaced with high efficiency fluorescent fixtures, and thousands of incandescent bulbs throughout campus have been replaced with compact fluorescents. The Laboratory has also embarked on a project that will replace all building management systems on campus, reducing heating and cooling costs by as much as twenty-five per cent.

Cold Spring Harbor Laboratory continues to explore new ways in which we can reduce our environmental footprint, including encouraging our visitors and employees to use reusable containers, conserve energy, and suggest areas in which the Laboratory's efforts can be improved. This book, for example, is printed on recycled paper.

1-800 Access Numbers

AT&T	**9-1-800-321-0288**
MCI	**9-1-800-674-7000**

Local Interest

Fish Hatchery	631-692-6768
Sagamore Hill	516-922-4447
Whaling Museum	631-367-3418
Heckscher Museum	631-351-3250
CSHL DNA Learning Center	x 5170

New York City

Helpful tip -

Take Syosset Taxi to Syosset Train Station ($8.00 per person, 15 minute ride), then catch Long Island Railroad to Penn Station (33rd Street & 7th Avenue). Train ride about one hour.

TRANSPORTATION

Limo, Taxi

Syosset Limousine	516-364-9681 (**1031**)
Super Shuttle	800-957-4533 (**1033**)
To head west of CSHL - Syosset train station	
Syosset Taxi	516-921-2141 (**1030**)
To head east of CSHL - Huntington Village	
Orange & White Taxi	631-271-3600 (**1032**)
Executive Limo	631-696-8000 (**1047**)

Trains

Long Island Rail Road	822-LIRR
Schedules available from the Meetings & Courses Office.	
Amtrak	800-872-7245
MetroNorth	800-638-7646
New Jersey Transit	201-762-5100

Ferries

Bridgeport / Port Jefferson	631-473-0286 **(1036)**
Orient Point/ New London	631-323-2525 **(1038)**

Car Rentals

Avis	631-271-9300
Enterprise	631-424-8300
Hertz	631-427-6106

Airlines

American	800-433-7300
America West	800-237-9292
British Airways	800-247-9297
Continental	800-525-0280
Delta	800-221-1212
Japan Airlines	800-525-3663
Jet Blue	800-538-2583
KLM	800-374-7747
Lufthansa	800-645-3880
Northwest	800-225-2525
United	800-241-6522
US Airways	800-428-4322