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Laser Tweezers Raman Spectroscopy Potential for Studies of Complex Dynamic Cellular Processes: Single Cell Bacterial Lysis

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The potential of laser tweezers Raman spectroscopy (LTRS) to study complex and dynamic cellular processes was investigated on the model of single *E. coli* cells lysed (1) from "outside" with egg white lysozyme and (2) from "within" by temperature-induced temperate bacteriophage λcI857. The two lysis processes differed in the final outcome (incomplete vs complete cell lysis) as revealed by the dynamic laser light scattering and exhibited distinctive dynamic Raman spectra changes. The technique enabled for the first time at the cellular level to observe and quantify real time interaction of lysozyme with *E. coli* cells, "visualize" a side effect of the process due to the presence of EDTA, and correlate the process of cell wall disruption, as evidenced by the onset and development of asymmetric speckle scattering patterns, with release/escape of intracellular material (ribosomes, nucleic acids, proteins, etc.) quantified by the intensity changes of Raman signatures. Raman spectra changes observed during the lysis from "within" suggest alleged production of heat shock proteins are consistent with the occurring synthesis of phage-related proteins and are in good agreement with the calculated potential contribution of the above proteins to the Raman spectra. It was also established and validated that the contribution of cellular DNA to the Raman spectra of bacterial cells is negligible compared to RNA. The results open new venues for LTRS research and strongly suggest that LTRS has a great potential especially in investigation of real-time processes.

Recent progress in laser physics, quantum optics, and other fields of physics has given a new impetus for the development and application in biology of more rapid, more sensitive, and more informative spectral analytical methods based on mass, Raman, infrared, and other spectroscopies. Furthermore, some of these new methods have the capability to allow study of live, individual cells. Because the single-cell studies are not subject to the averaging effects of population-scale methods, they offer a new level of analysis unavailable with traditional biochemical methods.

Among these new techniques is confocal Raman microscopy,[1] in which the Raman signal from only the focal region of the objective is collected, while background signals that are out of the focus are rejected. This technique allows high spatial resolution and enables detection of both the spectral features of single cells and interior organelles. The main drawback of the method is that the targeted cell must be immobilized by physical or chemical contact. However, by combination of this approach with optical tweezers (single-beam optical trapping),[2,3] it becomes possible to analyze single, live, moving cells in aqueous solutions. This new combined technique, called confocal laser tweezers Raman spectroscopy (LTRS), has been used in studies of single, optically trapped microparticles, different prokaryotic and eukaryotic cells, and liposomal membranes.[4–13] Because the method is based on Raman signatures yielding information on covalent and hydrogen bonding, hydrophobic and electrostatic interactions, etc. in real time, it may be especially valuable for the elucidation of mechanisms of different dynamic processes occurring within cells. However, until now very few LTRS studies attempted to address cellular processes. Among them were a complex process of Bacillus sporulation[10,11] and an induced synthesis of recombinant proteins in bacterial cells.[12,13] In both cases, however, the evolution of particular Raman markers specific for the process (dipicolinic acid band for spores and markers associated with protein vibrations in the case of protein synthesis), has been followed. To further test and explore the analytical potential of LTRS, it would be interesting to employ more complex cellular processes with fairly known molecular mechanisms. The latter could significantly help in interpretation, confirmation, and validation of the biological significance of observed Raman spectral changes (if any) at the cellular level. A good model of such cellular processes for LTRS analysis would be the process of bacterial cell lysis initiated by two different mechanisms: by exogenously added lysozyme and by induced temperate bacteriophage. There is a certain uniqueness of this biological model system for LTRS analysis in that it

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Comparison of Deterministic and Stochastic Models of the *lac* Operon Genetic Network

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ABSTRACT The *lac* operon has been a paradigm for genetic regulation with positive feedback, and several modeling studies have described its dynamics at various levels of detail. However, it has not yet been analyzed how stochasticity can enrich the system's behavior, creating effects that are not observed in the deterministic case. To address this problem we use a comparative approach. We develop a reaction network for the dynamics of the *lac* operon genetic switch and derive corresponding deterministic and stochastic models that incorporate biological details. We then analyze the effects of key biomolecular mechanisms, such as promoter strength and binding affinities, on the behavior of the models. No assumptions or approximations are made when building the models other than those utilized in the reaction network. Thus, we are able to carry out a meaningful comparison between the predictions of the two models to demonstrate genuine effects of stochasticity. Such a comparison reveals that in the presence of stochasticity, certain biomolecular mechanisms can profoundly influence the region where the system exhibits bistability, a key characteristic of the *lac* operon dynamics. For these cases, the temporal asymptotic behavior of the deterministic model remains unchanged, indicating a role of stochasticity in modulating the behavior of the system.

INTRODUCTION

The *lac* operon genetic switch is a paradigm for genetic regulation with positive feedback, and has been studied experimentally and theoretically for nearly half a century. Indeed, the operon concept, which pertains to a sequence of genes that function under the control of the same operator (1), was first introduced in 1960. The *lac* operon consists of three genes downstream of the *lac* promoter that encode for the proteins necessary for lactose metabolism. Specifically, *lacZ* encodes for β-galactosidase, which transforms lactose to the inducer allolactose; *lacY* encodes for LacY permease, which transports lactose into the cell; and *lacA* encodes for galactoside transacetylase (LacA), which transfers an acetyl group from acetyl-CoA to β-galactosides (2). Furthermore, upstream of the promoter, there exists the constitutively expressed *lacI* gene, which encodes for the LacI repressor protein.

The free LacI repressor is a tethered tetramer (dimer of dimers) (3) that has a high affinity for the *lacO* (*O1*) operator contained in the *lac* promoter. Therefore, in the absence of lactose, each LacI dimer binds to an operator and thus inhibits transcription of the *lacZ*, *lacY*, and *lacA* genes. LacI can also bind to pseudooperators that exist upstream (*O3*) and downstream (*O2*) of the promoter and create DNA loop structures (4–6). It has been suggested that binding of the LacI repressor to the pseudooperators results in a localization of LacI close to the main operator, thereby increasing its binding efficiency to the operator (7). Thus, more efficient suppression of the *lac* genes is attained.

However, if lactose is present in the extracellular medium, it gets transported into the cell, where one fraction is hydrolyzed to galactose and glucose and the other fraction is transformed to the inducer allolactose by β-galactosidase. The allolactose binds to the LacI repressor and forms a complex with reduced binding affinity to the operator. This process results in freeing the operator site(s). Induction of the LacI can also be achieved with a gratuitous inducer such as isopropyl-β-D-thiogalactopyranoside (IPTG), which does not require transformation by β-galactosidase. Instead, it can readily bind to the repressor with a stoichiometry of two IPTG molecules per LacI dimer (6).

Yet, for transcription of the *lac* operon genes to be initiated, the activator cAMP-CRP complex needs to bind to a sequence near the *lac* promoter, thereby enhancing the binding affinity of the RNA polymerase (8). High activator concentrations are brought about by low glucose concentrations. Hence, the *lac* operon genes are expressed only if the glucose concentration is low and simultaneously the lactose concentration is high. Therefore, glucose inhibits lactose metabolism in a dual manner: by reducing cAMP, and thus cAMP-CRP activator concentrations (catabolite repression); and by reducing inducer-allolactose concentrations, since it suppresses the *lac* operon genes (inducer exclusion).

Furthermore, since LacY facilitates lactose import, resulting in repressor inactivation, it follows that initial expression of the *lac* operon genes promotes further expression in an autocatalytic manner due to a positive feedback loop generated by the action of the permease. This positive genetic architecture is the cause of the experimentally observed all-or-none bistable response of the *lac* operon (9).

To reveal the role of the *lac* operon components, several experimental studies have introduced mutations to the promoter region or the coding sequences. Such mutations

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Kinetics of Genetic Switching into the State of Bacterial Competence

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ABSTRACT Nonlinear amplification of gene expression of master regulators is essential for cellular differentiation. Here we investigated determinants that control the kinetics of the genetic switching process from the vegetative state (B-state) to the competent state (K-state) of *Bacillus subtilis*, explicitly including the switching window which controls the probability for competence initiation in a cell population. For individual cells, we found that after initiation of switching, the levels of the master regulator [ComK](t) increased with sigmoid shape and saturation occurred at two distinct levels of [ComK]. We analyzed the switching kinetics into the state with highest [ComK] and found saturation after a switching period of length 1.4 ± 0.3 h. The duration of the switching period was robust against variations in the gene regulatory network of the master regulator, whereas the saturation levels showed large variations between individual isogenic cells. We developed a nonlinear dynamics model, taking into account low-number stochastic effects. The model quantitatively describes the probability and timescale of switching at the single cell level and explains why the ComK level in the K-state is highly sensitive to extrinsic parameter variations. Furthermore, the model predicts a transition from stochastic to deterministic switching at increased production rates of ComK in agreement with experimental data.

INTRODUCTION

Populations of genetically identical cells often maintain a diversity of phenotypes, characterized by different patterns of gene expression. This is usually triggered by stochastic fluctuations, which are amplified by the underlying gene regulatory networks (1–4). The benefits of such non-genotype-derived heterogeneity lie in the enhanced adaptability to environmental changes of the population as a whole (5–8). To analyze phenotypic heterogeneity it is necessary to monitor gene expression in individual cells (9). Real-time kinetics of gene expression have been extensively measured in individual cells to characterize noise in gene expression (10–14), multistability (1,5,15,16), oscillations (16–19), and timing of gene activities (20), but the determinants for genetic switching kinetics are not well characterized so far.

Competence development in *Bacillus subtilis* is one example in which a genetic switch determines cell fate. At low cell density, a homogeneous cell population undergoes exponential growth, but at high cell density (stationary growth phase), the cell population becomes heterogeneous in its phenotype, with a well-defined fraction of 15% expressing genes that code for a strong DNA import machine and recombination proteins (21). These cells are called competent for DNA transformation and they express the master regulator *comK* at high level (22–24). In this study, the state in which *comK* expression levels are high is denoted the K-state. The entry into the K-state is switchlike (25,26). The positive feedback loop in the genetic control circuit is important for the establishment of the competent phenotype in *Bacillus subtilis* (27,28). In noncompetent cells, the positive autoregulatory loop is not activated and *comK* expression is low (B-state). During exponential growth, ComK is kept at a basal level through degradation by the MecA/ClpC/ClpP protease complex, and by transcriptional repressors including Rok, AbrB, and CodY (Fig. 1). Due to a quorum-sensing mechanism, the concentration of ComS (an inhibitor of MecA/ClpC/ClpP) rises with increasing cell density. Work by Maamar et al. (29) revealed that at T_0 (i.e., at the entry into stationary phase, T_x=x h after transition point) the average number of mRNA coding for ComK per cell is of order 1. In this regime, small number fluctuations are, relative to the mean, of paramount importance. This is especially noteworthy since the reaction kinetics of ComK is highly nonlinear. Experiments and simulation have shown that the fraction of cells that switch into the K-state is determined by the magnitude of intrinsic fluctuations in *comK* expression (16,29). The second important determinant of the fraction of cells in the K-state is the length of a switching window in which basal *comK* expression rate is enhanced, which facilitates switching (30). Under conditions in which nutrient concentrations are constantly low, cycles of competence initiation and decay have been observed in real-time experiments in individual cells and a mathematical model described the system as an excitable regulatory circuit (15,16). Escape from the K-state has been attributed to negative feedback between ComK and the inhibitor of ComK proteolysis, ComS. Theoretical models of the competence decision system can be divided into two different categories, by the description of the system as excitable (15,16,31) as opposed to bistable (5,27–29,32).

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Cell

Resource

A Yeast Synthetic Network for In Vivo Assessment of Reverse-Engineering and Modeling Approaches

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SUMMARY

Systems biology approaches are extensively used to model and reverse engineer gene regulatory networks from experimental data. Conversely, synthetic biology allows "de novo" construction of a regulatory network to seed new functions in the cell. At present, the usefulness and predictive ability of modeling and reverse engineering cannot be assessed and compared rigorously. We built in the yeast *Saccharomyces cerevisiae* a synthetic network, IRMA, for in vivo "benchmarking" of reverse-engineering and modeling approaches. The network is composed of five genes regulating each other through a variety of regulatory interactions; it is negligibly affected by endogenous genes, and it is responsive to small molecules. We measured time series and steady-state expression data after multiple perturbations. These data were used to assess state-of-the-art modeling and reverse-engineering techniques. A semiquantitative model was able to capture and predict the behavior of the network. Reverse engineering based on differential equations and Bayesian networks correctly inferred regulatory interactions from the experimental data.

INTRODUCTION

Cellular complexity stems from the interactions among thousands of different molecular species. Thanks to the emerging fields of systems and synthetic biology (Hasty et al., 2002; Hayete et al., 2007; Kaern et al., 2003; Sprinzak and Elowitz, 2005), scientists are beginning to unravel these regulatory, signaling, and metabolic interactions and to understand their coordinated action.

Systems biology aims to develop a formal understanding of biological processes via the development of quantitative mathematical models. A model is a mathematical formalism to describe changes in concentration of each gene transcript and protein in a network, as a function of their regulatory interactions (gene regulatory network).

The usefulness of a model lies in its ability to formalize the knowledge about the biological process at hand, to identify inconsistencies between hypotheses and observations, and to predict the behavior of the biological process in yet untested conditions. There are a variety of mathematical formalisms proposed in literature (Di Ventura et al., 2006; Szallasi et al., 2006) to model biological circuits, with ordinary differential equations being the most common.

Synthetic biology aims to use such models to design unique biological "circuits" (synthetic networks) in the cell able to perform specific tasks (e.g., periodic expression of a gene of interest) or to change a biological process in a desired way (e.g., modify metabolism to produce a specific compound of interest) (Gardner et al., 2000; Khosla and Keasling, 2003; Ro et al., 2006).

Interactions among genes, when unknown, can be identified from gene expression data using reverse-engineering methods. Typically, the data consist of measurements at steady state after multiple perturbations (i.e., gene overexpression, knockdown, or drug treatment) or at multiple time points after one perturbation (i.e., time series data). Successful applications of these approaches have been demonstrated in bacteria, yeast, and, recently, in mammalian systems (Basso et al., 2005; Della Gatta et al., 2008; di Bernardo et al., 2005; Faith et al., 2007; Gardner et al., 2003). A plethora of reverse-engineering approaches is being proposed, and their assessment and evaluation is of critical importance (Stolovitzky et al., 2007). There are three well-established reverse-engineering approaches: ordinary differential equations (ODEs), Bayesian networks, and information theory.

ODEs relate changes in gene transcripts concentration to each other and to an external perturbation. The model consists of a differential equation for each of the genes in the network, describing the transcription rate of the gene as a function of the other genes and of the perturbation. The parameters of the equations have to be inferred from the expression data.

A Bayesian network is a graphical model of probabilistic relationships among a set of random variables, with each

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Resource

Functional Organization of the *S. cerevisiae* Phosphorylation Network

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SUMMARY

Reversible protein phosphorylation is a signaling mechanism involved in all cellular processes. To create a systems view of the signaling apparatus in budding yeast, we generated an epistatic miniarray profile (E-MAP) comprised of 100,000 pairwise, quantitative genetic interactions, including virtually all protein and small-molecule kinases and phosphatases as well as key cellular regulators. Quantitative genetic interaction mapping reveals factors working in compensatory pathways (negative genetic interactions) or those operating in linear pathways (positive genetic interactions). We found an enrichment of positive genetic interactions between kinases, phosphatases, and their substrates. In addition, we assembled a higher-order map from sets of three genes that display strong interactions with one another: triplets enriched for functional connectivity. The resulting network view provides insights into signaling pathway regulation and reveals a link between the cell-cycle kinase, Cak1, the Fus3 MAP kinase, and a pathway that regulates chromatin integrity during transcription by RNA polymerase II.

INTRODUCTION

Phosphate-based signaling is critical to almost all major cellular processes and is ubiquitously present across archea, prokaryota, and eukaryota (Kannan et al., 2007). Systems-wide studies in the post-genome era have provided unprecedented information about the activities of signaling proteins (Johnson and Hunter, 2005), and several thousand sites of protein phosphorylation have been mapped using mass spectrometry (Ficarro et al., 2002; Green and Pflum, 2007; Lee et al., 2006; Matsuoka et al., 2007; Olsen et al., 2006). The knowledge of kinase-substrate relationships has been expanded by both in vitro protein chip analysis (Ptacek et al., 2005) and large-scale genetic screens using a kinase overexpression strategy (Sopko et al., 2006). Bioinformatic efforts have focused on network-level analyses of phosphorylation, providing database resources for phosphorylation sites and signaling pathways (Diella et al., 2008; Lee et al., 2006; Zanzoni et al., 2007). In addition, the integration of context-dependent information (including protein interactions and cell-specific kinase expression) has helped to improve the specificity of phospho-consensus site assignments (Linding et al., 2007).

Despite these achievements, signaling networks have remained difficult to study. While phosphoproteomic datasets illuminate the magnitude and diversity of protein phosphorylation, the functional relevance for the majority of these phosphorylation sites remains unknown (Johnson and Hunter, 2005). Focused studies can elucidate the function of one specific kinase or one particular pathway but often overlook important connections to components that do not directly participate in the pathway. In particular, two hallmarks of kinase signaling are the linear cascades of kinases important for signal amplification and the abundant crosstalk between these pathways in order to coordinate multiple cellular inputs/outputs.

Genetic interactions report on the extent to which the function of one gene depends on the presence of a second gene and can illuminate the functional organization of protein networks. Negative genetic interactions (n; synthetic sick/lethal interactions) describe cases where two mutations in combination cause a stronger growth defect than expected from the two single mutations. In contrast, positive genetic interactions (p) correspond to cases where the double mutant is either no sicker (epistatic) or healthier (suppressive) than the sickest single mutant (Figure 1A). Negative genetic interactions are often found for proteins that work in compensatory pathways, while positive

Cell

A Systematic Survey Identifies Prions and Illuminates Sequence Features of Prionogenic Proteins

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SUMMARY

Prions are proteins that convert between structurally and functionally distinct states, one or more of which is transmissible. In yeast, this ability allows them to act as non-Mendelian elements of phenotypic inheritance. To further our understanding of prion biology, we conducted a bioinformatic proteome-wide survey for prionogenic proteins in *S. cerevisiae*, followed by experimental investigations of 100 prion candidates. We found an unexpected amino acid bias in aggregation-prone candidates and discovered that 19 of these could also form prions. At least one of these prion proteins, Mot3, produces a bona fide prion in its natural context that increases population-level phenotypic heterogeneity. The self-perpetuating states of these proteins present a vast source of heritable phenotypic variation that increases the adaptability of yeast populations to diverse environments.

INTRODUCTION

The prion hypothesis posits that biological information can be replicated solely through self-propagating conformations of proteins. Though it was initially conceived to explain baffling neurodegenerative diseases in mammals (Griffith, 1967; Prusiner, 1982), it has since grown to encompass a number of non-Mendelian traits in fungi (Ross et al., 2005b; Shorter and Lindquist, 2005; Shkundina and Ter-Avanesyan, 2007). All known prions, except for the initially discovered disease-causing prion PrP, are benign, and in some cases can confer selectable advantages (Saupe et al., 2000; True and Lindquist, 2000; True et al., 2004). The self-templating property of prions makes them both conformationally and epigenetically dominant, and positions prion-forming proteins as metastable cellular switches of protein function.

The realization that protein conformational switches could provide a means for inheritance of phenotypes dates back 15 years (Wickner, 1994), yet only a few proteins with this capacity have been discovered (Shorter and Lindquist, 2005; Du et al., 2008). Most of these have been found in the yeast *S. cerevisiae*, with the [*PSI*+] element being the best understood.

[*PSI*+] is caused by an amyloid-like aggregated state of the translation-termination factor Sup35p. In the prion state, the majority of Sup35p molecules are inactive, resulting in increased levels of nonsense suppression (Liebman and Sherman, 1979; Patino et al., 1996) and programmed frameshifting (Namy et al., 2008). This gives rise to RNA stability changes and functionally altered polypeptides and consequently to phenotypes that can be advantageous under diverse conditions (Eaglestone et al., 1999; True et al., 2004). Remarkably, the ability of Sup35p to switch into a prion conformation, and the regulation of that switch by the protein remodeling factor Hsp104p, have been conserved for over 800 million years of fungal evolution (Chernoff et al., 2000; Zenthon et al., 2006).

Three other amyloid-based prions, formed by the functionally diverse proteins Ure2p, Rnq1p, and Swi1p, have been described in *S. cerevisiae*. Ure2p regulates nitrogen catabolism; its prion state, [*URE3*], attenuates this activity resulting in the constitutive utilization of poor nitrogen sources (Aigle and Lacroute, 1975; Wickner, 1994). The Rnq1p protein in its prion state, [*RNQ*+] (also called [*PIN*+]), enhances the inducibility of other prions (Derkatch et al., 2000; Bradley et al., 2002). [*SWI*+], the most recently discovered prion, is caused by an inactive state of the chromatin remodeling factor Swi1p (Du et al., 2008). Intriguingly, [*SWI*+] represents the first established link between chromatin-based and prion-based epigenetics, although a biological relevance of this connection remains to be elucidated. Indeed, for all of these prion proteins, the putative functionality of their prion forms is highly debated (Nakayashiki et al., 2005).

The conformational duality of amyloid-based prions resides in structurally independent prion-forming domains (PrDs) (Edskes et al., 1999; Li and Lindquist, 2000; Santoso et al., 2000; Sondheimer and Lindquist, 2000). These PrDs are modular and can be transferred to other proteins to create novel prions (Li and Lindquist, 2000). They have a very unusual amino acid composition: enriched for polar residues such as glutamine (Q) and asparagine (N) and depleted of hydrophobic and charged residues. This composition promotes a disordered molten-globule-like

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The tail sheath structure of bacteriophage T4: a molecular machine for infecting bacteria

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The contractile tail of bacteriophage T4 is a molecular machine that facilitates very high viral infection efficiency. Its major component is a tail sheath, which contracts during infection to less than half of its initial length. The sheath consists of 138 copies of the tail sheath protein, gene product (gp) 18, which surrounds the central non-contractile tail tube. The contraction of the sheath drives the tail tube through the outer membrane, creating a channel for the viral genome delivery. A crystal structure of about three quarters of gp18 has been determined and was fitted into cryo-electron microscopy reconstructions of the tail sheath before and after contraction. It was shown that during contraction, gp18 subunits slide over each other with no apparent change in their structure.

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Introduction

For many bacteriophages, including T4, one viral particle is sufficient for infecting a single bacterial cell (Goldberg *et al*, 1994). A major reason for such high infection efficiency is a specialized organelle called a tail. It is present in about 96% of all bacteriophages and is designed to attach to bacteria, to penetrate their cell walls and to deliver the viral genome into the host (Ackermann, 2006). There are three groups of tailed bacteriophages based on the tail morphology: *Siphoviridae* (long, non-contractile tails), *Myoviridae* (contractile tails) and *Podoviridae* (short, non-contractile tails). In all three groups of phages, the signal that initiates genome ejection is passed through the tail by virtue of conformational changes. In *Siphoviridae*, these changes occur in the tail tube (Plisson *et al*, 2007), whereas in *Myoviridae*, the signal is transmitted through the tail sheath. In *Podoviridae*, the inner proteins might be ejected from the head to generate a delivery channel (Kemp *et al*, 2005).

Bacteriophage T4 belongs to the *Myoviridae* family. *Myoviridae* phages have the most complex tail structures, generally consisting of a baseplate with tail fibres and a long, non-contractible tube surrounded by a contractile sheath. Bacteriophage T4 has a tail sheath that is composed of 138 copies of gene product (gp) 18 (Leiman *et al*, 2004). The tail tube inside the sheath is estimated to be assembled from as many gp19 subunits as there are gp18 subunits in the sheath (Moody and Makowski, 1981).

During infection, following the attachment of the tail fibres to the host cell, the baseplate changes its conformation from a hexagonal dome-shaped to a planar star-shaped structure, causing the sheath to contract from an initial length of 925 Å to a final length of 420 Å, whereas its diameter increases from 240 to 330 Å (Leiman *et al*, 2004; Kostyuchenko *et al*, 2005). The contraction of the sheath drives the central tube through the outer membrane, creating a channel for DNA ejection from the capsid into the host cell (Leiman *et al*, 2003).

In the absence of the baseplate or the tail tube, the tail sheath protein can self-assemble both *in vivo* and *in vitro* into tubular structures of variable lengths called polysheaths that have the same helical parameters as the contracted tail sheath (Moody, 1967). Furthermore, different gp18 mutants with deletions of about 250 C-terminal amino-acid residues can still assemble into tubular structures, although with different helical parameters (Poglazov *et al*, 1999).

Here, we present a crystal structure of about $\frac{3}{4}$ of the tail sheath protein of bacteriophage T4. The structure is composed of three domains, each having a novel protein fold. To our knowledge, this is the first known atomic structure of a tail sheath protein. Combining the new structural information with the earlier cryo-electron microscopy (cryo-EM) reconstructions and biochemical data, we propose a mechanism for sheath contraction.

Results and discussion

Crystal structure of tail sheath mutants

Wild-type gp18 consists of 659 residues and assembles into tubular polymers of variable lengths, which makes crystallization difficult. Several C-terminal deletion mutants that lack polymerization properties (Efimov *et al*, 2002) were used in extensive crystallization screens. Crystal structures of two gp18 fragments have been determined. One of these is the protease-resistant fragment (gp18PR) and consists of residues 83–365 (Figure 1A), whereas the other deletion mutant (gp18M) consists of residues 1–510 with the last C-terminal residue replaced by a proline (Figure 1B and C) (Efimov *et al*, 2002). The crystal structure of gp18PR fragment was determined with the multiple anomalous dispersion (MAD) method, refined to 1.8 Å resolution, and subsequently used

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REVIEW

Positioning cytokinesis

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Cytokinesis is the terminal step of the cell cycle during which a mother cell divides into daughter cells. Often, the machinery of cytokinesis is positioned in such a way that daughter cells are born roughly equal in size. However, in many specialized cell types or under certain environmental conditions, the cell division machinery is placed at nonmedial positions to produce daughter cells of different sizes and in many cases of different fates. Here we review the different mechanisms that position the division machinery in prokaryotic and eukaryotic cell types. We also describe cytokinesis-positioning mechanisms that are not adequately explained by studies in model organisms and model cell types.

Cytokinesis is the terminal step in the cell cycle when barriers in the form of new membranes (and cell walls in some cases) are generated, so as to divide a mother cell into two daughter cells. Studies of cytokinesis have largely focused on three major questions: (1) What is the machinery that physically divides a cell? (2) How and where in the cell is this machinery positioned? and (3) What regulates the timing of assembly of new membranes (and cell wall) such that the process of cytokinesis does not cause inadvertent damage to the genetic material? Several excellent recent reviews focus on the description of the machinery that various cell types use for cytokinesis as well as the cell cycle regulation of cytokinesis (McCollum and Gould 2001; Guertin et al. 2002; Wang 2005). In this article, we will briefly introduce the cell division machinery but will largely focus on the mechanisms of its positioning.

The cytokinetic machinery

In all cell types, elements of the cytoskeleton assemble into distinct supramolecular assemblies to facilitate cell division (Fig. 1). A summary of molecules participating in cytokinesis in various organisms is provided in Table 1. In animal, yeast, and fungal cells filamentous actin (F-actin), type II myosin, and several other proteins assemble into a structure that resembles a ring or a belt in a plane perpendicular to the axis along which the chromosomes are segregated (Schroeder 1968, 1973; Fujiwara and Pollard 1976; Mabuchi and Okuno 1977; Marks et al. 1986; Balasubramanian et al. 1992; Bi et al. 1998). Constriction of this so-called actomyosin or contractile ring generates the forces necessary for the cleavage of a mother cell into two daughters. Constriction of the ring is precisely coordinated with membrane trafficking events such that the new membranes and cell walls (in fungi) are added in concert with ring constriction. In nearly all bacteria, the tubulin-like protein FtsZ assembles into a ring structure that is perpendicular to the axis of chromosome segregation (Bi and Lutkenhaus 1991). This so-called Z-ring is attached to the overlying plasma membrane via integral membrane proteins (Hale and de Boer 1997; Pichoff and Lutkenhaus 2005). Although molecular motors resembling the canonical eukaryotic microtubule-based motors, such as kinesins and dyneins, have not been discovered in bacteria, the Z-ring serves to recruit proteins important for division septum assembly. Septum assembly then ensues in coordination with constriction of the bacterial Z-ring. Although a contractile actomyosin-based apparatus has not been discovered in plants, microtubules organize into two mirrored bundles that serve to recruit machinery important for assembly of new membranes and cell wall. These dynamic microtubules interact with F-actin filaments to assemble the so-called phragmoplast, a structure essential for cytokinesis in plant cells (Gunning and Wick 1985). In contrast to cytokinesis events in bacteria, yeast, fungi, and animal cells, where membranes and cell wall material are added centripetally, new membrane assembly in plant cells occurs by a process of centrifugal expansion wherein the cell wall expands outward toward the cell cortex at the division site.

Spatial regulation of cytokinesis

In many cell types, prokaryotes and eukaryotes included, cytokinesis results in the formation of equally sized daughter cells. However, in many instances during development (both in single and multicellular organisms), cell division produces daughters of unequal sizes and differing fates. For example, under poor nutritional conditions, the soil bacterium *Bacillus subtilis* divides asymmetrically to produce a small spore, which is capable of withstanding harsher

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The Decay of the Chromosomally Encoded *ccdO157* Toxin–Antitoxin System in the *Escherichia coli* Species

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The origin and the evolution of toxin–antitoxin (TA) systems remain to be uncovered. TA systems are abundant in bacterial chromosomes and are thought to be part of the flexible genome that originates from horizontal gene transfer. To gain insight into TA system evolution, we analyzed the distribution of the chromosomally encoded *ccdO157* system in 395 natural isolates of *Escherichia coli*. It was discovered in the *E. coli* O157:H7 strain in which it constitutes a genomic islet between two core genes (*folA* and *apaH*). Our study revealed that the *folA–apaH* intergenic region is plastic and subject to insertion of foreign DNA. It could be composed (i) of a repetitive extragenic palindromic (REP) sequence, (ii) of the *ccdO157* system or subtle variants of it, (iii) of a large DNA piece that contained a *ccdAO157* antitoxin remnant in association with ORFs of unknown function, or (iv) of a variant of it containing an insertion sequence in the *ccdAO157* remnant. Sequence analysis and functional tests of the *ccdO157* variants revealed that 69% of the variants were composed of an active toxin and antitoxin, 29% were composed of an active antitoxin and an inactive toxin, and in 2% of the cases both ORFs were inactive. Molecular evolution analysis showed that *ccdBO157* is under neutral evolution, suggesting that this system is devoid of any biological role in the *E. coli* species.

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Episodic Selection and the Maintenance of Competence and Natural Transformation in *Bacillus subtilis*

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We present a new hypothesis for the selective pressures responsible for maintaining natural competence and transformation. Our hypothesis is based in part on the observation that in *Bacillus subtilis*, where transformation is widespread, competence is associated with periods of nongrowth in otherwise growing populations. As postulated for the phenomenon of persistence, the short-term fitness cost associated with the production of transiently nongrowing bacteria can be compensated for and the capacity to produce these competent cells can be favored due to episodes where the population encounters conditions that kill dividing bacteria. With the aid of a mathematical model, we demonstrate that under realistic conditions this "episodic selection" for transiently nongrowing (persisting) bacteria can maintain competence for the uptake and expression of exogenous DNA transformation. We also show that these conditions for maintaining competence are dramatically augmented even by rare episodes where selection favors transformants. Using experimental populations of *B. subtilis* and antibiotic-mediated episodic selection, we test and provide support for the validity of the assumptions behind this model and the predictions generated from our analysis of its properties. We discuss the potential generality of episodic selection for the maintenance of competence in other naturally transforming species of bacteria and critically evaluate other hypotheses for the maintenance (and evolution) of competence and their relationship to this hypothesis.

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Optically Modulated Fluorophores for Selective Fluorescence Signal Recovery

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Discriminating weak signals from large backgrounds confounds many fluorescence-based detection,[1−3] dynamics,[4,5] and structural applications.[6,7] Although fluorescence contrast is readily adaptable to both medical and biological imaging, sensitivity in deep tissue and intracellular imaging of low-copy-number proteins presents significant challenges. While great advances have been made,[4,5,8] available fluorophores too often access photostability- and emission-rate-impairing photoinduced dark states, thereby limiting interpretations.[9−11] However, controlled photoswitching to highly energy-recoverable, thermally stable, nonfluorescent isomers has been advantageously employed in the development of high-resolution optical serial localization methods,[6,7,12−14] and in stochastic switching-based optical lock-in detection (OLID) schemes.[15,16] Here we report dynamic photobrightening of fluorophores with photoaccessible but thermally *metastable* dark states that naturally decay on a 30 μs time frame. Under simultaneous near-IR (NIR) illumination, these metastable dark states are rapidly optically depopulated to directly and specifically modulate fluorescence at any externally applied frequency. Simultaneous demodulation of the entire epifluorescence image (i.e., digital signal processing-based lock-in detection for all pixels in parallel) specifically extracts weak-ensemble and even single-molecule fluorescence from high backgrounds.

Most photoswitching occurs through excited-state processes that stochastically trap a nonfluorescent isomer in a thermally stable configuration. Many applications of sequentially applied high-energy secondary excitation that stochastically recovers the original fluorescent state have been developed.[17,18] Unfortunately, the high-energy secondary beam required for switching simultaneously excites other emitters, generating significant additional background fluorescence unless applied when the sample is not being imaged. Additionally, switching is dependent on the laser intensity, forcing a compromise between bleaching and switching time to begin to approach biologically relevant time scales. Conversely, bulk fluorescence enhancement of organic dyes has been achieved via longer-wavelength but much less efficient optically induced reverse intersystem crossing.[19] Although potentially free of secondary-laser-induced additional background, such studies suffer from triplet reactivity, photoinstability, and exceedingly high, biologically incompatible secondary continuous-wave (cw) laser intensities (>1 MW/cm^2).[19] In contrast, single-stranded DNA (ssDNA)-encapsulated few-atom Ag nanodot-based fluorophores have all of the necessary components in place for such signal enhancements with vastly improved photostability and much lower incident intensities.[20−22] When encapsulated in ssDNA, nanoclusters or "nanodots" exhibit extremely bright fluorescence and excellent photophysics, yielding emission that is several-fold brighter and >10-fold more photostable than that obtainable from Cy3 or Cy5 while displaying only a single dark-state residence time of ~30 μs that itself shortens with increased primary excitation intensity.[22] In contrast to the use of alternating illumination with two high-energy wavelengths (relative to that of the detected fluorescence) and an internal standard to measure the demodulation waveform, as performed in OLID schemes, coillumination with an intensity-modulated, long-wavelength secondary laser dynamically photobrightens higher-energy nanodot emission. This secondary laser rapidly depopulates the dark state through transient absorption that regenerates the emissive manifold (see Figure 3S in the Supporting Information). This approach enables direct, noninterfering fluorescence modula-

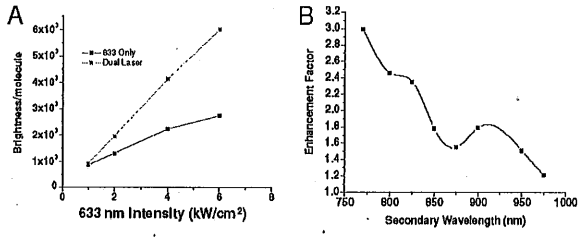

Figure 1. (A) Brightness per Ag nanodot under single-laser (633 nm, black) and dual-laser (633 nm + 805 nm, red) excitation, as determined by fluorescence correlation spectroscopy. Excitation becomes prematurely saturated as the 633 nm excitation intensity increases. Simultaneous 805 nm excitation (8 kW/cm^2) recovers the linearity between excitation and 710 nm emission. (B) Excitation scan of the secondary laser-based enhancement (4 kW/cm^2) relative to single-laser excitation (633 nm, 1.2 kW/cm^2).

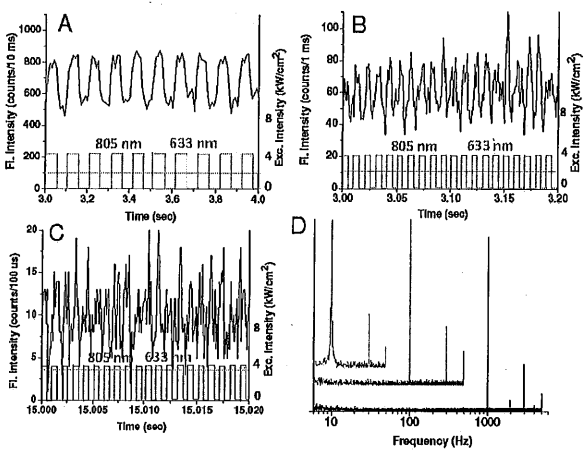

Figure 2. PVA-immobilized Ag nanodot emission collected with an APD in single-photon counting mode with constant 633 nm excitation and 805 nm excitation modulated at (A) 10, (B) 100, and (C) 1000 Hz. (D) Fourier transforms of the emission in (A−C) (red, blue, and green, respectively), each binned an order of magnitude faster than the modulation. The externally imposed 805 nm laser modulation results in the dynamic modulation of the fluorescence from the low-emission state to the high-emission state, with complete control down to short time regimes that is limited only by fluorophore brightness. With 1000 Hz modulation, the sample was cycled ~16 000 times with no decay in the fluorescence signal.

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J|A|C|S COMMUNICATIONS

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Transcription of an Expanded Genetic Alphabet

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The genetic alphabet is constrained by the four natural nucleotides and the two base pairs that they form. An unnatural base pair that is selectively replicated, transcribed, and translated would dramatically expand the information potential of the genetic alphabet.[1−4] This would also increase the potential of the already ubiquitous methodologies based on DNA and RNA and their sequence-specific amplification. Moreover, in vivo expansion of the genetic alphabet would serve as the foundation of a semisynthetic organism with an expanded genetic code.

Toward this goal, we have focused on developing unnatural base pairs formed between predominantly hydrophobic unnatural nucleotides. These unnatural base pairs are stable and selectively replicated by DNA polymerases on the basis of complementary shape and packing interactions rather than complementary hydrogen bonding. Through screening of a library of nucleotide analogues followed by hit optimization, we identified the unnatural base pair formed between d**5SICS** and d**MMO2** (Figure 1),[2a] which is

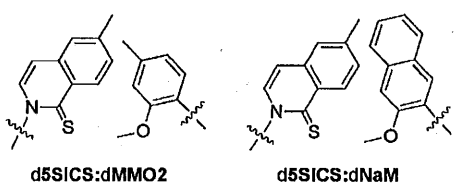

Figure 1. The d**5SICS**:d**MMO2** and d**5SICS**:d**NaM** base pairs. Sugar and phosphate backbone omitted for clarity.

relatively well recognized by a variety of different replicative DNA polymerases.[2b] Further optimization identified d**NaM**, which pairs with d**5SICS** to form an unnatural pair that is replicated with efficiencies and fidelities approaching those of a natural base pair.[2c]

Expansion of the genetic code requires unnatural base pairs that are not only replicable but also transcribed with good efficiency and selectivity in both strand contexts (i.e., d**X** must template **Y**TP insertion and d**Y** must template **X**TP insertion). Previous studies have examined the transcription of unnatural nucleotides bearing nucleobase analogues that pair on the basis of either orthogonal hydrogen bonding[1] or hydrogen bonding and hydrophobicity;[3a,b] however, in none of these cases was transcription of the unnatural base pair shown to be efficient and selective in both possible strand contexts. In addition, it remains unclear whether nucleobase shape and hydrophobicity alone are sufficient for transcription.

To characterize the transcription of the unnatural base pairs formed by d**5SICS** with d**MMO2** and d**NaM**, ribonucleotides and deoxynucleotides were synthesized and converted to the corresponding triphosphates or deoxyphosphoramidites, and the deoxyphorphoramidites were incorporated into DNA templates using automated DNA synthesis (see the Supporting Information). Transcription experiments were conducted with 100 nM DNA substrate, 1× Takara buffer [40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine], DEPC-treated and nuclease-free sterilized water (Fisher), T7 RNA polymerase (50 units), 20 μM each natural NTP, α-^{32}P-ATP (2.5 μCi, MP Biomedicals), and either 5 μM **5SICS**TP, 10 μM **MMO2**TP, or 10 μM **NaM**TP. After incubation for 2 h at 37 °C, the reaction was quenched by the addition of 10 μL of gel loading solution (10 M urea, 0.05% bromophenol blue), and the reaction mixture was loaded onto a 20% polyacrylamide-7 M urea gel, subjected to electrophoresis, and analyzed by phosphorimaging (see the Supporting Information).

We first characterized the ability of d**5SICS** to template the transcription of RNA containing **MMO2** or **NaM** (Figure 2). In

5′-dATAATACGACTCACTATAGGG
3′-dTATTATGCTGAGTGATATCCCTTAGGGCTCXTCAC

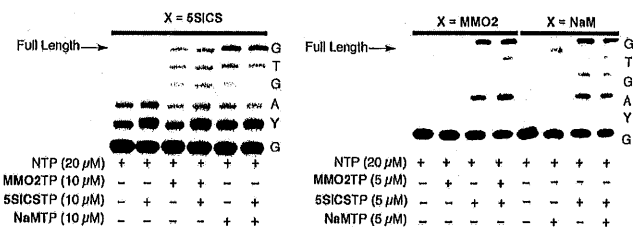

Figure 2. Full-length transcription of DNA containing **5SICS**, **MMO2**, or **NaM**. The template sequence is shown above. **X** and **Y** correspond to the indicated unnatural base in the template and transcript, respectively.

the absence of the unnatural triphosphates, no full length product was observed. Most of the truncated product corresponded to the termination of transcription immediately before d**5SICS** in the template, although a small amount corresponded to termination after mispairing or after single-nucleotide extension of a mispair. In contrast, in the presence of **MMO2**TP, a small amount of full-length transcript was observed, although a significant amount of truncated product remained after 2 h. In the presence **NaM**TP, significantly more full-length product was observed, revealing that d**5SICS** templates the incorporation of **NaM** into RNA more efficiently than it templates the incorporation of **MMO2**. This parallels the behavior observed with DNA polymerases,[2] suggesting that at least some aspects of unnatural base pair recognition are conserved between the two classes of enzymes. In the presence of either unnatural triphosphate, the major truncation products were those corresponding to termination immediately prior to and at the unnatural nucleotide in the template, which suggests that unnatural transcription is limited by both the rate at which the unnatural base pair is synthesized and the rate with which it is extended. This is again similar to what is observed with DNA polymerases.[2] Thus, we tentatively conclude that recognition of the unnatural base pairs with d**5SICS** in the template is similar for DNA and RNA polymerases. Importantly, the addition of **5SICS**TP did not alter the amount of transcript produced, suggesting that the self-pair does not inhibit transcription.

We next characterized transcription of the unnatural base pair in the opposite strand context by examining the ability of d**MMO2**

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Translation Initiation with Initiator tRNA Charged with Exotic Peptides

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The translation system evolved to polymerize 20 specific kinds of proteinogenic L-α-amino acids with extremely high accuracy according to the sequence information encoded in mRNA. Exclusion of nonproteinogenic amino acids from the polymerization is achieved by sophisticated mechanisms involving the multistep selection of correctly charged aminoacyl-tRNAs.[1] Despite the fact that techniques allowing researchers to manipulate the genetic code, so-called genetic code expansion[2] or genetic code reprogramming,[3] have been developed and many successes in incorporating nonproteinogenic L-α-amino acids into nascent peptide chain have been reported, nonproteinogenic amino acids containing more drastically altered structures (referred to as exotic amino acids), such as D- and β-amino acids, are notoriously difficult or often impossible to be elongated.[2a−c,3c,4] On the other hand, recent investigations have shown that the initiation apparatus is more tolerant to exotic amino acids than elongation event. In fact, it has been reported that "nonmethionine" amino acids, for example, various L-α-amino acids[5a] and some D-α-amino acids[5b] with or without *N*-acyl groups, were able to initiate translation, whereas formylmethionine (fMet) is a sole initiator in the ordinary prokaryotic translation system (Figure 1A).[6] The observed tolerance of initiation has prompted

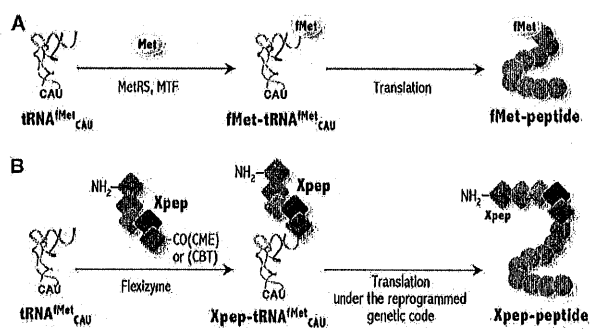

Figure 1. Reprogramming of the initiation event with exotic peptides. (A) Initiation in an ordinary prokaryotic translation system. The initiator tRNA (tRNA$^{fMet}_{CAU}$) is methionylated by methionyl-tRNA synthetase (MetRS) and its α-amino group is formylated by methionyl-tRNA formyltransferase (MTF) to afford fMet-tRNA$^{fMet}_{CAU}$. Initiation with fMet-tRNA$^{fMet}_{CAU}$ yields a peptide containing fMet at the N-terminus (fMet-peptide). (B) Outline of translation initiation with exotic peptides reported here. Flexizyme charges a short exotic peptide (Xpep) onto the tRNA$^{fMet}_{CAU}$. Reprogramming of the initiation event allows us to prime the translation reaction with Xpep-tRNA$^{fMet}_{CAU}$, yielding a peptide bearing several exotic amino acids at the N-terminus (Xpep-peptide).

us to further explore the repertoire expansion. Here we report the reprogramming of translation initiation with "exotic peptides" containing several exotic amino acids (Figure 1B).

To facilitate the reprogramming of initiation event, we utilized our original methodology in the combination of flexizyme and *w*PURE systems. The former system consists of a tRNA aminoacylation ribozyme, referred to as flexizyme, enabling us to charge a wide variety of nonproteinogenic amino acids activated with certain ester or thioester groups onto the 3′ hydroxyl group of any desired tRNAs, including the initiator tRNA$^{fMet}_{CAU}$.[3c] The latter system is based on a reconstituted *E. coli* cell-free translation system, so-called protein-translation using recombinant elements (PURE),[7] in which methionine (Met) is *withdrawn* to make the start codon vacant and thus it is called a *w*PURE system.[5,8] By using the integrated systems, that is, by adding an acylated tRNA$^{fMet}_{CAU}$ prepared by flexizyme to the *w*PURE system, the start codon AUG was reassigned from fMet to desirable non-fMet initiators.[5]

Although the flexizyme system has provided us a nearly unlimited opportunity for the synthesis of tRNA charged with nonproteinogenic amino acids (Xaa-tRNA),[3c,5,8] it is unknown if it can be applicable to tRNA peptidylation. We therefore first verified whether exotic peptides (Xpep) could be charged onto tRNA$^{fMet}_{CAU}$ by means of flexizyme. We designed eleven short peptides containing various combinations of proteinogenic amino acids and exotic amino acids including D-phenylalanine (^{D}Phe), D-glutamic acid (^{D}Glu), D-lysine (^{D}Lys), *N*-methyl-L-phenylalanine (^{Me}Phe), *N*-methyl-L-glutamic acid (^{Me}Glu), 4-aminobenzoic acid (^{4}Abz) and β-alanine (βAla) (the individual structures are shown in Supporting Information, Figure S1). To make the peptides to be accessible to the flexizyme system, those containing the C-terminal ^{L}Phe, ^{D}Phe, ^{Me}Phe, or ^{L}Met were derived to the cyanomethyl esters (CME) (Table 1, entries 1−8, 10, and 11), while one containing the C-terminal ^{L}Gln was derived to 4-cholorobenzyl thioester (CBT)

Table 1. The Sequences of Peptide Initiators Used in This Study and Their Acylation Yields and Translation Efficiencies

entry	initiator sequence[a]	activating group for flexizyme[b]	acylation yield (%)[c]	translation efficiency (%)[d]
1	^{D}Phe−^{L}Phe	CME	85	88
2	^{D}Phe−^{D}Phe−^{L}Phe	CME	44	68
3	^{D}Phe−^{4}Abz−^{L}Phe	CME	30	133
4	^{D}Phe−βAla−^{L}Phe	CME	69	115
5	^{D}Phe−^{Me}Glu−^{L}Phe	CME	47	39
6	^{D}Phe−βAla−^{D}Phe	CME	68	26
7	^{D}Phe−βAla−^{Me}Phe	CME	66	88
8	^{D}Phe−βAla−^{L}Met	CME	48	91
9	^{D}Phe−βAla−^{L}Gln	CBT	38	19
10	^{D}Glu−^{D}Lys−^{L}Phe	CME	55	25
11	^{D}Glu−^{D}Lys−^{D}Glu−^{D}Lys−^{L}Phe	CME	55	12

[a] ^{D}Xaa, ^{L}Xaa, ^{Me}Xaa, ^{4}Abz, and βAla denote D-amino acids, L-amino acids, *N*-methyl-*L*-amino acids, 4-aminobenzoic acid, and β-alanine, respectively. [b] CME and CBT denote cyanomethyl ester and 4-chlorobenzyl thioester activating groups, respectively. [c] Yields of the acylation were calculated based on the band intensity in acid PAGE (see Figure S2 for the detailed description). [d] Relative translation efficiencies of Xpep-peptides were determined by comparing with the band intensity of fMet-peptide in tricine-SDS PAGE (see Figure 2B). The expression quantity of the fMet-peptide was determined to be 7.5 pmol/μL based on the method reported elsewhere.[5a,8b]

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J|A|C|S COMMUNICATIONS

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Covalent Protein Labeling Based on Noncatalytic β-Lactamase and a Designed FRET Substrate

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Fluorescence microscopy is one of the most common techniques employed in the field of life science. With the rapid progress that has been achieved with regard to optical systems, fluorescent proteins (FPs) have acquired important roles for fluorescence microscopy experiments. In order to visualize the localization and behavior of particular proteins of interest, FPs such as green fluorescent protein (GFP) have conventionally been used.[1] More recently, techniques for labeling proteins with small molecules have attracted the attention of many life scientists because they can extend the range of natural FPs, for example, by incorporating near-infrared fluorescent dyes, MRI contrast agents, or biofunctional molecules such as biotin. Several approaches for modifying proteins with small molecules have been commercialized, including methods based on the tetracysteine tag,[2] HaloTag,[3] and SNAP-tag.[4] Other protein labeling methods involving the use of biotin ligase,[5] transglutaminase,[6] hexahistidine,[7] tetra-aspartic acid,[8] etc. have also been reported. Among the abovementioned labeling methods, only the tetracysteine tag exhibits fluorogenic properties. In the other labeling methods, it is necessary to wash the treated cells prior to microscopic measurements to eliminate background fluorescence. Thus, new labeling techniques that satisfy the dual criteria of specificity and fluorogenicity are desirable.

In this paper, we report a specific protein labeling system with an off-on fluorescence switch. It involves covalent modification of a genetically engineered hydrolytic enzyme with a rationally designed fluorogenic probe that exploits the principle of fluorescence resonance energy transfer (FRET). Using this system, we can achieve specific and fluorogenic protein labeling under physiological conditions.

First, we designed the tag protein. Plant or bacterial proteins are preferably used to achieve bioorthogonal labeling in mammalian cells. We focused on β-lactamase as the candidate tag because β-lactamases are small bacterial enzymes that hydrolyze antibiotics containing a β-lactam structure and have no endogenous counterpart among eukaryotic cells.[9] β-Lactamase has been widely used as a reporter enzyme for examining gene expression in living mammalian cells.[10] Class A β-lactamases such as the 29 kDa TEM-1[11] have been extensively investigated with regard to their structures, enzyme reaction kinetics, substrate specificity, inhibitors, etc.[12] The reaction of TEM-1 with β-lactams involves acylation and deacylation steps (Scheme 1). In the acylation step, Ser70 attacks the amide bond of the β-lactam ring to form an intermediate acyl-enzyme complex (ES*). In the deacylation step, an activated water molecule hydrolyzes the ester bond of the intermediate to yield the product. Previous studies have shown that Glu166 is essential for the deacylation step[13] and that the E166N mutant of TEM-1 (^{E166N}TEM) accumulates the acyl-enzyme intermediate by markedly slowing deacylation (k_3) relative to acylation (k_2).[14] We hoped to exploit the properties of the ^{E166N}TEM mutant to covalently attach a fluorescent substrate to β-lactamase.

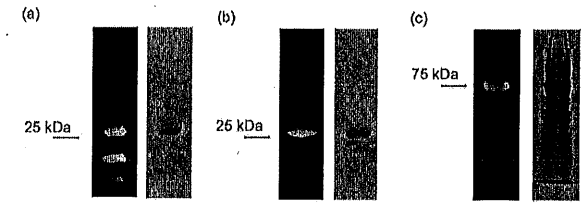
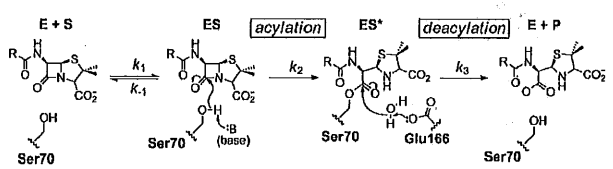
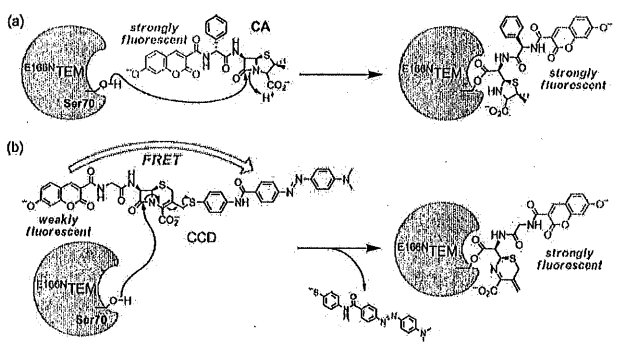


Figure 1. (a, b) Fluorescence (left) and CBB-stained (right) gel images of ^{E166N}TEM after incubation with (a) CA and (b) CCD. (c) Fluorescence and CBB-stained gel image of MBP-^{E166N}TEM mixed with HEK293T cell lysate after incubation with CCD.

Scheme 1. Mechanism of β-Lactam Cleavage by Class A β-Lactamases; (E) Enzyme, (S) Substrate, and (P) Product



Scheme 2. Structures and Labeling Mechanisms of the Fluorescent Probes (a) CA and (b) CCD



To investigate the feasibility of fluorescently labeling ^{E166N}TEM under physiological conditions, we designed and synthesized a penicillin-based fluorescent probe, coumarinyl ampicillin (CA). The labeling scheme is illustrated in Scheme 2a. Since CA contains 7-hydroxycoumarin, successfully labeled ^{E166N}TEM should exhibit cyan fluorescence. ^{E166N}TEM was incubated with CA in 10 mM Tris-HCl buffer (pH 7.0) at 25 °C, and protein labeling was assessed by SDS-PAGE. Fluorescent proteins were detected by irradiating the gels with UV light at 365 nm. When purified ^{E166N}TEM was mixed with CA, a protein band of ~29 kDa was observed that exhibited cyan fluorescence (Figure 1a); Coomassie Brilliant Blue (CBB) staining confirmed that this band corresponded to ^{E166N}TEM. In contrast, when wild-type (WT) TEM-1 was incubated with CA, no cyan fluorescence was seen (Figure S1a). Although CA successfully labels ^{E166N}TEM, other fluorescent bands were also observed on the gel. Since these bands were also seen when only

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The Rcs Two-Component System Regulates Expression of Lysozyme Inhibitors and Is Induced by Exposure to Lysozyme[▽]

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The *Escherichia coli* Rcs regulon is triggered by antibiotic-mediated peptidoglycan stress and encodes two lysozyme inhibitors, Ivy and MliC. We report activation of this pathway by lysozyme and increased lysozyme sensitivity when Rcs induction is genetically blocked. This lysozyme sensitivity could be alleviated by complementation with Ivy and MliC.

In gram-negative bacteria, the cell envelope represents an important functional compartment that extends from the cytoplasmic membrane to the outer membrane and supports a number of essential processes, such as solute transport, protein translocation, and respiratory energy generation (15). In addition, the cell envelope accommodates the bacterial peptidoglycan layer, a distinct and structurally vital element of the cell. Most recently, Laubacher and Ades (10) have demonstrated that the Rcs phosphorelay system of *Escherichia coli*, originally described as *r*egulator of *c*apsule *s*ynthesis, is activated by β-lactam antibiotics that inhibit penicillin-binding proteins and consequently interfere with peptidoglycan synthesis. Moreover, mutational activation of the Rcs pathway provided significant protection against these antibiotics, indicating that members of this regulon can prevent or repair the peptidoglycan damage caused by β-lactam antibiotics (10).

Interestingly, *ivy* and *ydhA*, two genes encoding specific lysozyme inhibitors, were found to reside under this Rcs regulon (8, 10). Ivy (*i*nhibitor of *v*ertebrate lysozyme, formerly known as YkfE) was discovered in 2001 as the first bacterial lysozyme inhibitor (1, 14), while the inhibitory activity of YdhA was only recently revealed by our research group (3). Although Ivy and YdhA are both able to inhibit c-type lysozymes, such as human lysozyme and hen egg white lysozyme (HEWL), they are structurally unrelated (1, 16). Interestingly, YdhA belongs to a group of proteins with a common conserved COG3895 domain that are widely spread among the *Proteobacteria* (3, 16). Unlike Ivy, which resides in the periplasm, YdhA is a lipoprotein and was therefore renamed MliC (*m*embrane-bound *l*ysozyme *i*nhibitor of *c*-type lysozyme) (3).

Given the elementary observation that the two currently known lysozyme inhibitors of *E. coli* are both part of the Rcs regulon that can in turn be induced by antibiotic-mediated peptidoglycan stress, we wondered whether Rcs induction could also result from exposure to lysozyme itself. To test this, we introduced a *tolA* knockout from MG1655 *tolA* (3) into strain DH300 that is equipped with a genomic *rprA-lacZ* fusion able to report Rcs activation (12), in order to increase outer membrane permeability for HEWL (Table 1 lists all strains). A stationary-phase culture of the resulting strain, designated LC100, was diluted 1/100 in 4 ml fresh LB medium with different final concentrations of HEWL (0, 5, 10, 25, and 50 μg/ml), and after 2.5 h of further growth at 37°C, β-galactosidase activity was measured (13). Interestingly, *rprA-lacZ* was significantly induced at HEWL concentrations of >10 μg/ml, up to 4.4-fold at 50 μg/ml (Fig. 1A). This induction could be completely abolished upon the additional introduction of a knockout of *rcsB* (strain LC102), the response regulator required to activate gene expression in the Rcs pathway. Moreover, knocking out *rcsF* (strain LC101), the outer membrane lipoprotein sensor that triggers the Rcs pathway upon antibiotic-mediated peptidoglycan stress (10), also resulted in a loss of lysozyme induction. As a comparison, *rprA-lacZ* induction in DH300 treated with amdinocillin (Sigma-Aldrich, Bornem, Belgium), as previously described (10), resulted in a 16-fold increase in β-galactosidase activity (Fig. 1B). Please note that the difference in basal β-galactosidase levels between LC100 and DH300 (Fig. 1A and B) is probably due to the *tolA* mutation in LC100, which is known to result in a higher basal expression of the Rcs pathway (5). These data clearly demonstrate that the Rcs phosphorelay can indeed be activated by exposure to lysozyme and that this induction is mediated by the outer membrane sensor *rcsF*. This also implies that the Rcs pathway responds to different types of peptidoglycan stress, as β-lactam antibiotics block the formation of peptide side-chain cross-links by binding irreversibly to the transpeptidases, while lysozyme hydrolyzes the heteropolysaccharide backbone.

We subsequently wondered whether an Rcs-compromised mutant would display a higher sensitivity to lysozyme due to its inability to induce lysozyme inhibitor production. In fact, during optimization of the previous experiment, we had already noticed that the RcsB$^-$ and RcsF$^-$ strains (LC102 and LC101) both showed a slight concentration-dependent growth retardation compared to the growth of the Rcs$^+$ strain (LC100) in the presence of HEWL (data not shown). To further investigate this effect of the Rcs pathway on growth inhibition by HEWL, and especially the role of lysozyme inhibitors in this phenotype, the rates of growth of strains LC100, LC101, and LC102 carrying a plasmid that enables arabinose-induced expression of

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DIALOG

The Modern Concept of the Procaryote[▽]

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In their seminal work, Stanier and van Niel (47) proposed that bacteria are distinguished from other forms of life, including viruses, protists, fungi, algae, plants, and animals, by their procaryotic cell structure. They defined the procaryotic cell by three major criteria: the absence of internal membranes that compartmentalize the nuclear material and the enzymatic machinery for respiration and photosynthesis, nuclear division which occurs by fission and not mitosis, and the presence of peptidoglycan in the cell wall. They also recognized that procaryotes possess enormous diversity, similar in scope to that of eucaryotic protists, and further proposed that the procaryotes represent a distinct mode of evolutionary diversification parallel to that found in the eucaryotes (47). Thus, the procaryotic-eucaryotic dichotomy was founded upon the recognition of two very different types of cellular organisms and not the phylogenetic relationships between them.

Recently, five major criticisms of the concept of the procaryote have been proposed (36, 55): it fundamentally contradicts the three-domain model of life, the procaryotes are not monophyletic, the procaryotes are defined by negative characteristics, the term procaryote "sustain[s] the concept that procaryotes evolved into eucaryotes," and the term is imprecise. As shown below, these criticisms are misstatements of the original proposal and modern descriptions of the concept or otherwise erroneous (47, 27). In fact, since its original proposal in 1962, the experimental evidence for the procaryote concept has been enormously enriched.

Today, the concept of the procaryote includes a much greater understanding of the molecular basis for the differences between procaryotic and eucaryotic cells. In addition, it recognizes the antiquity, abundance, and diversity of procaryotes. Procaryotes likely dominated life on the early earth for over a billion years prior to the appearance of eucaryotes. Today, the biomass of the procaryotes is comparable to that of eucaryotes. The procaryotes are also extremely diverse, and representatives of two ancient domains, *Bacteria* and *Archaea*, are common today. Each domain includes organisms with many different metabolic and physiological capabilities, and the number of species is correspondingly so large that it has never been estimated accurately.

The procaryotic cell. The cellular organization of procaryotes is of fundamental importance to their physiological and biochemical processes, and their differences from those of eucaryotes are well described (27). Three features are especially relevant. (i) Nuclear membranes are absent, which allows coupled transcription and translation (13, 33). Because the DNA is not segregated to the nucleus, it is also possible to regulate transcription with repressors and activators that bind metabolites. In this sense, transcriptional regulation is further coupled to metabolism. In the eucaryotes, the major metabolic processes occur in the mitochondria, chloroplast, and the cytoplasm and are isolated from transcription in the nucleus.

(ii) Procaryotic cells are usually smaller than eucaryotic cells. There are some notable exceptions. The sulfur-oxidizing bacterium *Thiomargarita* has a diameter up to 750 μm (44), which is larger than that of many protists. The eucaryotic marine picoalgae, which are 1 to 2 μm in diameter, are similar in size to many procaryotes (41). In spite of this diversity, size remains an important distinguishing characteristic (58). Size establishes the surface-to-volume ratio of the cell, which limits the rate and type of nutrient uptake. It also allows for rapid diffusion of small molecules and proteins throughout the entire cell, which provides a mechanism for coupling metabolism and regulation.

(iii) The cytoplasmic membrane is multifunctional in procaryotes and represents the defining structure of the cell. A proton motive force is generated on the cytoplasmic membrane by respiration, photosynthesis, or ATP hydrolysis to empower key cellular processes such as ATP biosynthesis, NAD^+ reduction by reverse electron transport, nutrient uptake, motility, and secretion. Procaryotes utilize membrane transporters on the cell surface to assimilate nutrients dissolved in their environment. In many procaryotes, the cytoplasmic membrane possesses a complex topology composed of lamellae, tubules, or other cytoplasmic intrusions (27). In contrast, the cytoplasmic membrane of eucaryotes is very different in structure and function. Eucaryotes commonly take up particulate material by phagocytosis, a process that does not occur in procaryotes.

Evolution of procaryotes. Geochemical and fossil evidence indicates that life on earth is at least 3.5 billion years old (1, 42, 43). While the form of ancient microfossils resembles that of modern procaryotes, there is little additional evidence in the fossil record for their molecular nature. However, by 2.5 billion years ago, there is evidence for abundant procaryotic life, including widespread microfossils and stromatolites or fossilized microbial mats, and major signatures of biological processes in the geochemical record, such as depletion of inorganic carbonates for ^{12}C and deposits of complex organic carbon enriched in ^{12}C (42). By this time, the oxygenation of the earth was also well under way (9), and it is likely that oxygenic photosynthesis was fully evolved within the domain *Bacteria*.

Molecular clocks based upon both rRNA and protein-en-

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Contact-Dependent Growth Inhibition Causes Reversible Metabolic Downregulation in *Escherichia coli*[▽]

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Contact-dependent growth inhibition (CDI) is a mechanism identified in *Escherichia coli* by which bacteria expressing two-partner secretion proteins encoded by *cdiA* and *cdiB* bind to BamA in the outer membranes of target cells and inhibit their growth. A third gene in the cluster, *cdiI*, encodes a small protein that is necessary and sufficient to confer immunity to CDI, thereby preventing cells expressing the *cdiBA* genes from inhibiting their own growth. In this study, the *cdiI* gene was placed under *araBAD* promoter control to modulate levels of the immunity protein and thereby induce CDI by removal of arabinose. This CDI autoinhibition system was used for metabolic analyses of a single population of *E. coli* cells undergoing CDI. Contact-inhibited cells showed altered cell morphology, including the presence of filaments. Notably, CDI was reversible, as evidenced by resumption of cell growth and normal cellular morphology following induction of the CdiI immunity protein. Recovery of cells from CDI also required an energy source. Cells undergoing CDI showed a significant, reversible downregulation of metabolic parameters, including aerobic respiration, proton motive force (Δp), and steady-state ATP levels. It is unclear whether the decrease in respiration and/or Δp is directly involved in growth inhibition, but a role for ATP in the CDI mechanism was ruled out using an *atp* mutant. Consistent with the observed decrease in Δp, the phage shock response was induced in cells undergoing CDI but not in recovering cells, based on analysis of levels of *pspA* mRNA.

Intercellular communication mechanisms enable bacteria to coordinate biological phenomena such as DNA uptake, differentiation for fruiting body development, light production, and swarming (7, 8). These cell-to-cell interactions enable individual bacteria to form a multicellular community, such as in a biofilm on a solid surface, under specific environmental conditions (20, 52). Similarly to multicellular organisms, bacteria have signal transduction mechanisms to facilitate cellular cross talk, including two-component regulatory systems and other cell surface ligand-receptor interactions that control cellular processes.

We previously described a cross talk phenomenon designated as contact-dependent growth inhibition (CDI) in which one bacterial isolate (CDI^+) blocks the growth of another bacterium when mixed together (4). CDI requires two contiguous genes, *cdiB* and *cdiA*, which encode proteins that are in the two-partner secretion (TPS) family. Overlapping the stop codon of *cdiA* is a downstream open reading frame designated *cdiI*, which encodes a 79-amino-acid protein that provides immunity to growth inhibition from cells expressing *cdiBA* (4). Evidence strongly indicates that cell-to-cell contact is required for growth inhibition. First, separation of CDI^+ inhibitor cells and target cells by a 0.4-μm nitrocellulose membrane blocked CDI, distinguishing CDI from the class of soluble bacterial growth inhibitors known as bacteriocins. Second, to address the possibility that a very short-lived bacteriocin-like molecule might be released from CDI^+ inhibitor cells, we separated inhibitor-target cell aggregates by fluorescence-activated cell sorting. Target cells within aggregates with CDI^+ inhibitor cells lost viability, as measured by growth on LB medium, more rapidly than did unbound target cells, supporting the conclusion that cell-to-cell contact is required for CDI (4).

In our previous work, analysis of CDI was carried out with a bipartite system using *Escherichia coli* inhibitor cells containing *cdiB*, *-A*, and *-I* on a multicopy plasmid that constitutively expressed CDI activity, cocultured with *E. coli* K-12 target cells. Mixing inhibitor cells with *E. coli* K-12 target cells resulted in a 5- to 6-log decrease in target cell number after only 1 to 2 h (4). Because this bipartite CDI assay contains both inhibitor and target cells, monitoring of target cell growth in real time is not possible. Thus, we have not been able to determine if CDI is a reversible process or a nonreversible toxin-like system. This is important in assessing the role of CDI in the biology of *E. coli* as well as its potential role in many gram-negative bacteria, including uropathogenic *E. coli*, *Burkholderia pseudomallei*, and *Yersinia pestis* that contain genes with significant sequence identity to *cdiB* and *cdiA* (4).

Recently, in collaboration with J. Malinverni and T. Silhavy (Princeton University), we showed that the target cell receptor for CDI is BamA, an essential outer membrane protein (OMP). Homologues of *bamA* are conserved in genomes from bacteria to mitochondria (3). BamA is a key component of the β-barrel assembly machine required for biogenesis of many other OMPs (34, 43, 53). Our results indicated that the BamA receptor facilitates CdiB/CdiA-dependent cell-to-cell binding and growth inhibition since antibodies to BamA blocked formation of inhibitor-target cell aggregates and CDI (3). The ligand for BamA is not known, but it seems probable that it is CdiA, which is at the surface of inhibitor cells (4), and may form a short 40- to 50-nm fiber, such as filamentous hemagglutinin in *Bordetella pertussis*, based on sequence similarities

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Isolation and Characterization of Superdormant Spores of *Bacillus* Species[▽]

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Superdormant spores of *Bacillus subtilis* and *Bacillus megaterium* were isolated in 4 to 12% yields following germination with high nutrient levels that activated one or two germinant receptors. These superdormant spores did not germinate with the initial nutrients or those that stimulated other germinant receptors, and the superdormant spores' defect was not genetic. The superdormant spores did, however, germinate with Ca^{2+}-dipicolinic acid or dodecylamine. Although these superdormant spores did not germinate with high levels of nutrients that activated one or two nutrient germinant receptors, they germinated with nutrient mixtures that activated more receptors, and using high levels of nutrient mixtures activating more germinant receptors decreased superdormant spore yields. The use of moderate nutrient levels to isolate superdormant spores increased their yields; the resultant spores germinated poorly with the initial moderate nutrient concentrations, but they germinated well with high nutrient concentrations. These findings suggest that the levels of superdormant spores in populations depend on the germination conditions used, with fewer superdormant spores isolated when better germination conditions are used. These findings further suggest that superdormant spores require an increased signal for triggering spore germination compared to most spores in populations. One factor determining whether a spore is superdormant is its level of germinant receptors, since spore populations with higher levels of germinant receptors yielded lower levels of superdormant spores. A second important factor may be heat activation of spore populations, since yields of superdormant spores from non-heat-activated spore populations were higher than those from optimally activated spores.

Spores of various *Bacillus* species are formed in sporulation and are metabolically dormant and very resistant to environmental stress factors (21, 37). While such spores can remain in this dormant, resistant state for long periods, they can return to life rapidly through the process of germination, during which the spore's dormancy and extreme resistance are lost (36). Spore germination has long been of intrinsic interest, and continues to attract applied interest, because (i) spores of a number of *Bacillus* species are major agents of food spoilage and food-borne disease and (ii) spores of *Bacillus anthracis* are a major bioterrorism agent. Since spores are much easier to kill after they have germinated, it would be advantageous to trigger germination of spores in foods or the environment and then readily inactivate the much less resistant germinated spores. However, this simple strategy has been largely nullified because germination of spore populations is heterogeneous, with some spores, often called superdormant spores, germinating extremely slowly and potentially coming back to life long after treatments are applied to inactivate germinated spores (8, 9, 16). The concern over superdormant spores in populations also affects decisions such as how long individuals exposed to *B. anthracis* spores should continue to take antibiotics, since spores could remain dormant in an individual for long periods and then germinate and cause disease (3, 11).

In many species, spore germination can be increased by a prior activation step, generally a sublethal heat treatment, although the changes taking place during heat activation are not known (16). Spore germination in *Bacillus* species is normally triggered by nutrients such as glucose, amino acids, or purine ribosides (27, 36). These agents bind to germinant receptors located in the spore's inner membrane that are specific for particular nutrients. In *Bacillus subtilis*, the GerA receptor responds to L-alanine or L-valine, while the GerB and GerK receptors act cooperatively to respond to a mixture of L-asparagine (or L-alanine), D-glucose, D-fructose and K^+ ions (AGFK [or Ala-GFK]) (1, 27, 36). There are even more functional germinant receptors in *Bacillus megaterium* spores, and these respond to D-glucose, L-proline, L-leucine, L-valine, or even salts, such as KBr (6). Glucose appears to trigger germination of *B. megaterium* spores through either of two germinant receptors, GerU or GerVB, while L-proline triggers germination through only the GerVB receptor, and KBr germination is greatly decreased by the loss of either GerU or GerVB (6). Nutrient binding to the germinant receptors triggers the release of small molecules from the spore core, most notably the huge depot (~10% of spore dry weight) of pyridine–2,6-dicarboxylic acid (dipicolinic acid [DPA]) present in spores predominantly as a 1:1 diluted chelate with Ca^{2+} (Ca-DPA) (35, 36). Ca-DPA release then triggers the activation of one of two redundant cortex lytic enzymes (CLEs) that degrade the spore's peptidoglycan cortex, and cortex degradation completes spore germination and allows progression into outgrowth and then vegetative growth (27, 33, 36).

Spore germination can also be triggered by nonnutrient agents, including Ca-DPA and cationic surfactants (27, 33, 36). With *B. subtilis* spores, Ca-DPA triggers germination by acti-

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The Conserved Sporulation Protein YneE inhibits DNA replication in *Bacillus subtilis*

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Cells of *Bacillus subtilis* triggered to sporulate under conditions of rapid growth undergo a marked decrease in chromosome copy number, which was partially relieved by a mutation in the sporulation-induced gene *yneE*. Cells engineered to express *yneE* during growth were impaired in viability and produced anucleate cells. We conclude that YneE is an inhibitor of DNA replication.

The ability of bacteria to adapt to a variety of growth conditions demands mechanisms for controlling DNA replication and coordinating chromosome duplication with the cell cycle. Bacteria initiate one and only one round of DNA replication at the origin of chromosomal DNA replication, *oriC*, for each cycle of cell division (1). During slow growth the single copy of *oriC* is duplicated to create two copies of *oriC*. In contrast, under conditions of rapid growth, when the time required to duplicate a chromosome is longer than the generation time, bacteria inherit chromosomes undergoing active replication with multiple replication forks. In this case, replication initiation occurs synchronously across all origins, leading to multifork replication with 2^n (n=1,2,3) copies of *oriC* present in each cell (15). Multifork replication ensures that each newly divided cell will receive at least one chromosome. The transition from one growth condition to another, such as when nutrients become limiting and cells enter stationary phase, requires mechanisms to adjust the frequency of replication initiation (10). One noteworthy but poorly understood example of replication control is exhibited by *Bacillus subtilis* during the entry into sporulation. Sporulating cells complete a final round of replication before undergoing a process of asymmetric division that results in a forespore and a mother cell (11, 14). Each of these progeny cells ordinarily inherits a single chromosome, and mature spores, which are derived from the forespore, are known to only contain one chromosome (9, 14).

Cells normally enter the pathway to sporulate in response to nutrient limitation. We have previously shown, however, that artificial induction of synthesis of the kinase KinA causes efficient sporulation even during growth in rich medium (7). KinA transfers phosphate groups into a multi-component phosphorelay that phosphorylates the response regulator Spo0A, the master regulator for entry into sporulation (2). The discovery that rapidly growing cells can be triggered to sporulate with high efficiency implies that *B. subtilis* must have an active mechanism to shut down DNA replication, given that rapidly growing cells contain multiple replication forks. An appealing hypothesis for how such a mechanism might work stems from the observation that the *B. subtilis* origin of

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The Inhibitory Mechanism of Protein Synthesis by YoeB, an *Escherichia coli* Toxin*

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YoeB is a toxin encoded by the *yefM-yoeB* antitoxin-toxin operon in the *Escherichia coli* genome. Here we show that YoeB, a highly potent protein synthesis inhibitor, specifically blocks translation initiation. In *in vivo* primer extension experiments using two different mRNAs, a major band was detected after YoeB induction at three bases downstream of the initiation codon at 2.5 min. An identical band was also detected in *in vitro* toeprinting experiments after the addition of YoeB to the reaction mixtures containing 70 S ribosomes and the same mRNAs, even in the absence of $tRNA_f^{Met}$. Notably, this band was not detected in the presence of YoeB alone, indicating that YoeB by itself does not have endoribonuclease activity under the conditions used. The 70 S ribosomes increased upon YoeB induction, and YoeB was found to be specifically associated with 50 S subunits. Using tetracycline and hygromycin B, we demonstrated that YoeB binds to the 50 S ribosomal subunit in 70 S ribosomes and interacts with the A site leading to mRNA cleavage at this site. As a result, the 3'-end portion of the mRNA was released from ribosomes, and translation initiation was effectively inhibited. These results demonstrate that YoeB primarily inhibits translation initiation.

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Minireview

Unstable Nucleotide Repeat Minireview Series: A Molecular Biography of Unstable Repeat Disorders*

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Expansion of an unstable nucleotide repeat is a mutational mechanism that is apparently unique to humans and is known to cause a variety of neurological disorders. This collection of minireviews examines several of these unstable repeats, focusing on those where there is considerable molecular information on how the mutation alters function.

* This minireview will be reprinted in the 2009 Minireview Compendium, which will be available in January, 2010.

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The multinomial simulation algorithm for discrete stochastic simulation of reaction-diffusion systems

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The Inhomogeneous Stochastic Simulation Algorithm (ISSA) is a variant of the stochastic simulation algorithm in which the spatially inhomogeneous volume of the system is divided into homogeneous subvolumes, and the chemical reactions in those subvolumes are augmented by diffusive transfers of molecules between adjacent subvolumes. The ISSA can be prohibitively slow when the system is such that diffusive transfers occur much more frequently than chemical reactions. In this paper we present the Multinomial Simulation Algorithm (MSA), which is designed to, on the one hand, outperform the ISSA when diffusive transfer events outnumber reaction events, and on the other, to handle small reactant populations with greater accuracy than deterministic-stochastic hybrid algorithms. The MSA treats reactions in the usual ISSA fashion, but uses appropriately conditioned binomial random variables for representing the net numbers of molecules diffusing from any given subvolume to a neighbor within a prescribed distance. Simulation results illustrate the benefits of the algorithm. © *2009 American Institute of Physics.* [DOI: 10.1063/1.3074302]

I. INTRODUCTION

The idea of treating a spatially inhomogeneous chemically reacting system as a collection of smaller interacting subsystems has appeared in literature since the 1970s under a few names, notably the "Reaction Diffusion Master Equation"[1] (RDME) and the "Multivariate Master Equation."[2] The theory was explored by Nicolis and Prigogine.[2] The reaction-diffusion approach has been confirmed against results obtained by direct simulation Monte Carlo[3] and reactive hard sphere molecular dynamics.[4] Widespread awareness of the multivariate master equation approach was achieved through its inclusion in the classic texts of Gardiner[5] and Van Kampen.[6]

At this mesoscopic level of description, a system consists of a list of molecular species, and reactions which couple them as reactants or products. The system state, $\boldsymbol{x}$, is given by the number of molecules of each species. It evolves from the initial condition through the firing of reactions, whose stochastic rates are known as *propensity functions*. The forward Kolmogorov equation governing the flow of probability from one state to another in time is called the *Master Equation*.

The Stochastic Simulation Algorithm[7,8] (SSA) is the technique commonly used to sample the *Chemical Master Equation* (CME), which governs the evolution of *homogeneous*, or well-stirred, systems. There exist several implementations of the exact SSA, for example, the direct method, the first reaction method,[8] and the next reaction method.[9] Much effort has gone into developing approximations to the exact SSA, e.g., τ-leaping[10] and the Slow Scale SSA.[11]

In the *inhomogeneous* setting, a system is divided into subvolumes, each of which is assumed to be homogeneous. Reactions occur in each subvolume as in the homogeneous case, and the populations in neighboring subvolumes are coupled by diffusive transfers, treated as unimolecular reactions.[1,12,13] The probability of the system being in any given state at any time is then given by the MME[2,3] or RDME.[1] We call the SSA as applied to the inhomogeneous setting the "Inhomogeneous SSA" (ISSA). Analogously to the SSA, the ISSA can also be implemented in different ways. An implementation based on the Next Reaction Method was used by Isaacson and Peskin,[14] the Next Subvolume Method was developed by Elf and co-workers,[15,16] and the null process technique was developed by Hanusse and Blanche.[17] However, even optimized versions of the ISSA can be prohibitively slow for some systems and, in particular, in the presence of fast diffusion.

In this paper we present the Multinomial Simulation Algorithm (MSA), which is designed to outperform the ISSA in just this type of scenario: when diffusive transfers greatly outnumber reaction events. The MSA is a stochastic-stochastic hybrid method which is based on separating chemical reactions, which are treated in the usual SSA way, from diffusive transfers, which are treated by an approximate stochastic process. The MSA computes the *net* diffusive transfer from each subvolume to its neighbors in a given time step. In this sense it is similar to the τ-leaping method, but with some important differences. In τ-leaping, each reaction channel which consumes a given species fires independently of the other channels consuming that species, so it is possible that the sum of the molecules of a species removed by all channels which consume it will be greater than the number of molecules that were present in the beginning

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An exact accelerated stochastic simulation algorithm

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An exact method for stochastic simulation of chemical reaction networks, which accelerates the stochastic simulation algorithm (SSA), is proposed. The present "ER-leap" algorithm is derived from analytic upper and lower bounds on the multireaction probabilities sampled by SSA, together with rejection sampling and an adaptive multiplicity for reactions. The algorithm is tested on a number of well-quantified reaction networks and is found experimentally to be very accurate on test problems including a chaotic reaction network. At the same time ER-leap offers a substantial speedup over SSA with a simulation time proportional to the 2/3 power of the number of reaction events in a Galton–Watson process. © *2009 American Institute of Physics.*
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I. INTRODUCTION

The stochastic simulation algorithm[1] (SSA) is a widely used method for simulating the stochastic dynamics of chemical reaction networks. SSA executes every reaction event and provides an accurate view of the system dynamics, albeit at a significant computational cost over the corresponding mass-action differential equations that approximate the mean numbers of each molecular species. A number of algorithms have been proposed for the acceleration of the SSA at the expense of its accuracy. The *τ-leaping* algorithm[2] and its recent variants[3–5] simulate leaps over several reaction events during a preselected time increment. Further developments include multiscale SSAs such as "nested stochastic simulation,"[6] the multiscale methods,[7,8] and the "slow-scale stochastic simulation" algorithm.[9] Another acceleration method[10] uses rejection sampling to achieve constant time scaling with the number of reaction channels; this differs from the present work which uses rejection sampling to improve scaling with respect to the number of reaction events.

A related work is the *R-leaping* algorithm[11] which proposes the simulation of preselected numbers of reaction firings that occur over time intervals sampled from an Erlang distribution. An essential aspect of these approximate methods is the requirement that the changes to the reaction rate or "propensity" functions are small during each step.

We present a SSA which, similar to R-leap, accelerates SSA by executing multiple reactions per algorithmic step, but which samples the reactant trajectories from the same probability distribution as the SSA. This "exact R-leap" or "ER-leap" algorithm is a modification of the R-leap algorithm which is both exact and capable of substantial speedup over SSA. The simplest versions of both τ-leap and R-leap have difficulties with the potential of producing negative numbers of reactants, which can be fixed by modifications such as binomial tau-leap[3] and modified tau-leap.[4] Since ER-leap is exact, it intrinsically avoids this potential pitfall; stochastic moves to negative reactant states have zero probability and will be rejected. We demonstrate by computational experiments that ER-leap can execute in time sublinear in the number of reaction events to be simulated, while remaining exact. The algorithm is based on the rejection sampling concept, using efficiently computable bounds on the SSA probability distribution.

The paper is organized as follows. In Sec. II we derive upper and lower bounds on the SSA reaction probabilities after multiple reactions, expressed using matrix notation for Markov processes, and use rejection sampling to derive the ER-leap algorithm. The algorithm itself is stated, analyzed for cost, and illustrated in Sec. II E. In Sec. III we report on a series of numerical experiments designed to evaluate the accuracy and speedup of the ER-leap algorithm. In Sec. IV we discuss the results and conclude with an assessment of the method in the context of related works and an outline of directions for future work.

II. THEORY

This section is organized as follows. Sections II A–II C introduce the required notations, reaction probabilities, and bounds on these probabilities, respectively. The ER-leap algorithm's key update equations are derived from these probability bounds in the calculations of Sec. II D. The resulting algorithm is assembled from the key update equations, analyzed for cost, and illustrated in the case of a simple reaction network in Sec. II E.

We consider a set of reactions, indexed by r, among chemical species C_a, indexed by a,

$$\{m_a^r C_a\} \rightarrow \{m_a'^r C_a\}, \quad \text{with reaction rate } \rho_r. \tag{1}$$

Here $\boldsymbol{m}^r=[m_a^r]$ and $\boldsymbol{m}'^r=[m_a'^r]$ are the input and output stoichiometries of the reaction r. In the following we derive an expression for the probability of states after a number of such reaction events.

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Dynamics of the interlocked positive feedback loops explaining the robust epigenetic switching in *Candida albicans*

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ABSTRACT

The two element mutual activation and inhibitory positive feedback loops are a common motifs that occur in many biological systems in both isolated and interlocked form, as for example, in the cell division cycle and thymus differentiation in eukaryotes. The properties of three element interlocked positive feedback loops that embeds both mutual activation and inhibition are studied in depth for their bistable properties by performing bifurcation and stochastic simulations. Codimension one and two bifurcations reveal important properties like robustness to parameter variations and adaptability under various conditions by its ability to fine tune the threshold to a wide range of values and to maintain a wide bistable regime. Furthermore, we show that in the interlocked circuit, mutual inhibition *controls* the decision to switch from OFF to ON state, while mutual activation *enforces* the decision. This view is supported through a concrete biological example *Candida albicans*, a human fungal pathogen that can exist in two distinctive cell types; one in the default white state and the other in an opaque form. Stochastic switching between these two forms takes place due to the epigenetic alternation induced by the transcriptional regulators in the circuit, albeit without any rearrangement of the nuclear chromosomes. The transcriptional regulators constitute interlocked mutual activation and inhibition feedback circuits that provide adaptable threshold and wide bistable regime. These positive feedback loops are shown to be responsible for robust noise induced transitions without chattering, persistence of particular phenotypes for many generations and selective exhibition of one particular form of phenotype when mutated. Finally, we propose for synthetic biology constructs to use interlocked positive feedback loops instead of two element positive feedback loops because they are better controlled than isolated mutual activation and mutual inhibition feedback circuits.

1. Introduction

In biological systems, positive feedback loops are an ubiquitous motif that plays a strong dominant role in converting graded signals into binary one (Brandman et al., 2005), acts as a buffer for propagating noise while at the same time increasing the sensitivity of the system (Hornung and Barkai, 2007), exhibits ultrasensitivity (Ferrell and Machleder, 1998), is involved in the amplification of signals (Freeman, 2000) and memory generation (Hayer and Bhalla, 2005), induces phenotypic diversity (Smits et al., 2006) and genetic competence (Maamar and Dubnau, 2005), and most importantly is a necessary condition for multistability (Thomas, 1981; Fages and Soliman, 2008; Soule, 2003). Bistability is the dynamical property observed in many biological systems that toggle between two stable steady states separated by an intermediate unstable steady state when appropriate parameters of the system are changed.

This transition, and multistability in general, play a significant role in epigenetic modifications and inheritance (Keller, 1994; Thomas, 1998). Bistable systems can be reversible (hysteretic) or irreversible, which provides threshold response and memory. These properties are observed in many experimental systems (see Ferrell, 2002, for a review) and have a tremendous implication in robustness to parameter variations and fluctuations due to both internal and external noise. Even though the role of positive feedback loop motifs in gene regulatory networks has been extensively studied both from experimental and modeling point of views (Ferrell and Xiong, 2001; Becksei et al., 2001), their study continues to attract a lot of interest to the systems biology community (Kim et al., 2006, 2007).

Feedback loops are classified based on the parity of positive and negative interactions among the dynamical elements. If the number of negative interactions are even, the feedback loop is positive. Two interesting positive feedback loops that are often encountered in both prokaryotic and eukaryotic biological systems (Fig. 1a, b, c, and d) are mutual inhibition (MI) and activation (MA) and predominantly their interlocked form (MAMI, see Fig. 1e). MI is also called double negative feedback loop (Fig. 1g) and MA

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Long run coexistence in the chemostat with multiple species ☆

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ABSTRACT

In this work we analyze the transient behavior of the dynamics of multiple species competing in a chemostat for a single resource, presenting slow/fast characteristics. We prove that coexistence among a subset of species, with growth functions close to each other, can last for a substantially long time. For these cases, we also show that the proportion of non-dominant species can be increasing before decreasing, under certain conditions on the initial distribution.

1. Introduction

A popular concept in microbial ecology is the competitive exclusion principle (CEP) which expresses the fact that when two or more microbial species grow on the same substrate in a chemostat, at most one species, i.e. the species that has the best affinity with the limiting substrate, will eventually survive. This concept has been first introduced by Hardin (1960) and has been widely mathematically studied in the literature since (e.g. Aris and Humphrey, 1977; Stephanopoulos et al., 1979; Armstrong and McGehee, 1980; Butler and Wolkowicz, 1985). However coexistence of multiple species in chemostat is largely encountered in practical situations. Many efforts have been done to emphasize mathematically such coexistence behavior, either via periodic inputs (e.g. Smith, 1981; Butler et al., 1985) or via model rewriting (e.g. Cenens et al., 2000 that considers the filamentous backbone theory to emphasize the coexistence of flocks and filaments, or Lobry et al., 2004; Lobry and Harmand, 2006; Lobry et al., 2006 where the specific growth rate models are also dependent on the biomass, via in particular ratio dependence).

One should have in mind that the CEP characterizes an asymptotic property of the system, but does not provide any information on the transient dynamics, that has not yet been thoroughly investigated, to our knowledge. In the present paper, we propose to study the transient dynamics of multiple species growing on the same substrate, depending on the initial species distribution. When some of the species have close growth functions, on may observe a *practical* coexistence in the following sense: even if the species with best affinity will finally be the only surviving one, the transient stage before the other species have almost disappeared may eventually be substantially long. It appears that the different species may coexist for a long time before the competitive exclusion practically applies. More precisely, some of species may be first increasing (before finally decreasing) depending on the initial distribution.

The motivation of considering many species with close growth functions comes from the observations made by recent molecular approaches. In microbial ecosystems, thousands of species are present whereas the number of functions is limited (Curtis and Sloan, 2004; Pace, 1997). Moreover, the structural instability of microbial communities shows that same function can be carried out by several different species (Zumstein et al., 2000). It is also well known that constant mutation rates lead to occurring new individuals with different traits and with different but close growth functions, that can be consider as new species from the modeling point of view. In chemostat-like systems, the main function under consideration is usually the degradation of a given substrate, which is measured by the growth functions of each species. But only about 1% of the overall micro-organisms observed in real ecosystems can be isolated and cultivated in laboratory (Amann et al., 1995). Thus micro-organisms whose growth functions can be clearly identified represent only a tip of the iceberg and it is most probable that among a huge number of species, many should have growth functions close to each other.

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Plastic–PDMS bonding for high pressure hydrolytically stable active microfluidics

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We explore the application of organofunctional silanes for bonding plastic substrates to PDMS membranes. Such devices would enable actuated membrane microfluidics in plastic devices. Bond strength degradation in aqueous environments can be reduced by using bis-silanes with larger alkoxy end groups to promote organofunctional bond formation with the plastic substrate. Hydrolytic failure can also result from low silane crosslink density or interface hydrophilicity. A test device consisting of three-valve peristaltic pumps is fabricated out of polycarbonate (PC) and bonded to PDMS through isopropoxy modified bis-trimethoxy-silyl-propyl-amine. Valves operated up to 60 psi in aqueous environments without failure. Solutions of DI water and 1 M HCl were also pumped through the device *via* peristaltic actuation at 18 psi for 2 weeks without bond failure. 1 M NaOH was also tested but resulted in bond failure after 115 hours.

Introduction

PDMS has greatly reduced the entrance barrier for research in microfluidics based chemistry and biology. The introduction of the elastic microvalve has led to the creation of highly integrated systems capable of automated experimentation, with examples such as whole blood PCR analysis,[1] microbial cell culture,[2,3] protein crystallization,[4] and multicellular manipulation and analysis,[5] and particle production.[6] However, for actuated microfluidics to transition from customized prototype devices to industrial scale device production, a transition must be made from elastomers to plastics. Plastics can be manufactured using mass fabrication technologies such as injection molding and hot embossing with well established bonding processes.[7] Plastics are also more dimensionally stable, rigid, and chemically resistant.[8]

Plastics will provide many benefits for microfluidic devices not offered by PDMS. Rigidity enables a variety of reliable external interface options, such as manifold integration, direct barbed tubing connections, and gasket connectors. Additionally, integrating flexible membranes into rigid plastics will enable a variety of new devices currently not possible in PDMS due to chip elasticity such as large area or high pressure membrane deformation, on-chip pressure regulators, full volume pumps, and reliable square channel membrane valve particle filters.

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Microfluidic means of achieving attomolar detection limits with molecular beacon probes†

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We used inline, micro-evaporators to concentrate and transport DNA targets to a nanoliter single molecule fluorescence detection chamber for subsequent molecular beacon probe hybridization and analysis. This use of solvent removal as a unique means of target transport in a microanalytical platform led to a greater than 5000-fold concentration enhancement and detection limits that pushed below the femtomolar barrier commonly reported using confocal fluorescence detection. This simple microliter-to-nanoliter interconnect for single molecule counting analysis resolved several common limitations, including the need for excessive fluorescent probe concentrations at low target levels and inefficiencies in direct handling of highly dilute biological samples. In this report, the hundreds of bacteria-specific DNA molecules contained in ~25 microliters of a 50 aM sample were shuttled to a four nanoliter detection chamber through micro-evaporation. Here, the previously undetectable targets were enhanced to the pM regime and underwent probe hybridization and highly-efficient fluorescent event analysis *via* microfluidic recirculation through the confocal detection volume. This use of microfluidics in a single molecule detection (SMD) platform delivered unmatched sensitivity and introduced compliment technologies that may serve to bring SMD to more widespread use in replacing conventional methodologies for detecting rare target biomolecules in both research and clinical labs.

1 Introduction

The development of microanalytical systems for biosensing is driven by advances in microfluidic control technologies for handling nano- to picoliter sample volumes.[1–3] However, the use of small sample volumes in these platforms also requires highly sensitive analyte detection schemes and it is the development and

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Water-ice chip with liquid-core waveguide functionality. Toward lab on ice†

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A liquid-core waveguide is fabricated with water-ice, which has lower refractive index than most of the solvents, as cladding, and provides a possibility as an ice chip for flow analyses.

Liquid-core waveguide (LCW) provides various options in separation and spectroscopic measurements. Enhanced sensitivity based on a long optical path is a typical example of analytical use.[1–3] Also, whole capillary imaging in electrophoretic systems has been enabled in various fashions; *i.e.* axial irradiation with radial detection and radial irradiation with axial detection for fluorescence modes and axial irradiation with axial absorption detection.[4–7] In addition, Raman spectroscopic measurements have been attempted with the LCW, which allows versatile optical alignments.[8] These methodological advancements strongly rely on the development of an efficient cladding material, which should have a smooth surface and can guide the light over a long distance without the significant loss of intensity. Liquid cladding has also been devised and has proved efficient for probing a liquid/liquid interface.[9] Although this method may open a new facet of analytical chemistry, solid cladding is much easier to deal with in various applications of the LCW. The refractive index of the clad should be smaller than that of a core to propagate the incident light effectively. The refractive indexes of solid materials (*e.g.* n_D = 1.458, 1.517, 1.51 and 2.418 for silica glass, optical glass, polyethylene, and diamond) are generally larger than those of the usual solvent (*e.g.* n_D = 1.333, 1.362, and 1.375 for water, ethanol, and hexane).[10] Thus, common solid materials cannot be used as cladding for LCW. The invention of Teflon AF-2400 has allowed the exploitation of the LCW with the usual solvents including water as a core; actually, most of the current LCW devices are fabricated with this material. Various types of Teflon AF-2400 capillaries are now commercially available and have allowed the application of the LCW to a number of analytical methods. However, this material adsorbs various substances on the surface and gets damaged even by weak mechanical contact; these severely reduce light guiding efficiency. A high cost as well as these disadvantages of Teflon AF-2400 has restricted the extensive utilization of the LCW.

Water-ice can be a potential alternative to Teflon AF-2400 because of its relatively low refractive index (*e.g.* n_D = 1.309).[11] Ice is ubiquitous, environmentally friendly, and readily available and utilizable anytime

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Microfluidics for cryopreservation†

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Minimizing cell damage throughout the cryopreservation process is critical to enhance the overall outcome. Osmotic shock sustained during the loading and unloading of cryoprotectants (CPAs) is a major source of cell damage during the cryopreservation process. We introduce a microfluidic approach to minimize osmotic shock to cells during cryopreservation. This approach allows us to control the loading and unloading of CPAs in microfluidic channels using diffusion and laminar flow. We provide a theoretical explanation of how the microfluidic approach minimizes osmotic shock in comparison to conventional cryopreservation protocols *via* cell membrane transport modeling. Finally, we show that biological experiments are consistent with the proposed mathematical model. The results indicate that our novel microfluidic-based approach improves post-thaw cell survivability by up to 25% on average over conventional cryopreservation protocols. The method developed in this study provides a platform to cryopreserve cells with higher viability, functionality, and minimal inter-technician variability. This method introduces microfluidic technologies to the field of biopreservation, opening the door to future advancements at the interface of these fields.

Introduction

Over the past decade, microfluidic systems have led to significant breakthroughs in a variety of biological applications such as point-of-care diagnostics, bioterrorism detection, and drug discovery through an interdisciplinary approach.[1–7] In particular, microfluidic technology represents a new tool to manipulate biological samples such as blood, living cells, DNA, proteins, and small molecules by exploiting the dynamic properties of microscale transport phenomena.[8–14] Microfluidic technologies can impact the field of biopreservation as well.[13,15–18] For instance, microfluidic channels have recently been used to isolate healthy sperm cells.[17] Also, innovative approaches were introduced to biopreserve oocytes in quartz capillary channels[18] and to eject cell-encapsulating cryoprotectant (CPA) droplets directly into liquid nitrogen for cryopreservation.[13] Several advances in biopreservation such as the use of electron microscopy grids, cryoloops, and nylon loops enable the cryopreservation of germ cells in particular at increasing heat transfer rates.[16,19,20]

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Nucleation and solidification in static arrays of monodisperse drops†

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The precise measurement of nucleation and non-equilibrium solidification are vital to fields as diverse as atmospheric science, food processing, cryopreservation and metallurgy. The emulsion technique, where the phase under study is partitioned into many droplets suspended within an immiscible continuous phase, is a powerful method for uncovering rates of nucleation and dynamics of phase changes as it isolates nucleation events to single droplets. However, averaging the behavior of many drops in a bulk emulsion leads to the loss of any drop-specific information, and drop polydispersity clouds the analysis. Here we adapt a microfluidic technique for trapping monodisperse drops in planar arrays to characterize solidification of highly supercooled aqueous solutions of glycerol. This system measured rates of nucleation between 10^{-5} and 10^{-2} pL^{-1} s^{-1}, yielded an ice-water interfacial energy of 33.4 mJ m^{-2} between −38 and −35 °C, and enabled the specific dynamics of solidification to be observed for over a hundred drops in parallel without any loss of specificity. In addition to the physical insights gained, the ability to observe the time and temperature of nucleation and subsequent growth of the solid phase in static arrays of uniform drops provides a powerful tool to discover thermodynamic protocols that generate desirable crystal structures.

Introduction

Although solidification from the melt is favorable at temperatures below the equilibrium melting point (T_m), some super-cooling typically occurs before a stable solid nucleus forms. This is because the positive Gibbs free energy associated with the solid–liquid interface inhibits solidification until a critical number of liquid molecules have arranged stochastically into the correct packing of the solid phase and then gain an additional molecule. From the point of nucleation onwards, the negative Gibbs free energy of phase change drives the system towards the equilibrium solid state. The rate of homogeneous nucleation (J, in m^{-3} s^{-1}) is defined as the average number of stable solid nuclei that are produced during an increment of time and within a defined volume of the super-cooled fluid, without the aid of surface- or impurity-mediated (heterogeneous) nucleation.

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Microwave dielectric heating of drops in microfluidic devices

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We present a technique to locally and rapidly heat water drops in microfluidic devices with microwave dielectric heating. Water absorbs microwave power more efficiently than polymers, glass, and oils due to its permanent molecular dipole moment that has large dielectric loss at GHz frequencies. The relevant heat capacity of the system is a single thermally isolated picolitre-scale drop of water, enabling very fast thermal cycling. We demonstrate microwave dielectric heating in a microfluidic device that integrates a flow-focusing drop maker, drop splitters, and metal electrodes to locally deliver microwave power from an inexpensive, commercially available 3.0 GHz source and amplifier. The temperature change of the drops is measured by observing the temperature dependent fluorescence intensity of cadmium selenide nanocrystals suspended in the water drops. We demonstrate characteristic heating times as short as 15 ms to steady-state temperature changes as large as 30 °C above the base temperature of the microfluidic device. Many common biological and chemical applications require rapid and local control of temperature and can benefit from this new technique.

Introduction

The miniaturization of the handling of liquid and biological samples has enabled great advances in fields such as drug discovery, genetic sequencing and synthesis, cell sorting, single cell gene expression studies, and low-cost portable medicine.[1–7] At the forefront of this technology are the micro-fabricated pipes, valves, pumps, and mixers of microfluidics that are leading to integrated lab-on-a-chip devices. These integrated microfluidic devices are causing a paradigm shift in fluid handling that is analogous to what integrated circuit technology did for electronics half of a century ago.[6] A growing library of elements for lab-on-a-chip systems have been developed in recent years for tasks such as the mixing of reagents, detecting and counting of cells, sorting cells, genetic analysis, and protein detection.[1–7] There is one function, however,

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Nanomechanical Properties of Dead or Alive Single-Patterned Bacteria

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The main goal of this paper is to probe mechanical properties of living and dead bacteria via atomic force microscopy (AFM) indentation experimentations. Nevertheless, the prerequisite for bioAFM study is the adhesion of the biological sample on a surface. Although AFM has now been used in microbiology for 20 years, the immobilization of micro-organisms is still challenging. Immobilizing a single cell, without the need for chemical fixation has therefore constituted our second purpose. Highly ordered arrays of single living bacteria were generated over the millimeter scale by selective adsorption of bacteria onto micrometric chemical patterns. The chemically engineered template surfaces were prepared with a microcontact printing process, and different functionalizations of the patterns by incubation were investigated. Thanks to this original immobilization strategy, the Young moduli of the same cell were measured using force spectroscopy before and after heating (45 °C, 20 min). The cells with a damaged membrane (after heating) present a Young modulus twice as high as that of healthy bacteria.

Introduction

A major concern in bacteriology is to determine whether a bacterium is dead or alive. Especially when cells are starved, or have been subjected to an oxidative stress[1] (e.g., in tap water networks), it is very difficult to make the difference between a cell that is unable to grow and a dead cell. Several techniques were developed to overcome this problem, such as methods based on the ability of a fluorophore to penetrate the bacterial cell wall. Nevertheless, the question of viability is still under debate, and we present, in this paper a new way to probe *Escherichia coli* viability, using an atomic force microscope (AFM) conducted in the force spectroscopy mode. Thanks to the AFM, operated in liquid, it is now possible to explore living cells at the nansoscale. Nanoindentation experiments have been applied on human platelets,[2] living macrophages,[3] and red blood cells[4] as well as on diatoms[5] and different bacteria or fungi.[6−11] In 2007, Cross et al.[12] showed that mechanical analysis can distinguish cancerous cells from normal ones, even when the cells show similar shape. They found a Young modulus of 1.97 ± 0.70 kPa on benign mesothelial cells, but of only 0.53 ± 0.10 kPa for tumor cells. As far as *E. coli* is concerned, Eaton et al.[13] have recently tested the impact of chitosans (an antibacterial compound) on the elasticity of the cell wall. As the preparation of the AFM sample included a drying step, they found Young moduli in the range of 180−220 MPa. In our study, the cells are never allowed to dry, and the force spectroscopy experiments are made in phosphate-buffered saline (PBS) buffer to avoid any electrostatic interactions that can occur in low ionic strength buffers.[14] Nevertheless, their nanoindentation experiments revealed mechanical changes in the bacterial cell wall induced by the treatment. In another paper, Volle et al.[15] worked on the elasticity of *E. coli* as it is predated by *Bdellovibrio bacteriovorus*. They show that the invaded cells are softer (spring constant: 0.064 N/m) than the healthy cells (0.23 N/m). To measure the spring constant, one has to focus on the end of the indentation curve, on high loading forces. The behavior of the cell at high loading forces is known to be caused by osmotic pressure.[16,17] As a consequence the modification observed by Volle et al. is essentially due to a dramatic decrease of the cell turgor pressure during the predation process, and not only to the cell wall modification. However, those three studies clearly demonstrate that mechanical properties of cells, which can be probed by force spectroscopy (cell wall elasticity or cell turgor pressure), are specific for a condition (eg.: benign−cancerous, antibacterial or not, predated or not). On this basis we have decided to explore the modifications that could occur in the nanomechanical properties of a single *E. coli* bacterium, while it is alive and while it is dead. To reach this goal, it has been of first importance to immobilize the living bacteria in an aqueous environment to avoid any cell wall modifications due to a drying step. In the literature, this problem has been overcome by creating positively charged surfaces where negatively charged micro-organisms can be electrostatically immobilized.[18] Another solution, very convenient for round shape cells, is to use an Isopore polycarbonate membrane.[19] The

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MicroReview

A chance at survival: gene expression noise and phenotypic diversification strategies

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Summary

Phenotypic diversification plays a central role in evolution and provides species with a capacity to survive environmental adversity. The profound impact of random molecular events on the shaping of life is well accepted in the context of chance mutations and genetic drift; however, the evolution of the regulatory networks encoding microorganismal stress response and survival strategies might also have been significantly influenced by gene expression noise. This likelihood has inspired numerous investigations to characterize the sources of phenotypic diversity within isogenic populations, and to explore their direct and potential biological implications. Here, we discuss different scenarios where gene expression noise might bestow a selective advantage under stress, highlighting a potentially fundamental role of stochastic mechanisms in the evolution of microbial survival strategies.

Introduction

The term 'gene expression noise' is typically used in broad reference to observed variation in protein content among seemingly identical cells experiencing the same environment. This variation can be divided into intrinsic and extrinsic components (Elowitz *et al.*, 2002; Swain *et al.*, 2002; Raser and O'Shea, 2004; Kaern *et al.*, 2005; Raser and O'Shea, 2005; Pedraza and van Oudenaarden, 2005; Rosenfeld *et al.*, 2005; Volfson *et al.*, 2006; Maheshri and O'Shea, 2007). Extrinsic gene expression noise arises from variations in cell-specific factors that can be physiological and global in nature, such as the metabolic state of the cell, cell-cycle phase and cell age, or specific to certain subsets of genes, such as the variability in upstream signal transduction. Intrinsic gene expression noise refers to variation that arises from 'finite-number' molecular-level fluctuations inherent to reaction kinetics in the nanomolar range during the expression of individual alleles.

While many molecular-level details remain to be uncovered, two mechanisms – transcriptional and translational bursting – are important sources of intrinsic gene expression noise that have been directly linked to DNA-encoded parameters. In the translational bursting model, variability in protein content is a direct consequence of finite-number fluctuations in mRNA abundance. When the average protein content is kept fixed, inefficient translation of highly abundant mRNA results in steady protein production while efficient translation of low-abundance mRNA results in large random bursts of protein synthesis. In the transcriptional bursting model, slow reaction kinetics cause infrequent transitions between active and inactive promoter states, which, in turn, cause multiple mRNA templates to be synthesized in rapid succession at irregular intervals. Noise generated by both of these mechanisms has been shown to depend on DNA-encoded parameters. Specifically, ribosome binding site mutations (Ozbudak *et al.*, 2002) and codon usages (Blake *et al.*, 2003) modulating translational efficiency, as well as promoter mutations modulating the stability of active promoter states (Raser, 2004; Blake *et al.*, 2006) directly impact cell–cell variability.

The direct evidence that genome sequence contributes to cell–cell variability indicates that gene expression noise, like other genome-encoded traits, is inheritable and evolvable; subject to selective pressures during the course of evolution. Indeed, the identification in yeast of quantitative trait loci influencing stochastic cell–cell variability supports the concept that gene expression noise is a heritable genetic trait (Ansel *et al.*, 2008). Moreover, the notion that gene expression noise has undergone significant evolutionary drifts is supported by differences in

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Complex topology rather than complex membership is a determinant of protein dosage sensitivity

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The 'balance hypothesis' predicts that non-stoichiometric variations in concentrations of proteins participating in complexes should be deleterious. As a corollary, heterozygous deletions and overexpression of protein complex members should have measurable fitness effects. However, genome-wide studies of heterozygous deletions in *Saccharomyces cerevisiae* and overexpression have been unable to unambiguously relate complex membership to dosage sensitivity. We test the hypothesis that it is not complex membership alone but rather the topology of interactions within a complex that is a predictor of dosage sensitivity. We develop a model that uses the law of mass action to consider how complex formation might be affected by varying protein concentrations given a protein's topological positioning within the complex. Although we find little evidence for combinatorial inhibition of complex formation playing a major role in overexpression phenotypes, consistent with previous results, we show significant correlations between predicted sensitivity of complex formation to protein concentrations and both heterozygous deletion fitness and protein abundance noise levels. Our model suggests a mechanism for dosage sensitivity and provides testable predictions for the effect of alterations in protein abundance noise.
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Subject Categories: bioinformatics; proteins
Keywords: balance hypothesis; dosage sensitivity; heterozygous deletion; protein abundance noise; protein interaction networks

Introduction

Essentially all biological processes involve proteins frequently acting as multi-component complexes (Eisenberg *et al*, 2000; Vidal, 2005; Gavin *et al*, 2006; Krogan *et al*, 2006). However, it remains a challenge to characterize how quantitative interaction parameters, such as rates, affinities and protein concentrations, affect function at the cellular and organismal levels (Kuriyan and Eisenberg, 2007). The balance hypothesis posits that an imbalance in the relative concentrations of proteins involved in a protein complex can disrupt complex formation and should thus be deleterious. As a corollary, it has been suggested that proteins involved in complexes should be more likely to be dosage sensitive than other proteins (Papp *et al*, 2003).

Several means exist by which stoichiometric imbalances could disrupt complex formation and lead to adverse phenotypic effects: first, reducing the abundance of a component of a protein complex, as might occur through a heterozygous deletion mutation, would be predicted to have a measurable effect on fitness. Accordingly, it has been shown that a twofold reduction in the amount of a component protein can result in a many fold reduction in complex formation, and thus have an amplified effect on cell phenotype (Veitia, 2002, 2003). A second, somewhat less intuitive mechanism is referred to as the pro-zone effect or combinatorial inhibition (CI) (Bray and Lay, 1997; Burack and Shaw, 2000; Ferrell, 2000; Levchenko *et al*, 2000). CI can occur when a stoichiometric excess of one component of a protein complex is added to a solution containing only moderate amounts of the other components. If the component in excess satisfies certain topological conditions in its interaction with other components from the complex, and particularly if it acts as a bridge between two separate parts of the complex, then this excess will typically inhibit the formation of the full complex, by instead favoring the formation of many incomplete subspecies

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Force- and kinesin-8-dependent effects in the spatial regulation of fission yeast microtubule dynamics

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Microtubules (MTs) are central to the organisation of the eukaryotic intracellular space and are involved in the control of cell morphology. For these purposes, MT polymerisation dynamics are tightly regulated. Using automated image analysis software, we investigate the spatial dependence of MT dynamics in interphase fission yeast cells with unprecedented statistical accuracy. We find that MT catastrophe frequencies (switches from polymerisation to depolymerisation) strongly depend on intracellular position. We provide evidence that compressive forces generated by MTs growing against the cell pole locally reduce MT growth velocities and enhance catastrophe frequencies. Furthermore, we find evidence for an MT length-dependent increase in the catastrophe frequency that is mediated by kinesin-8 proteins (Klp5/6). Given the intrinsic susceptibility of MT dynamics to compressive forces and the widespread importance of kinesin-8 proteins, we propose that similar spatial regulation of MT dynamics plays a role in other cell types as well. In addition, our systematic and quantitative data should provide valuable input for (mathematical) models of MT organisation in living cells.
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Subject Categories: simulation and data analysis; cell and tissue architecture
Keywords: catastrophes; fission yeast; forces; kinesin-8; microtubules

Introduction

Microtubules (MTs) are dynamic protein polymers that change their length by switching between growing and shrinking states in a process termed 'dynamic instability' (Mitchison and Kirschner, 1984; Desai and Mitchison, 1997). It is important to understand how dynamic instability is regulated, because this affects MT length (Verde *et al*, 1992; Dogterom and Leibler, 1993) and intracellular organisation (Kirschner and Mitchison, 1986; Hayles and Nurse, 2001), as well as the ability of MTs to exert pushing and pulling forces (Inoue and Salmon, 1995; Dogterom *et al*, 2005). Several proteins have been characterised that globally affect MT dynamics and catastrophe rates (Howard and Hyman, 2007), but it is a largely open question how such regulation is achieved locally, in response to spatially varying biochemical cues and/or mechanical effects induced by the shape and size of cells. In fact, in addition to global regulation, it has been reported that catastrophe frequencies can be locally enhanced, for example close to the periphery of animal cells (Komarova *et al*, 2002; Mimori-Kiyosue *et al*, 2005). There are several ways by which such a local catastrophe enhancement could be accomplished: (activity) gradients of destabilising MT-associated proteins (Niethammer *et al*, 2004) and/or MT length-dependent mechanisms (Dogterom *et al*, 1996) would lead to a gradual catastrophe enhancement when approaching the cell boundary. On the other hand, force-induced effects that may result from growing MTs pushing against the cell boundary (Dogterom and Yurke, 1997; Janson *et al*, 2003; Janson and Dogterom, 2004) would lead to an abrupt catastrophe enhancement once contact with the cell boundary has been made. A similar abrupt change would be expected in case there is an MT-destabilising factor associated locally with the membrane (Mimori-Kiyosue *et al*, 2005). To distinguish between long- and short-range mechanisms requires quantitative and spatially resolved measurements of the catastrophe frequency inside living cells. In mammalian cells, this is often a challenging task because MT networks are quite dense and reference points, such as the cell boundary in interphase or chromosomes in mitosis are dynamic structures themselves.

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REPORT

Force- and length-dependent catastrophe activities explain interphase microtubule organization in fission yeast

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The cytoskeleton is essential for the maintenance of cell morphology in eukaryotes. In fission yeast, for example, polarized growth sites are organized by actin, whereas microtubules (MTs) acting upstream control where growth occurs. Growth is limited to the cell poles when MTs undergo catastrophes there and not elsewhere on the cortex. Here, we report that the modulation of MT dynamics by forces as observed *in vitro* can quantitatively explain the localization of MT catastrophes in *Schizosaccharomyces pombe*. However, we found that it is necessary to add length-dependent catastrophe rates to make the model fully consistent with other previously measured traits of MTs. We explain the measured statistical distribution of MT–cortex contact times and re-examine the curling behavior of MTs in unbranched straight *tea1*Δ cells. Importantly, the model demonstrates that MTs together with associated proteins such as depolymerizing kinesins are, in principle, sufficient to mark the cell poles.
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Subject Categories: cell and tissue architecture
Keywords: cell; cytoskeleton; force; mechanics; simulations

The fission yeast *Schizosaccharomyces pombe* is a convenient model to study cell morphogenesis (Hayles and Nurse, 2001). Wild-type cells are simple elongated rods growing at the cell poles and dividing in the middle. Yet, previous studies have outlined an interesting interplay between shape, growth and cytoskeletal organization. The first component is the rigid cell wall surrounding yeast cells that maintains cell shape independently of the cytoskeleton. Second, the actin cytoskeleton is essential for cell growth and cell wall remodeling (La Carbona *et al*, 2006). Lastly, although microtubules (MTs) are not required for growth *per se*, they control the location of growth sites by depositing specific marker proteins (Mata and Nurse, 1997; Brunner and Nurse, 2000; Sawin and Snaith, 2004). Abnormal deposition, occurring for example in mutants where MTs are shorter, results in cells that are either bent or branched (Sawin and Nurse, 1998; Snaith and Sawin, 2005). MTs also position the nucleus (Tran *et al*, 2001; Loiodice *et al*, 2005) and thus define the site of cytokinesis (Daga and Chang, 2005; Tolic-Norrelykke *et al*, 2005) and the partitioning of the cell into daughter cells. Hence, by controlling cell growth and division, MTs impact the evolution of shape in the cell lineage. As MTs are constrained within the cell, the converse is also true with MT organization being dependent on cell shape. For the rigid *S. pombe* cells, the two processes occur on very different timescales; with MT lifetimes being in the order of minutes, whereas cells typically double in size after 3 h. Consequently, individual MTs are enclosed in a boundary that is effectively constant during their lifetime. This means that it is valid to first study how MTs depend on cell shape, and to later include cell shape changes. We use here computer simulation for the first step, calculating the dynamic spatial organization of MTs within a fixed cell shape. This approach complements other efforts where cell morphogenesis is modeled with reaction–diffusion equations (Csikasz-Nagy *et al*, 2008) by focusing on the MT cytoskeleton.

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Temporal switching and cell-to-cell variability in Ca^{2+} release activity in mammalian cells

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Genetically identical cells in a uniform external environment can exhibit different phenotypes, which are often masked by conventional measurements that average over cell populations. Although most studies on this topic have used microorganisms, differentiated mammalian cells have rarely been explored. Here, we report that only approximately 40% of clonal human embryonic kidney 293 cells respond with an intracellular Ca^{2+} increase when ryanodine receptor Ca^{2+} release channels in the endoplasmic reticulum are maximally activated by caffeine. On the other hand, the expression levels of ryanodine receptor showed a unimodal distribution. We showed that the difference in the caffeine sensitivity depends on a critical balance between Ca^{2+} release and Ca^{2+} uptake activities, which is amplified by the regenerative nature of the Ca^{2+} release mechanism. Furthermore, individual cells switched between the caffeine-sensitive and caffeine-insensitive states with an average transition time of approximately 65 h, suggestive of temporal fluctuation in endogenous protein expression levels associated with caffeine response. These results suggest the significance of regenerative mechanisms that amplify protein expression noise and induce cell-to-cell phenotypic variation in mammalian cells.
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Subject Categories: simulation and data analysis; signal transduction
Keywords: Ca^{2+}-induced Ca^{2+} release; cell-to-cell phenotypic variability; mathematical modelling; positive feedback

Introduction

Biochemical processes in cells inevitably fluctuate owing in part to the stochastic nature of gene expression systems, which typically involve small numbers of molecules such as DNA, mRNA and proteins (Kaern *et al*, 2005; Kaufmann and van Oudenaarden, 2007; Pedraza and Paulsson, 2008). In addition to such 'intrinsic noise', the internal states of cells and the structure of the signalling pathway also contribute to the fluctuation in the concentration of molecules, collectively termed 'extrinsic noise' (Hooshangi *et al*, 2005; Pedraza and van Oudenaarden, 2005; Rosenfeld *et al*, 2005; Shahrezaei *et al*, 2008). In some cases, intracellular noise in individual cells is filtered so that the system as a whole is precisely regulated, as is observed in the segmentation of a *Drosophila melanogaster* embryo (Houchmandzadeh *et al*, 2002; Gregor *et al*, 2007) and in circadian rhythms (Forger and Peskin, 2005; Gonze and Goldbeter, 2006). Yet in other cases, intracellular noise can be exploited to play roles such as in the amplification of signals, the divergence of cell fates and the diversification of phenotypes, as seen in the lysis/lysogeny decision circuit of the bacteriophage lambda (Arkin *et al*, 1998; Skupin *et al*, 2008). In support of this idea, several recent reports suggested that individual clonal cells in the same external environment can exhibit qualitatively different phenotypes, which may confer a selective advantage in adapting to changing external environments (Rao *et al*, 2002; Raser and O'Shea, 2005; Acar *et al*, 2008). Such phenomena question the implicit assumption behind cell-population-wide experiments that genetically identical cells are phenotypically identical (Levsky and Singer, 2003) and highlight the need for measuring individual cells.

Thus far, most studies of intracellular noise have focused on unicellular organisms such as *Escherichia coli* or *Saccharomyces cerevisiae*. Earlier studies explored the origin of intracellular noise using artificial gene circuits (Elowitz *et al*, 2002; Ozbudak *et al*, 2002; Blake *et al*, 2003; Raser and O'Shea, 2004), whereas recent studies showed various phenotypic diversities in naturally arising biological systems (Samadani *et al*, 2006; Di Talia *et al*, 2007; Maamar *et al*, 2007; Nachman

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Quantifying the Integration of Quorum-Sensing Signals with Single-Cell Resolution

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Cell-to-cell communication in bacteria is a process known as quorum sensing that relies on the production, detection, and response to the extracellular accumulation of signaling molecules called autoinducers. Often, bacteria use multiple autoinducers to obtain information about the vicinal cell density. However, how cells integrate and interpret the information contained within multiple autoinducers remains a mystery. Using single-cell fluorescence microscopy, we quantified the signaling responses to and analyzed the integration of multiple autoinducers by the model quorum-sensing bacterium *Vibrio harveyi*. Our results revealed that signals from two distinct autoinducers, AI-1 and AI-2, are combined strictly additively in a shared phosphorelay pathway, with each autoinducer contributing nearly equally to the total response. We found a coherent response across the population with little cell-to-cell variation, indicating that the entire population of cells can reliably distinguish several distinct conditions of external autoinducer concentration. We speculate that the use of multiple autoinducers allows a growing population of cells to synchronize gene expression during a series of distinct developmental stages.

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Introduction

In a process called quorum sensing, bacteria communicate with one another using extracellular signaling molecules called autoinducers. Quorum sensing allows groups of bacteria to track their cell numbers, synchronize gene expression on a population-wide scale, and thereby carry out collective activities. In quorum sensing, bacteria produce, release, and detect autoinducers that accumulate in a cell-density–dependent manner, and, thus, autoinducer concentration serves as a proxy for cell number. Quorum-sensing systems are widespread in the bacterial world, existing in both Gram-negative and Gram-positive bacteria, and quorum sensing is used to control such diverse functions as bioluminescence, virulence-factor secretion, biofilm formation, conjugation, and antibiotic production [1–3].

Typically, Gram-negative bacteria use acyl-homoserine lactones and Gram-positive bacteria use peptides as autoinducers. To our knowledge, these two kinds of molecules most often promote intraspecies cell–cell communication, because a particular acyl-homoserine lactone or particular peptide can be detected only by the bacterial species that produces it [2]. In addition, a non–species-specific autoinducer called AI-2, which is a family of interconverting molecules all derived from the same precursor 4,5-dihydroxy 2,3-pentanedione, is produced and detected by a large variety of both Gram-negative and Gram-positive bacteria [4,5]. Interestingly, many bacterial species use more than a single autoinducer molecule for quorum sensing. For example, Gram-negative bacteria (e.g., *Rhizobium*) can use multiple homoserine lactones and likewise, Gram-positive bacteria (e.g., *Bacillus*) can use several peptides for communication [2,6]. These bacteria have evolved sophisticated quorum-sensing circuits to detect and integrate the information contained in multiple autoinducers.

It remains a mystery how and why bacteria integrate multiple autoinducer signals and what additional information multiple autoinducers reveal about the cells' environment that one autoinducer cannot reveal [7]. Furthermore, while in principle, quorum sensing enables bacteria to act in synchrony, the behavior of the entire population is ultimately dictated by events inside single cells. Recent single-cell studies of gene expression in bacteria have revealed that noise is inevitable even for isogenic cells in essentially homogeneous environments, and that noise can result in heterogeneous phenotypes within a population [8–14]. Likewise, in quorum sensing, noise could make individual cells behave differently from one another even if they receive identical autoinducer inputs. To understand quorum-sensing signal integration and, ultimately, the evolution of cooperative behaviors at the population level, it is imperative to understand how cells behave individually. Specifically, do cells respond in unison or do they maintain population diversity? Bulk measurements—which focus on the population's response—generally mask the behavior of individual cells and thus lose information about cell-to-cell variation. To fully understand the molecular mechanism underlying quorum sensing as well as the general principles underlying bacterial communication and cooperation, we must study this process at the single-cell level.

To begin to explore the above questions, we investigated the network of the model quorum-sensing bacterium *Vibrio harveyi*, the first bacterium shown to use more than one autoinducer for quorum sensing [15,16]. *V. harveyi* has a

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Abbreviations: AI, autoinducer; GFP, green fluorescent protein; sRNA, small RNA

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Transcriptional Infidelity Promotes Heritable Phenotypic Change in a Bistable Gene Network

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Bistable epigenetic switches are fundamental for cell fate determination in unicellular and multicellular organisms. Regulatory proteins associated with bistable switches are often present in low numbers and subject to molecular noise. It is becoming clear that noise in gene expression can influence cell fate. Although the origins and consequences of noise have been studied, the stochastic and transient nature of RNA errors during transcription has not been considered in the origin or modeling of noise nor has the capacity for such transient errors in information transfer to generate heritable phenotypic change been discussed. We used a classic bistable memory module to monitor and capture transient RNA errors: the *lac* operon of *Escherichia coli* comprises an autocatalytic positive feedback loop producing a heritable all-or-none epigenetic switch that is sensitive to molecular noise. Using single-cell analysis, we show that the frequency of epigenetic switching from one expression state to the other is increased when the fidelity of RNA transcription is decreased due to error-prone RNA polymerases or to the absence of auxiliary RNA fidelity factors GreA and GreB (functional analogues of eukaryotic TFIIS). Therefore, transcription infidelity contributes to molecular noise and can effect heritable phenotypic change in genetically identical cells in the same environment. Whereas DNA errors allow genetic space to be explored, RNA errors may allow epigenetic or expression space to be sampled. Thus, RNA infidelity should also be considered in the heritable origin of altered or aberrant cell behaviour.

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Introduction

Altered proteins can result from errors incurred at any step during information transfer from DNA to protein. Errors in DNA, RNA, and protein synthesis occur at rates of, very roughly, 10^{-9}, 10^{-5}, and 10^{-4} errors per residue, respectively [1]. Although rare, errors in DNA synthesis can be fixed as permanent errors—mutations—which can generate heritable change in cellular phenotype. Transcription and translation errors occur more frequently, but are considered transient and their effects fleeting, since the altered molecules are present for a limited time. It has been shown that transcription over a damaged DNA template can generate altered proteins in nondividing DNA repair-deficient cells [2], and it has been suggested that transient errors can produce transient mutators, thereby generating phenotypic change by introducing mutations [3,4]. However, the capacity for transient errors to generate heritable epigenetic phenotypic change has not been considered.

The stochastic nature of gene expression results in random fluctuations in protein numbers per cell [5,6]. Theoretical and experimental studies have culminated in stochastic chemical kinetic models that describe the statistics of molecular noise [7–9]. Many aspects of gene expression have been considered, including rates of transcription and translation and rates of destruction of the corresponding mRNA and protein products. These models address protein quantity; the quality of the protein produced is not considered with transcription and translation deemed error-free processes. However, due to RNA transcription errors, approximately 1% of all mRNAs encoding polypeptides of 300 amino acids will encode erroneous messages [3]. It has been shown in bacteria, yeast, and mammalian cells that gene expression, and the accompanying noise, occurs in stochastic bursts dominated by the production of mRNAs [10–12]. Since one mRNA is translated many times, RNA errors become amplified, challenging the cell with erroneous proteins that may exhibit partial function, loss-of-function, gain-of-function, or dominant-negative properties. Therefore, any cell at any time may be transiently impaired for a function encoded in a rarely made transcript [3].

As first suggested by Delbrück [13], epigenetic differences can be understood in terms of multistability: a given cell can persist in one of many stable steady states, which differ from each other by the genes that are ON and those that are OFF. This multistable nature of biological switches is fundamental for the determination of cell fate in unicellular and multicellular organisms [14–21]. Bistability can arise in gene networks that contain a positive-feedback loop [15]. Such gene networks are often regulated by transcription factors

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Abbreviations: Pgal, phenyl-β-D-galactoside; RNAP, RNA polymerase; TMG, thiomethylgalactoside

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Cell Lineages and the Logic of Proliferative Control

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It is widely accepted that the growth and regeneration of tissues and organs is tightly controlled. Although experimental studies are beginning to reveal molecular mechanisms underlying such control, there is still very little known about the control strategies themselves. Here, we consider how secreted negative feedback factors ("chalones") may be used to control the output of multistage cell lineages, as exemplified by the actions of GDF11 and activin in a self-renewing neural tissue, the mammalian olfactory epithelium (OE). We begin by specifying performance objectives—what, precisely, is being controlled, and to what degree—and go on to calculate how well different types of feedback configurations, feedback sensitivities, and tissue architectures achieve control. Ultimately, we show that many features of the OE—the number of feedback loops, the cellular processes targeted by feedback, even the location of progenitor cells within the tissue—fit with expectations for the best possible control. In so doing, we also show that certain distinctions that are commonly drawn among cells and molecules—such as whether a cell is a stem cell or transit-amplifying cell, or whether a molecule is a growth inhibitor or stimulator—may be the consequences of control, and not a reflection of intrinsic differences in cellular or molecular character.

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Introduction

In recent decades, biologists have come to view cell lineages as fundamental units of tissue and organ development, maintenance, and regeneration. The highly differentiated, often nondividing cells that characterize the mature functions of tissues are seen as end products of orderly, tissue-specific sequences of cell divisions, during which progenitor cells pass through distinct stages, marked by expression of stage-specific genes (e.g., [1–4]). At the starting points of lineages—particularly those in self-renewing tissues such as blood, epidermis, and the intestinal lining—one finds stem cells, characterized both by multipotency (ability to produce many cell types) and their ability to maintain their own numbers through self-replication [5–8]. As scientists and clinicians have become increasingly interested in harnessing these features of stem cells to repair injury and cure disease, there has been a resurgence of interest in the mechanisms underlying the execution and regulation of cell lineages (e.g., [9–12]).

The functions of lineages are often presented in terms of progressive allocation of developmental potential: Thus, pluripotent stem cells often give rise to oligopotent progenitors, which in turn give rise to unipotent (committed) progenitors. The sequential expression of marker genes at different lineage stages may be related to transcriptional "priming" events needed to lock cells into specific patterns of gene expression [13,14].

Not all lineage stages correlate with restriction of cell fate, however, raising the question of what else lineages do. The fact that lineage intermediates often display "transit-amplifying" behavior, i.e., are capable of at least some degree of self-replication, has led to the suggestion that lineage stages play essential roles in the control of tissue and organ growth (with growth referring in this case to increase in cell number). Here, we seek to discover what those roles are. We approach this question from the perspective of lineages in general, and within the context of the mammalian olfactory epithelium (OE), the neural tissue that senses odor and transmits olfactory information to the brain. The OE is a continually self-renewing tissue, even in man, and is capable of rapid regeneration [15]. As discussed below, a wealth of experimental data on the OE lineage and the molecules that regulate it makes the OE an attractive system in which to investigate the relationship between lineages and growth control.

Performance Objectives of Growing Tissues

In biology, "control" is often used interchangeably with "regulation," but in engineering, control has a precise meaning: It refers to the strategies that enable a system to

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Abbreviations: BrdU, bromodeoxyuridine; FST, follistatin; GDF8, growth and differentiation factor 8; GDF11, growth and differentiation factor 11; INP, immediate neuronal precursor; Ngn1, Neurogenin1; OE, olfactory epithelium; ORN, olfactory receptor neuron; TGFβ, transforming growth factor β

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Robustness in Glyoxylate Bypass Regulation

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Abstract

The glyoxylate bypass allows *Escherichia coli* to grow on carbon sources with only two carbons by bypassing the loss of carbons as CO_2 in the tricarboxylic acid cycle. The flux toward this bypass is regulated by the phosphorylation of the enzyme isocitrate dehydrogenase (IDH) by a bifunctional kinase–phosphatase called IDHKP. In this system, IDH activity has been found to be remarkably robust with respect to wide variations in the total IDH protein concentration. Here, we examine possible mechanisms to explain this robustness. Explanations in which IDHKP works simultaneously as a first-order kinase and as a zero-order phosphatase with a single IDH binding site are found to be inconsistent with robustness. Instead, we suggest a robust mechanism where both substrates bind the bifunctional enzyme to form a ternary complex.

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Introduction

Robustness in biological systems has seen a renewal of research interest in recent years [1–12]. To define robustness, one needs to specify what feature is robust and with respect to which variations. Classic experimental studies have shown that metabolic fluxes are often insensitive to the levels of enzymes in the pathway, as reviewed in [13]. Metabolic control theory addresses this by suggesting that control of flux is distributed amongst many enzymes, and thus no single enzyme is rate limiting.

In the last decade, studies have added a new level of understanding on robustness by providing detailed molecular mechanisms that can preserve the essential function of a system in the face of large variations in the protein levels. For example, specific mechanisms explain how exact adaptation in bacterial chemotaxis is robust with respect to chemotaxis protein levels [2,3], and how patterning in drosophila embryos is robust with respect to morphogen production rates [12,14,15]. A recent review summarizes experiments and theoretical mechanisms for robustness [10].

Recently, an intriguing class of robust mechanisms has been found, based on bifunctional enzymes that carry out two opposing reactions (such as both modifying a target protein, and removing the modification) [8,11]. These robust mechanisms seem to apply to a class of bacterial two-component signaling system. These systems show robustness of input-output relations, in the sense that output responds to input signals in a way that is not disrupted by variations in protein levels.

Here, we extend this line of research to one of the best studied regulation steps in *E. coli* metabolism, the IDHKP/IDH system. This system raised our interest because it employs a bifunctional enzyme that carries out two opposing reactions, hinting at a robust mechanism. However, it has several biochemical differences from previously studied systems [8,11], suggesting that it may show a new type of robust mechanism.

The need for precise regulation in the IDHKP/IDH system is evident from its biological function. The IDH system regulates the partitioning of carbon flux between the TCA cycle and the glyoxylate bypass (Figure 1). Precise regulation of flux to the glyoxylate bypass is essential when the bacterium grows on substances such as acetate that contain only two carbon atoms. Without the glyoxylate bypass, both carbon atoms would be converted to CO_2 by the TCA cycle, thereby leaving no carbon available for biosynthesis of cell constituents. Hence, growth on acetate and other two-carbon compounds requires directing some of the carbon flux to the glyoxylate bypass, thereby avoiding carbon loss.

The precise partitioning of carbon flux between the cycle and the bypass is achieved by regulating the activity of the enzyme IDH (isocitrate dehydrogenase), which stands at the entry to the bypass. The activity of IDH is determined by its phosphorylation state: only unphosphorylated IDH is active. During growth on substances with more than two carbon atoms, IDH is mostly unphosphorylated and hence active. Thus, most of the carbon flux is directed to the more efficient TCA cycle. On the contrary, during growth on acetate, most of IDH is phosphorylated and hence inactive, so that a large part of the carbon flux is directed to the bypass [16–19].

To regulate the IDH phosphorylation level, *E. coli* employs a bifunctional enzyme. This enzyme catalyzes both the phosphorylation of IDH, and its dephosphorylation, and is called IDHKP (IDH Kinase/Phosphatase) [20]. IDHKP uses ATP as the phosphoryl donor for the kinase reaction, and also requires ATP as a cofactor for the dephosphorylation reaction [20–22]. The activity of IDHKP is allosterically regulated by the levels of various metabolites in the cell that act as the input signals to this system [21].

The robustness of IDH activity has been experimentally tested by Laporte et. al. [23]. It was found that during growth on acetate, the concentration of active (unphosphorylated) IDH is extremely

Developmental Robustness by Obligate Interaction of Class B Floral Homeotic Genes and Proteins

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Abstract

DEF-like and *GLO*-like class B floral homeotic genes encode closely related MADS-domain transcription factors that act as developmental switches involved in specifying the identity of petals and stamens during flower development. Class B gene function requires transcriptional upregulation by an autoregulatory loop that depends on obligate heterodimerization of DEF-like and GLO-like proteins. Because switch-like behavior of gene expression can be displayed by single genes already, the functional relevance of this complex circuitry has remained enigmatic. On the basis of a stochastic *in silico* model of class B gene and protein interactions, we suggest that obligate heterodimerization of class B floral homeotic proteins is not simply the result of neutral drift but enhanced the robustness of cell-fate organ identity decisions in the presence of stochastic noise. This finding strongly corroborates the view that the appearance of this regulatory mechanism during angiosperm phylogeny led to a canalization of flower development and evolution.

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Introduction

Depending on the nature of the interactions of their constituents, gene regulatory circuits can display a variety of dynamical behaviors ranging from simple steady states, to switching and multistability, to oscillations. Temporal or spatial patterning during development requires activation of genes at a particular time or position, respectively, and the inhibition in the remaining time or part. Regulatory genes involved in such processes often show a switch-like temporal or spatial dynamics, which requires a direct or indirect positive non-linear feedback of the genes on their own expression, e.g. via dimers of their own product [1]. Switch-like behavior can be displayed by a single gene [2,3], but many gene regulatory switches have a more complex structure. Due to the small number of molecules involved, these switches are inherently stochastic and their behavior under noisy conditions can strongly depend on their genetic architecture [4–6]. In some cases the complex regulatory interactions have been quite well documented, but the functional implications of the corresponding regulatory circuitry have remained enigmatic. A good case in point is provided by some floral homeotic (or organ identity) genes from model plants such as *Arabidopsis thaliana* (thale cress; henceforth termed *Arabidopsis*) and *Antirrhinum majus* (snapdragon; henceforth called *Antirrhinum*).

Floral homeotic genes act as developmental switches involved in specifying organ identity during flower development. According to the 'ABC model', three classes of floral organ identity (or homeotic) genes act in a combinatorial way to specify the identity of four types of floral organs, with class A genes specifying sepals in the first floral whorl, A+B petals in the second whorl, B+C stamens (male reproductive organs) in the third whorl, and C alone carpels (female organs) in the fourth floral whorl [7]. The combinatorial genetic interaction of floral homeotic genes may involve the formation of multimeric transcription factor complexes that also include class E (or SEPALLATA) proteins, as outlined by the 'floral quartet' model [8].

In *Antirrhinum*, there are two different class B genes termed *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*). In *Arabidopsis* these genes are represented by *APETALA3* (*AP3*), the putative orthologue of *DEF*, and *PISTILLATA* (*PI*), the putative *GLO* orthologue. For simplicity, we will refer to *DEF*-like and *GLO*-like genes from here on. *DEF*-like and *GLO*-like genes represent paralogous gene clades that originated by the duplication of a class B gene precursor 200–300 million years ago [9,10]. All class B genes identified so far, like most other floral homeotic genes, belong to the family of MADS-box genes, encoding MADS-domain transcription factors [11,12].

Mutant phenotypes reveal that *DEF*-like and *GLO*-like genes are essential for the development of petals and stamens, since *def* and *glo* loss-of-function mutants all produce flowers with petals converted into sepals and stamens transformed into carpels [13–17]. When co-expressed in the context of a flower, DEF and GLO are not only required, but even sufficient for specifying petal and stamen identity, as revealed by transgenic studies (e.g., [18]).

Induction and stable maintenance of switch-gene expression are typically two independent processes, depending on a transient external signal and autoregulation, respectively [19]. Whenever a transient activating signal is above a threshold, the gene activity switches from the OFF- to the ON-state. The signal is required only for initiation, but not for maintenance of gene activity. Due to the autoregulation, the gene's response becomes in a wide range independent of the exact strength of the input signal. During later stages of flower development (in *Arabidopsis* from stage 5 on),

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Precise Regulation of Gene Expression Dynamics Favors Complex Promoter Architectures

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Abstract

Promoters process signals through recruitment of transcription factors and RNA polymerase, and dynamic changes in promoter activity constitute a major noise source in gene expression. However, it is barely understood how complex promoter architectures determine key features of promoter dynamics. Here, we employ prototypical promoters of yeast ribosomal protein genes as well as simplified versions thereof to analyze the relations among promoter design, complexity, and function. These promoters combine the action of a general regulatory factor with that of specific transcription factors, a common motif of many eukaryotic promoters. By comprehensively analyzing stationary and dynamic promoter properties, this model-based approach enables us to pinpoint the structural characteristics underlying the observed behavior. Functional tradeoffs impose constraints on the promoter architecture of ribosomal protein genes. We find that a stable scaffold in the natural design results in low transcriptional noise and strong co-regulation of target genes in the presence of gene silencing. This configuration also exhibits superior shut-off properties, and it can serve as a tunable switch in living cells. Model validation with independent experimental data suggests that the models are sufficiently realistic. When combined, our results offer a mechanistic explanation for why specific factors are associated with low protein noise in vivo. Many of these findings hold for a broad range of model parameters and likely apply to other eukaryotic promoters of similar structure.

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Introduction

Combinatorial regulation of gene expression is an important mechanism for signal integration in prokaryotes and eukaryotes (reviewed in [1]). Typically, specific motifs in the DNA sequence favor binding of particular transcription factors (TFs) and thus encode a *cis*-regulatory input function [2]. Protein-protein interactions among different TFs, which do not necessarily involve direct contacts with DNA, contribute to—frequently synergistic—regulatory function [1]. This is a very versatile mechanism for hierarchical control, e.g., when TFs can only be recruited in a predefined sequence or when they are excluded under specific conditions [3]. Chromatin state and chromatin-modifying activities provide yet another layer of regulation, and recruitment of the latter is typically also mediated by TFs [4]. Hence, multiple, complex levels of combinatorial control characterize transcriptional regulation [5].

New high-throughput measurement methods have generated a wealth of information on transcriptional regulatory circuits at different levels such as chromatin states, promoter occupancy by TFs, and mRNA expression dynamics as the system's output. Analysis of combinatorial regulation at the genome-scale points to a modular organization of transcriptional regulatory networks, which could facilitate data integration. However, this requires a multi-level analysis [6] and dynamic processes may lead to large functional re-arrangements of transcriptional regulatory networks. Concomitantly, understanding network design principles needs a detailed investigation of dynamic processes [7,8].

Corresponding computational models aid in disentangling transcriptional network structures and in quantitatively analyzing the impact of promoter architecture on the regulatory outcome. Depending on network size, available experimental data, and model purpose, model types range from qualitative logical models to quantitative approaches based on thermodynamic considerations or ordinary differential equations (ODEs) (reviewed in [9–11]). However, most previous work focused on stationary gene-regulatory input functions in real-life organisms and in rational promoter design [2,12–14]. Recently, stochastic kinetic models have received increased attention because we lack a deeper understanding of how gene network architecture shapes gene expression noise [15]. Stochasticity in gene expression arises from environmental effects and from intrinsic sources. It can have benefits and adverse effects for gene network function (reviewed in [16,17]). Hence, noise in gene expression may be an evolvable trait that is intimately linked to promoter architecture [16]. For eukaryotic systems, irregular promoter activation due to chromatin modifications or transcriptional re-initiation are the main intrinsic noise sources [15,18]. Despite recent progress [15,18,19], our understanding of how the dynamic interplay of transcription factors, chromosomal positioning, epigenetic control, and *cis*-regulatory promoter elements shapes expression dynamics and noise properties is still limited. Moreover, much of our knowledge

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Steady-State Kinetic Modeling Constrains Cellular Resting States and Dynamic Behavior

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Abstract

A defining characteristic of living cells is the ability to respond dynamically to external stimuli while maintaining homeostasis under resting conditions. Capturing both of these features in a single kinetic model is difficult because the model must be able to reproduce both behaviors using the same set of molecular components. Here, we show how combining small, well-defined steady-state networks provides an efficient means of constructing large-scale kinetic models that exhibit realistic resting and dynamic behaviors. By requiring each kinetic module to be homeostatic (at steady state under resting conditions), the method proceeds by (*i*) computing steady-state solutions to a system of ordinary differential equations for each module, (*ii*) applying principal component analysis to each set of solutions to capture the steady-state solution space of each module network, and (*iii*) combining optimal search directions from all modules to form a global steady-state space that is searched for accurate simulation of the time-dependent behavior of the whole system upon perturbation. Importantly, this stepwise approach retains the nonlinear rate expressions that govern each reaction in the system and enforces constraints on the range of allowable concentration states for the full-scale model. These constraints not only reduce the computational cost of fitting experimental time-series data but can also provide insight into limitations on system concentrations and architecture. To demonstrate application of the method, we show how small kinetic perturbations in a modular model of platelet $P2Y_1$ signaling can cause widespread compensatory effects on cellular resting states.

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Introduction

Computational models help quantify the reaction dynamics and regulatory modes in complex biochemical systems [1–5], particularly when a system is so intricate that its behavior cannot be predicted by intuition alone. The building blocks for constructing large reaction networks are often available in numerous databases [6–9] and journal archives. Here, one can obtain many of the experimentally-derived elementary reaction steps, kinetic constants, or rate laws for individual steps in a given biochemical system or pathway. Despite this wealth of information, however, compiling these data to construct models with accurate system-wide behavior represents a significant challenge in systems biology [10,11]. Comprehensive models of metabolism have been successfully developed for microbial systems [5,12,13] and certain eukaryotic cell types [14–16]. These constraint-based models [17] are often represented by stoichiometric networks that lack an explicit description of substrate concentrations, reaction mechanisms, or the transient behavior of the system. Although various strategies have been proposed to incorporate these features into large-scale models [18,19], the task of assembling complex kinetic models with nonlinear dynamics remains a difficult problem. One of the major obstacles to building accurate kinetic models is the number of unknown parameters in the model that must be estimated using experimental datasets [19], which themselves are often massive, incomplete, noisy, and/or imperfect [20]. A number of parameter estimation methods, such as genetic programming, simulated annealing, and various gradient-based routines [21,22], have been proposed to infer unknown quantities in biochemical models. Most of these methods address the problem of estimation in purely abstract terms and do not take into account the unique mathematical features of biochemical systems, such as a well-characterized kinetic subsystem (e.g., the dynamics properties of an ion channel [23]). Estimated parameters must still meet constraints imposed by the other experimentally measured parameters in the model.

To address these challenges, we propose a strategy for assembling large kinetic networks that retain the nonlinear dynamics governing individual reactions in the system. The key features of the method are: (*i*) restriction of steady-state values by subsystem kinetics, (*ii*) reduction of the steady-state solution space by principal component analysis (PCA), and (*iii*) combination of independently constructed submodels (modules). The first feature is a Monte Carlo sampling over unknown concentrations with fixed kinetic parameters derived from the literature. The opposite strategy has been used in microbial systems to restrict kinetic parameters based on species concentrations [12]. The second feature, reduction of the steady-state space by PCA, has been

A Simple Screen to Identify Promoters Conferring High Levels of Phenotypic Noise

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Abstract

Genetically identical populations of unicellular organisms often show marked variation in some phenotypic traits. To investigate the molecular causes and possible biological functions of this phenotypic noise, it would be useful to have a method to identify genes whose expression varies stochastically on a certain time scale. Here, we developed such a method and used it for identifying genes with high levels of phenotypic noise in *Salmonella enterica* ssp. I serovar Typhimurium (*S.* Typhimurium). We created a genomic plasmid library fused to a green fluorescent protein (GFP) reporter and subjected replicate populations harboring this library to fluctuating selection for GFP expression using fluorescent-activated cell sorting (FACS). After seven rounds of fluctuating selection, the populations were strongly enriched for promoters that showed a high amount of noise in gene expression. Our results indicate that the activity of some promoters of *S.* Typhimurium varies on such a short time scale that these promoters can absorb rapid fluctuations in the direction of selection, as imposed during our experiment. The genomic fragments that conferred the highest levels of phenotypic variation were promoters controlling the synthesis of flagella, which are associated with virulence and host–pathogen interactions. This confirms earlier reports that phenotypic noise may play a role in pathogenesis and indicates that these promoters have among the highest levels of noise in the *S.* Typhimurium genome. This approach can be applied to many other bacterial and eukaryotic systems as a simple method for identifying genes with noisy expression.

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Introduction

Clonal populations of unicellular organisms growing under constant conditions often show substantial variation in phenotypic traits. The rate at which some of these traits vary is so high that it cannot result from mutational change. Rather, this phenotypic noise has been shown to result from chance events in the cells, namely random fluctuation in the transcription and translation of genes [1–3]. Most of the research on phenotypic noise focuses on two questions. First, what are the molecular processes underlying this phenomenon? Second, are there cases in which phenotypic noise is beneficial? Can it provide a genotype with new biological functions and improve the chance that it will survive and reproduce?

To further our understanding of the biological significance of phenotypic noise, it would be helpful to have a simple method to identify genes whose expression varies stochastically at a given timescale and under specific environmental conditions. So far, most of the research on phenotypic noise was based on the detailed analysis of individual biological traits [4–6]. It is interesting to complement these studies with a global analysis, so that one can ask whether the traits studied so far are indeed particularly noisy, or whether a substantial fraction of all genes show such high levels of noise. One possibility for a global analysis of phenotypic noise is the exhaustive characterization of ordered libraries of strains marked with reporter proteins [7]. Here, we have established a simple alternative that allows identifying promoters whose activity varies on a specific time-scale; we used this method to identify promoters in the bacterial pathogen *S.* Typhimurium that switch between active and inactive over the course of a few generations.

The method is based on subjecting a promoter library to selection for high levels of random variation on a short time scale. The screen was initiated with a genomic library consisting of short genomic fragments upstream of a gene encoding green fluorescent protein (GFP). Cells carrying a fragment with an active promoter expressed GFP. In order to select for promoters with a high level of phenotypic noise, we used fluorescence-activated cell sorting to select cells based on the cellular concentration of GFP, and alternated between selecting for high levels of GFP, and selecting for low levels of GFP. There was no signal indicating the direction of selection during a given round of the selection experiment; one would thus expect that promoters that randomly switch between expressing and not expressing GFP would increase in frequency.

Versatile selection technology for intracellular protein–protein interactions mediated by a unique bacterial hitchhiker transport mechanism

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We have developed a reliable genetic selection strategy for isolating interacting proteins based on the "hitchhiker" mechanism of the *Escherichia coli* twin-arginine translocation (Tat) pathway. This method, designated FLI-TRAP (functional ligand-binding identification by Tat-based recognition of associating proteins), is based on the unique ability of the Tat system to efficiently cotranslocate noncovalent complexes of 2 folded polypeptides. In the FLI-TRAP assay, the protein to be screened for interactions is engineered with an N-terminal Tat signal peptide, whereas the known or putative partner protein is fused to mature TEM-1 β-lactamase (Bla). Using a series of c-Jun and c-Fos leucine zipper (JunLZ and FosLZ) variants of known affinities, we observed that only those chimeras that expressed well and interacted strongly in the cytoplasm were able to colocalize Bla into the periplasm and confer β-lactam antibiotic resistance to cells. Likewise, the assay was able to efficiently detect interactions between intracellular single-chain Fv (scFv) antibodies and their cognate antigens. The utility of FLI-TRAP was then demonstrated through random library selections of amino acid substitutions that restored (*i*) heterodimerization to a noninteracting FosLZ variant, and (*ii*) antigen binding to a low-affinity scFv antibody. Because Tat substrates must be correctly folded before transport, FLI-TRAP favors the identification of soluble, nonaggregating, protease-resistant protein pairs and, thus, provides a powerful tool for routine selection of interacting partners (e.g., antibody-antigen), without the need for purification or immobilization of the binding target.

ligand binding proteins | protein folding quality control | signal peptide | twin-arginine translocation | 2-hybrid system

Protein–protein interactions are key molecular events that integrate multiple gene products into functional complexes in virtually every cellular process. Because such interactions mediate numerous disease states and biological mechanisms underlying the pathogenesis of bacterial and viral infections, identification of protein–protein interactions remains one of the most important challenges in the postgenomics era. The yeast 2-hybrid (Y2H) system (1) has been the tool of choice for revealing numerous protein–protein interactions, underlying diverse protein networks and complex protein machinery inside living cells. To date, Y2H has been used to generate protein interaction maps for humans (2), *Drosophila melanogaster* (3), *Caenorhabditis elegans* (4), *Saccharomyces cerivisiae* (5, 6), vaccinia virus (7), and *Escherichia coli* bacteriophage T7 (8). Another important application of the Y2H methodology is the discovery of diagnostic and therapeutic proteins, whose mode of action is high-affinity binding to a target peptide or protein. For example, several groups have isolated antibody fragments that are readily expressed in the cytoplasm of cells where they bind specifically to a desired target (9, 10), and in certain instances ablate protein function (11, 12).

Although the 2H system was initially developed by using yeast as a host organism, numerous bacterial (B)2H systems are now common laboratory tools and represent an experimental alternative with certain advantages over the yeast-based systems (13, 14). A number of these bacterial approaches employ split activator/repressor proteins; thus, they are functionally analogous to the GAL4-based yeast system (15–17). Unfortunately, both Y2H and B2H GAL4-type assays are prone to a high frequency of false positives that arise from spurious transcriptional activation (18), and complicate the interpretation of interaction data. As proof, a comparative assessment revealed that >50% of the data generated using Y2H were likely to be false positives (19). To address this shortcoming, several groups have exploited oligomerization-assisted reassembly of split enzymes such as adenylate cyclase (20), β-lactamase (Bla) (21), and dihydrofolate reductase (22, 23), as well as split fluorescent proteins (24, 25). Alternatively, a number of methodologies for detecting interacting proteins in bacteria have been developed that do not rely on interaction-induced complementation of protein fragments, but instead use phage display (26), FRET (27), and cytolocalization of GFP (28).

In this study, we have developed a genetic selection for protein–protein interactions in *E. coli* based on the native ability of the twin-arginine translocation (Tat) pathway to both proofread (29–31) and colocalize (32) folded protein complexes across biological membranes. In the latter instance, Wu and coworkers (32) revealed that the Tat pathway exports heterodimers whereby only 1 protein subunit carries an N-terminal Tat signal peptide (ssTorA), a process referred to as "hitchhiker" export. More recently, we exploited this natural hitchhiker mechanism for the periplasmic expression of a murine F_{AB} antibody fragment (30). In this earlier study, an ssTorA was fused to the F_{AB} heavy chain, whereas the F_{AB} light chain was expressed without any signal peptide. After coexpression of the individual heavy and light chains in *E. coli*, the F_{AB} subunits assembled in the cytoplasm and were colocalized to the periplasm via the Tat machinery. Along similar lines, a genetic reporter of protein–protein interactions using the Tat system was described recently by Strauch and Georgiou (33); however, the assay relied on complex phenotypic complementation that potentially limits the effectiveness of the approach. Here, we show that Tat-mediated colocalization of the reporter enzyme Bla enabled semiquantitative, high-throughput selection of a wide range of interacting polypeptide pairs that were stably expressed, and interacted with high affinity in the bacterial cytoplasm. By using this method, termed FLI-TRAP (functional ligand-binding identification by Tat-based recognition of associating proteins), we were able to efficiently isolate high-affinity binding proteins from large combinatorial libraries after just a single round of mutagenesis and selection.

Results

Reconstitution of the Tat-Dependent Hitchhiker Mechanism for a Native Tat Substrate. To verify whether the Tat hitchhiker mechanism could be developed as a general platform for detecting intracellular protein–protein interactions (Fig. 1*A*), we first at-

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ARTICLES

Kinetochore geometry defined by cohesion within the centromere

Takeshi Sakuno[1,2], Kenji Tada[1,3] & Yoshinori Watanabe[1,3]

During cell division microtubules capture chromosomes by binding to the kinetochore assembled in the centromeric region of chromosomes. In mitosis sister chromatids are captured by microtubules emanating from both spindle poles, a process called bipolar attachment, whereas in meiosis I sisters are attached to microtubules originating from one spindle pole, called monopolar attachment. For determining chromosome orientation, kinetochore geometry or structure might be an important target of regulation. However, the molecular basis of this regulation has remained elusive. Here we show the link between kinetochore orientation and cohesion within the centromere in fission yeast *Schizosaccharomyces pombe* by strategies developed to visualize the concealed cohesion within the centromere, and to introduce artificial tethers that can influence kinetochore geometry. Our data imply that cohesion at the core centromere induces the mono-orientation of kinetochores whereas cohesion at the peri-centromeric region promotes bi-orientation. Our study may reveal a general mechanism for the geometric regulation of kinetochores, which collaborates with previously defined tension-dependent reorientation machinery.

A well-defined mechanism for the regulation for kinetochore orientation is the tension-dependent stabilization of kinetochore–microtubule attachment; unstable spindle microtubules repeatedly attach and release (reorient) kinetochores until they capture paired kinetochores from opposite sides, thus generating tension across cohered centromeres[1,2]. During meiosis I, however, homologous chromosomes are connected by a reciprocal recombination (chiasmata) and, therefore, tension can be generated not only when sister chromatids are captured from opposite sides but also when homologous chromosomes are pulled from opposite sides[1,3,4]. Indeed, meiotic cells always take the latter option at the first meiotic division. Importantly, even if recombination is genetically abolished during meiosis, sister kinetochores apparently refuse bipolar attachment, although tension-generating attachment can be established only in this way. Therefore, the structure or geometry of sister kinetochores may be modified to allow orientation towards the same surface at this stage of meiosis.

The geometric aspect of kinetochore orientation has long been recognized in vertebrates[5]. The staining of human interphase nuclei with anti-centromere antibodies revealed that the centromere is duplicated and resolved by the end of interphase[6]. This physical separation or resolution of sister centromeres would be important for back-to-back assembly of sister kinetochores, thus facilitating bipolar attachment to microtubules in mitosis. In contrast to mitosis, electron microscopic analyses of several animal germ cells have shown that sister kinetochores orient side-by-side and fuse in meiosis I[3,7–9]. Despite its paramount importance for chromosome segregation, the molecular mechanism underlying the regulation of kinetochore geometry has remained largely elusive[10].

Fission yeast centromeres, like metazoan centromeres, are composed of two domains, a kinetochore-assembling core centromere and heterochromatic peri-centromeric regions; a single kinetochore can attach several microtubules[11,12]. One plausible model for the geometric regulation of kinetochores arose from the analysis of the sister chromatid cohesion molecule (cohesin) in this organism[13]. Mitotic Rad21-containing cohesin localizes preferentially to the peri-centromeric regions[14–16], whereas meiotic Rec8-containing cohesin localizes additionally to the core centromere[13]. Crucially, the abolishment of Rec8 only at the core centromere results in equational rather than reductional division at meiosis I, advocating a model whereby the establishment of cohesion at the core centromere conjoins the two kinetochore domains at meiosis I, whereas the core regions may open to opposite sides when not establishing this cohesion[17]. Fission yeast Moa1, a meiosis-specific kinetochore protein, also has an essential role in establishing the mono-orientation of kinetochores, whereas centromeric Rec8 localization increases, rather than decreases, in *moa1Δ* cells[17]. Moreover, the contribution of cohesin to kinetochore orientation has been disputed in budding yeast[18,19]. Thus, the lack of observation of actual cohesion at the core centromere in any organism leaves the cohesion-mediated mono-orientation model largely hypothetical.

Visualization of core centromere cohesion in meiosis

To visualize the cohesion of DNA sequences lying under kinetochores, we inserted a *lac* operator (*lacO*) array into the centre of the core centromere and expressed green fluorescent protein–lactose repressor (GFP–LacI) fusion proteins (*cnt1*–GFP) within the cell (Fig. 1a). The engineered centromere was shown to preserve intact function (Supplementary Fig. 1). However, this fluorescence marker might not reveal the cohesion state at the core centromere in the context of the chromosome because of the intact cohesion at neighbouring sites. To overcome this problem, we excised the DNA from the neighbouring chromosomal domains by a site-specific recombinase (R recombinase of *Zygosaccharomyces rouxii*) and circularized it[20,21] (Fig. 1a). Given that cohesion is usually established during S phase, we excised the DNA region during post-DNA replication in meiosis by expressing R recombinase with a meiosis-specific $spo5^{+}$ promoter[13]. A Southern blot assay confirmed the proper excision of the DNA duplex as a circle in zygotes (Fig. 1a). To visualize the uncoupling of the core centromere marked with GFP signals, we coloured the outer flanking *dh1L* site differently with the insertion

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LETTERS

Cdc14 inhibits transcription by RNA polymerase I during anaphase

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Chromosome condensation and the global repression of gene transcription[1] are features of mitosis in most eukaryotes. The logic behind this phenomenon is that chromosome condensation prevents the activity of RNA polymerases. In budding yeast, however, transcription was proposed to be continuous during mitosis[2]. Here we show that Cdc14, a protein phosphatase required for nucleolar segregation[3] and mitotic exit[4], inhibits transcription of yeast ribosomal genes (rDNA) during anaphase. The phosphatase activity of Cdc14 is required for RNA polymerase I (Pol I) inhibition *in vitro* and *in vivo*. Moreover Cdc14-dependent inhibition involves nucleolar exclusion of Pol I subunits. We demonstrate that transcription inhibition is necessary for complete chromosome disjunction, because ribosomal RNA (rRNA) transcripts block condensin binding to rDNA, and show that bypassing the role of Cdc14 in nucleolar segregation requires *in vivo* degradation of nascent transcripts. Our results show that transcription interferes with chromosome condensation, not the reverse. We conclude that budding yeast, like most eukaryotes, inhibit Pol I transcription before segregation as a prerequisite for chromosome condensation and faithful genome separation.

In most eukaryotic cells, a dramatic structural reorganization of the genetic material into highly condensed chromosomes and the global silencing of gene transcription[1] accompany entry into mitosis. This might reflect an incompatibility between transcription and chromosome condensation and/or segregation processes. The most highly transcribed regions in eukaryotic genomes are the ribosomal gene arrays (rDNA), which require a dedicated polymerase named RNA Pol I. A study in budding yeast using cell size as an indicator of cell-cycle stage and cellular RNA transcript levels established that transcription, including rDNA, is not inhibited at any stage during mitosis[2]. This is surprising because rDNA becomes hyper-condensed during anaphase[5,6]. We thus revisited whether transcription is inhibited during yeast mitosis using more sensitive assays.

We measured total RNA synthesis in synchronised yeast cultures undergoing an entire cell cycle using incorporation of [^{3}H]uracil into total RNA and found that cells downregulate RNA synthesis during anaphase (Fig. 1a). Analysis of nascent 35S rRNA transcripts also showed significant reduction during anaphase (Fig. 1b; 75 min and Supplementary Fig. 1). The anaphase inhibition of rRNA transcription correlates with the exclusion of the Pol I subunit Rpa43 from the 35S gene region (Fig. 1d and Supplementary Figs 2 and 3). Therefore, yeast cells, like most eukaryotes, inhibit transcription during mitosis; however, whereas transcription inhibition in most eukaryotic cells takes place in metaphase, in yeast it occurs during anaphase.

In early anaphase the conserved phosphatase Cdc14 becomes activated[7]. Cdc14 is required for the resolution of transcription-dependent linkages in the ribosomal gene array[8,9]. Expression of *CDC14* from the *GAL1-10* promoter in metaphase cells causes a four-fold reduction in rRNA synthesis (Fig. 2a) and the effect is dependent on its phosphate activity (Fig. 2a). Cfi1 (also known as Net1), the nucleolar inhibitor of Cdc14 and a *CDK1* target[10–12], interacts directly with Pol I and stimulates transcription *in vitro*[13]. Expression of a stabilized form of cyclin B2, *CLB2-DK*[14], causes a mitotic arrest where Cdc14 is maintained at the fully released and active stage[12]. *CLB2-DK* expression also causes reduction in rRNA synthesis in the presence of wild-type Cdc14 (Fig. 2b), confirming that rDNA transcription inhibition is directly dependent on the phosphatase and not Cfi1. Moreover, *CLB2-DK* expression in the presence of Cdc14 causes delocalization of the essential Pol I subunit Rpa43 from the nucleolus (Fig. 2c, d and Supplementary Fig. 4). However, this is not the case for the entire Pol I holocomplex because the Rpa190 subunit is not delocalized when *CLB2-DK* is expressed in the presence of Cdc14 (Supplementary Fig. 5), despite the fact that Pol I transcription is inhibited (Fig. 2b). Therefore Cdc14 probably inhibits Pol I transcription by destabilization of specific subunits. These findings suggest that Cdc14 is a Pol I transcriptional repressor. Indeed, purified Cdc14 inhibits Pol I transcription *in vitro* (Fig. 3a) whereas the phosphatase-dead mutant does not (Fig. 3a). Cdc14 does not prevent stimulation Pol I transcription by Cfi1 (Fig. 3b). Therefore the activities of Cfi1 and Cdc14 in the activation and repression of Pol I transcription are independent.

Phosphorylation of Pol I has been linked to active transcription[15]. In metaphase-arrested cells at least two phospho-forms of Rpa43 can be detected (Supplementary Fig. 6). Expression of Cdc14 causes the loss of one of the Rpa43 phospho-bands (Supplementary Fig. 6). Therefore Cdc14 promotes dephosphorylation of Rpa43. Deletions in the carboxy-terminal region of Rpa43, where most phospho-sites are located[16], prevent Cdc14-dependent nucleolar delocalization but do not affect transcription inhibition (Supplementary Fig. 7). Therefore additional Cdc14 targets in the regulation of Pol I transcription repression must exist. Nonetheless, Rpa43 seems to be a key factor because a phospho-mimicking mutant of Rpa43 shows defects in transcription inhibition (Supplementary Fig. 8).

The requirement of Cdc14 for rDNA condensation[17,18] and segregation[17–20] is thought to stem from a defect in condensin localization[17,20]. Condensin binding is incompatible with rDNA transcription[21], and inactivation of Pol I allows rDNA segregation in the absence of Cdc14 (refs 8 and 9). Therefore transcription inhibition by Cdc14 is probably sufficient to promote condensin binding to rDNA. Indeed, in the absence of Cdc14 removal of rRNA transcripts by expression of the RntA RNase T1 from *Aspergillus oryzae*[22] (Supplementary Fig. 9) promotes condensin binding to rDNA (Fig. 4a).

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nature cell biology

The yeast global transcriptional co-repressor protein Cyc8 can propagate as a prion

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Although many proteins can misfold into a self-seeding amyloid-like conformation[1], only six are known to be infectious, that is prions. The prions [*PSI*$^+$], [*PIN*$^+$], [URE3], [*SWI*$^+$] and [HET-s] cause distinct heritable physiological changes in fungi[2–4], whereas PrP^{Sc} causes infectious encephalopathies in mammals[5]. It is unknown whether 'protein-only' inheritance is limited to these exceptional cases or whether it represents a widespread mechanism of epigenetic control. Towards this goal, we now describe a new prion formed by the Cyc8 (Ssn6) protein of *Saccharomyces cerevisiae*. Analogously to other yeast prions, transient overproduction of a glutamine-rich region of Cyc8 induced a heritable dominant *cyc8*$^-$ phenotype that is transmitted cytoplasmically and is dependent on the chaperone Hsp104 and the continued presence of the Cyc8 protein. The evolutionarily conserved Cyc8–Tup1 global transcriptional repressor complex[6] forms one of the largest gene regulatory circuits, controlling the expression of more than 7% of yeast genes[7]. Our finding that Cyc8 can propagate as a prion, together with a recent report that Swi1 of the Swi–Snf global transcriptional regulatory complex also has a prion form[4], shows that prionization can lead to mass activation or repression of yeast genes and is suggestive of a link between the epigenetic phenomena of chromatin remodelling and prion formation.

We previously identified 11 yeast proteins whose overexpression facilitated the *de novo* appearance of the [*PSI*$^+$] prion in an unbiased screen: Cyc8, Lsm4, New1, Nup116, Pin2, Pin3, Pin4, Ste18, Swi1, Ure2 and Yck1 (ref. 8). As the appearance of [*PSI*$^+$] was also facilitated by the presence of the established prions [*PIN*$^+$] or [URE3], and the protein determinant of [URE3] was among the 11 proteins identified, we hypothesized that some of the other 10 proteins could also form prions[8]. This was further supported by the observation that all 11 proteins contained domains with an unusually high glutamine and asparagine (QN) content. Such QN-rich domains are found in all known yeast prions as part of the 'prion domain' required for prion formation and propagation[3,4]. Here we show that one of these proteins, Cyc8, can indeed form a prion.

Mutations in *CYC8* cause slow growth, defects in sporulation and mating, high iso-2-cytochrome *c*, flocculation, and invertase derepression[6,9,10]. Because inactivation of Cyc8 either by mutation or prion formation should be manifested as a loss-of-function phenotype[2], we used the *cyc8* mutant phenotype of increased levels of iso-2-cytochrome *c* (ref. 9) to select initially for the prion. Yeast needs cytochrome *c* to grow on the non-fermentable carbon source lactate[9]. Because 95% of a cell's cytochrome *c* (iso-1) is encoded by *CYC1*, *cyc1* mutants cannot grow if lactate is the only carbon source. However, a *cyc1* mutant can grow on lactate if the level of iso-2-cytochrome *c*, encoded by *CYC7*, is increased by inactivating the Cyc8–Tup1 repressor complex that represses the synthesis of iso-2-cytochrome *c* (refs 7, 9; Fig. 1a). Thus, to screen for cells propagating a Cyc8 prion, we selected for *cyc1Δ* yeast cells that grew well on lactate.

The appearance of a prion *de novo* is generally a rare event, but the transient overproduction of the protein's prion domain greatly enhances its chance of misfolding into a prion[2,3]. Thus, we overexpressed the C-domain of Cyc8 (residues 465–966), which is not essential for Cyc8 function and contains a highly Q-rich segment (52% of residues 491–668 are Q)[11,12]. This domain organization is reminiscent of the known yeast prion proteins, in which the functional and QN-rich prion domains are separate and distinct[3]. Although lactate$^+$ (Lac$^+$) colonies appeared spontaneously in a *cyc1Δ* haploid strain, presumably as a result of recessive spontaneous Lac$^+$ mutations such as *cyc8* mutations[9], we found that overproduction of Cyc8 (465–966) enhanced the appearance of Lac$^+$ derivatives more than 100-fold (data not shown). When the screen was repeated in a *cyc1Δ/cyc1Δ* diploid, Lac$^+$ derivatives appeared at a frequency of $(1.7 \pm 0.4) \times 10^{-5}$ ($n = 3$), whereas virtually no Lac$^+$ derivatives were seen in control plates without Cyc8 overexpression (Fig. 1b) because recessive spontaneous Lac$^+$ mutations were hidden in the diploid. The proposed, Lac$^+$, prion form of Cyc8 is designated as [*OCT*$^+$]. Because inactivation of Cyc8, either by mutation or by prion formation, would inhibit the sporulation of *MAT***a**/*MATα* diploid cells[6] and compromise the ability of *MATα*/*MATα* cells to mate[6], we isolated [*OCT*$^+$] candidates from a *cyc1Δ/cyc1Δ MAT***a**/*MAT***a** diploid, which can mate and thus allow genetic manipulations to examine prion propagation. We examined three independent [*OCT*$^+$] isolates from *MAT***a**/*MAT***a** cells, and

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Prions hijack tunnelling nanotubes for intercellular spread

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In variant Creutzfeldt–Jakob disease, prions (PrP^{Sc}) enter the body with contaminated foodstuffs and can spread from the intestinal entry site to the central nervous system (CNS) by intercellular transfer from the lymphoid system to the peripheral nervous system (PNS)[1]. Although several means[2–4] and different cell types[5–7] have been proposed to have a role, the mechanism of cell-to-cell spreading remains elusive. Tunnelling nanotubes (TNTs) have been identified between cells[8–12], both *in vitro* and *in vivo*[10,11,13], and may represent a conserved means of cell-to-cell communication[14–16]. Here we show that TNTs allow transfer of exogenous and endogenous PrP^{Sc} between infected and naive neuronal CAD cells[17]. Significantly, transfer of endogenous PrP^{Sc} aggregates was detected exclusively when cells chronically infected with the 139A mouse prion strain were connected to mouse CAD cells by means of TNTs, identifying TNTs as an efficient route for PrP^{Sc} spreading in neuronal cells. In addition, we detected the transfer of labelled PrP^{Sc} from bone marrow-derived dendritic cells to primary neurons connected through TNTs. Because dendritic cells can interact with peripheral neurons in lymphoid organs, TNT-mediated intercellular transfer would allow neurons to transport prions retrogradely to the CNS[1]. We therefore propose that TNTs are involved in the spreading of PrP^{Sc} within neurons in the CNS and from the peripheral site of entry to the PNS by neuroimmune interactions with dendritic cells.

To examine whether TNTs represent a biological method for prion spreading in neuronal cells we used CAD cells, a mouse neuronal cell line of catecholaminergic origin that expresses neuron-specific proteins[17]. These cells have been shown to be an excellent neuronal cell culture model because they can differentiate[17] and they have also been shown to efficiently replicate prion strains[18].

We found that CAD cells readily form membrane bridges[19] that contain actin and do not touch the substratum[9–12] (Fig. 1a). To determine the frequency of these structures and to exclude incomplete cytokinesis of daughter cells, we co-cultured cells transfected with green fluorescent protein (GFP)–actin and Cherry–actin (Fig. 1b). Overall, these experiments indicated that 44 ± 6% (s.d.) of the observed CAD cells could efficiently form actin-containing membrane bridges of different lengths and diameters between differentially labelled populations (Fig. 1b–d; see Supplementary Methods). To determine whether some of these connections were tunnelling nanotubes that would permit intercellular vesicle transfer, we co-cultured untransfected CAD cells with GFP–actin-transfected cells. After 24 h, the cells were labelled with LysoTracker red and imaged by spinning-disk confocal microscopy (Fig. 2a). Figure 2a shows a GFP–actin-transfected CAD cell (green) that has extended a tubular structure to a cell stained with LysoTracker (red). Over time, a LysoTracker-positive vesicle can be seen moving toward the GFP–actin-transfected cell and entering its cytoplasm (Fig. 2b; Supplementary Movie S1), indicating that this tube was in fact a functional TNT. Measurements of the mean square displacement of vesicles demonstrate that the vesicle has a directed movement towards the recipient cell, as opposed to random Brownian movement (Supplementary Information, Fig. S1a). The estimated velocity of the tracked vesicle (Fig. 2c; Supplementary Movie S2) is very similar to that calculated for a complex of myosin VI and the GLUT1 transporter binding protein[20], indicating the possible involvement of actin-mediated motors, as previously suggested for TNT vesicular transport[8]. Thus, in CAD cells, vesicles of lysosomal origin are actively transferred intercellularly through TNTs.

To analyse whether CAD cells could use TNTs to transfer PrP^{C} in live conditions, we transfected them with GFP-tagged wild-type PrP (GFP–PrPwt). Interestingly, GFP–PrPwt could be seen in TNTs, including in networks bridging multiple cells (Fig. 2d), both at the surface and

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Nuclear transport factor directs localization of protein synthesis during mitosis

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Export of messenger RNA from the transcription site in the nucleus and mRNA targeting to the translation site in the cytoplasm are key regulatory processes in protein synthesis. In yeast, the mRNA-binding proteins Nab2p and Nab4p/Hrp1p accompany transcripts to their translation site, where the karyopherin Kap104p mediates both their dissociation from the mRNA and their transport back into the nucleus. We found that Kap104p localized to the distal bud tip and the bud neck during cell division, resulting in a localized release of translation-competent mRNA and increased protein synthesis in the emerging daughter cell. Temporally and spatially coordinated localization of Kap104p is a new mechanism for the asymmetric distribution of protein synthesis in dividing cells.

During its maturation, mRNA is complexed with a changing repertoire of proteins, a fraction of which act co-transcriptionally. These proteins are involved in the modification of mRNA, including 5′-end capping, splicing, 3′-end cleavage and poly(A) addition, and only fully processed transcripts are exported from the nucleus[1]. In addition to mRNA processing, a complex of proteins bound to a single mRNA, called messenger ribonucleoprotein (mRNP), also regulates the export, targeting, stability and translatability of the transcript[2–6]. Trafficking of mRNP to distinct cellular sites is a common mechanism for ensuring protein synthesis at defined subcellular sites, and it has been found to regulate cell polarity as well as asymmetry during development and differentiation in animals, plants and fungi[7].

The transport of molecules between the cytoplasmic and nuclear compartments occurs through nuclear pore complexes (NPCs)[8] and involves the formation of a carrier–cargo complex, translocation through the NPC, release of the cargo molecule at the *trans* side, and recycling of the carrier. Most nuclear transport involves the binding of a carrier of the β-karyopherin superfamily to a nuclear localization signal (NLS) present on the cargo. In contrast, mRNA export employs several proteins unrelated to karyopherins, such as the Mex67–Mtr2 heterodimer[2,3] and nuclear polyadenylated RNA-binding proteins 2 and 4 (Nab2p and Nab4p/Hrp1p). Nab2p is a protein related to human heterogeneous nuclear RNP (hnRNP) that binds poly(A) RNA with high affinity, as demonstrated by its dissociation constant of about 30 nM (refs 9–12). Nab2p is essential for mRNA export of a subset of transcripts, and its absence leads to the accumulation of poly(A) RNA in the nucleus[12–14]. Nab4p/Hrp1p is the yeast protein most similar in sequence to mammalian hnRNP A1. In addition to being a factor for the export of mRNA, Nab4p is a component of the cleavage factor I complex[15]. Another abundant mRNP component is poly(U)-binding protein (Pub1p). In the cytoplasm, the bulk of Nab2p interacts directly with Pub1p, and this interaction modulates transcript stability[16]. Before translation, Nab2p and Nab4p are released from the mRNA. The binding of cytosolic karyopherin Kap104p (Kapβ2/Trn in metazoans) to the NLSs of Nab2p (rgNLS) and Nab4p has been implicated in this release[9,17–19]. The release of Nab2p from the mRNA is also mediated by the ADP-bound form of the DEAD-box RNA helicase Dbp5p[12]. After the release of Nab2p and Nab4p, the Kap104–cargo complex is imported into the nucleus, where RanGTP and mRNA act cooperatively to dissociate both Nab2p and Nab4p from Kap104p, probably resulting in only a small fraction of Nab2p not being bound to mRNA or Kap104p[9].

Because of its function as a nuclear transport factor, Kap104p was expected to be uniformly distributed in the cytoplasm. We determined the subcellular localization of Kap104p in a *Saccharomyces cerevisiae* strain that produces a carboxy-terminally tagged Kap104p–GFP (green fluorescent protein) from the genomic *KAP104* locus. Surprisingly, Kap104p–GFP accumulated roughly 10-fold compared with the cytoplasm at the distal tip of the daughter cell during early mitotic phase of the cell cycle (referred to hereafter as M phase) and about 50-fold at the bud neck during late M phase; that is, at and after nuclear division (Fig. 1a–c; Supplementary Information, Movie S1). Kap104p was the only one of the 14 known karyopherins in yeast that localized to the bud neck or distal tip (not shown). A FRAP (fluorescence recovery after photobleaching) study indicated that Kap104p–GFP was still mobile at the bud neck and the bud tip, with a half time of recovery ($t_{1/2}$) of 4–8 s (Fig. 1d). The cytoplasmic concentration of Kap104p–GFP during G1 phase (no bud present) was about 1 μM (Fig. 1e) as determined from the fluorescence intensity, which corresponds to about 25,000 mol-

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Monomeric fluorescent timers that change color from blue to red report on cellular trafficking

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Based on the mechanism for chromophore formation in red fluorescent proteins, we developed three mCherry-derived monomeric variants, called fluorescent timers (FTs), that change their fluorescence from the blue to red over time. These variants exhibit distinctive fast, medium and slow blue-to-red chromophore maturation rates that depend on the temperature. At 37 °C, the maxima of the blue fluorescence are observed at 0.25, 1.2 and 9.8 h for the purified fast-FT, medium-FT and slow-FT, respectively. The half-maxima of the red fluorescence are reached at 7.1, 3.9 and 28 h, respectively. The FTs show similar timing behavior in bacteria, insect and mammalian cells. Medium-FT allowed for tracking of the intracellular dynamics of the lysosome-associated membrane protein type 2A (LAMP-2A) and determination of its age in the targeted compartments. The results indicate that LAMP-2A transport through the plasma membrane and early or recycling endosomes to lysosomes is a major pathway for LAMP-2A trafficking.

Monomeric fluorescent proteins of various emission wavelengths have become invaluable tools for studying the spatial behavior of intracellular molecules, including their localization and interaction[1]. To visualize temporal and spatial molecular events, FTs[2], which change their emission wavelengths over time, could be especially valuable. The only currently available FT is DsRed-Timer FT (also known as DsRed-E5)[3]; however, it is a tetramer, which prevents its application as a protein fusion tag. Nevertheless, the tetrameric state of the DsRed-Timer does not limit its use to study gene activities[4], relative age of organelles[5] and cell differentiation[3].

It has been suggested that a red DsRed-like chromophore in the red fluorescent proteins (RFPs) results from an oxidation of a protonated blue form of the GFP-like chromophore, not from the green anionic form, which is a dead-end product[6]. This suggested scheme for red chromophore maturation provides a basis for developing monomeric FTs that change their color from blue to red. The most suitable templates for this appear to be the monomeric variants of DsRed[7]. One of these variants, mCherry, was chosen for a directed molecular evolution to develop three monomeric FTs with different maturation rates between the protonated blue GFP-like and the anionic red DsRed-like chromophore states.

FTs can be used as molecular genetically encoded tools to study trafficking of different cellular proteins and to provide accurate insight into the timing of intracellular processes. The sequence of events during trafficking of different cellular proteins before they reach their final compartment has often been the subject of contradictory investigations. An example of a long-standing dilemma is the contribution of different pathways to trafficking and final delivery of LAMP-2A to lysosomes. LAMP-2A is encoded by a spliced variant of the *lamp2* gene and acts as a lysosomal receptor for chaperone-mediated autophagy (CMA)[8,9]. LAMP-2A is sorted to endosomes and lysosomes through a tyrosine-based signal in its cytosolic C terminus, but a fraction of the protein is also observed at the plasma membrane[10]. This dual location led investigators to propose the existence of both direct (Golgi to lysosomes) and indirect (through endocytosis from the plasma membrane) targeting of LAMP-2A to lysosomes[11]. The use of FTs has allowed us to study the sequence of events involved in transport of LAMP-2A to lysosomes and to determine the preferential pathway followed by LAMP-2A for its trafficking.

RESULTS

Development of monomeric FTs

The gene encoding mCherry was used as a template for the multiple saturated mutagenesis at positions 42, 44, 65, 69, 106, 148, 203 and 224 (amino acid numbering follows that of *Aequorea victoria* GFP; see **Fig. 1a**). These positions were identified either according to the X-ray structure of mCherry[12] or using experimental data regarding amino acid substitutions affecting maturation of DsRed variants[13–15]. It has been shown that substitutions at these positions slow or accelerate formation of the red chromophore. According to the mCherry structure, residues 42 and 44 are close to the Met65 residue of the mCherry chromophore, and residues 69, 148 and 203 are close to the phenolic and/or imidazolinone rings of the chromophore. Residue 224 was found in close proximity to residue 203 and has been suggested to indirectly influence chromophore positioning. Therefore, a bacterial

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nature structural & molecular biology

Transient ribosomal attenuation coordinates protein synthesis and co-translational folding

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Clustered codons that pair to low-abundance tRNA isoacceptors can form slow-translating regions in the mRNA and cause transient ribosomal arrest. We report that folding efficiency of the *Escherichia coli* multidomain protein SufI can be severely perturbed by alterations in ribosome-mediated translational attenuation. Such alterations were achieved by global acceleration of the translation rate with tRNA excess *in vitro* or by synonymous substitutions to codons with highly abundant tRNAs both *in vitro* and *in vivo*. Conversely, the global slow-down of the translation rate modulated by low temperature suppresses the deleterious effect of the altered translational attenuation pattern. We propose that local discontinuous translation temporally separates the translation of segments of the peptide chain and actively coordinates their co-translational folding.

The full set of 20 amino acids is encoded by 61 sense codons and, except for methionine and tryptophan, all other amino acids are encoded by several synonymous triplets. These are read by several isoaccepting tRNAs that vary substantially (at least ten-fold) in their cellular concentrations[1,2]. Consequently, the asymmetric tRNA abundance can cause variation in the rate of translation of each single codon. The translation rate is maximized at codons with highly abundant cognate tRNA and minimized at codons read by rare tRNAs[3]. Importantly, the concentration of isoaccepting tRNAs for a set of synonymous codons varies among organisms, tissues and stages of differentiation[2,4], and the codon-reading program can be dramatically altered upon amino acid starvation[5].

The concentrations of the full set of tRNAs are known for only few organisms, among them *Escherichia coli* and *Bacillus subtilis*[1,6]; however, the tRNA copy number has been shown in general to positively correlate with the frequency of synonymous codon usage[2], and the effect of translational rate on any process downstream of translation has been interpreted in the context of codon usage. Some codons from each synonymous set of codons are more frequently used ('codon bias') and tend to dominate in highly expressed genes[7]. Codon bias differs in various organisms, and the failure of recombinant gene expression in heterologous hosts is often attributed to incompatibility of the codon-usage programs among the organisms[3,8].

Although it is not clear why some codons are preferred over others, single-nucleotide polymorphisms causing synonymous substitutions (that is, changing the codon-usage pattern without any amino acid substitution) in one or more codons can alter substrate specificity, viral virulence or differentiation-dependent protein expression[3,9,10]. The codon distributions might be related to secondary-structural elements in proteins: α-helices are encoded by more frequent codons, whereas β-structures, loops and disordered structures are enriched in infrequent codons[11]. The underrepresented triplets pair to low-abundance isoacceptor tRNAs, and therefore it is likely that they will be slowly translated[12]. Local slow-down of the elongation rate is involved in translational regulation[13], or specific sequences in the mRNA may have coevolved to cause transient translational arrest, which can guide interactions with other proteins and regulate expression or facilitate membrane insertion[14,15]. Previous work proposed that discontinuous translation might be needed to temporally separate the translation of defined protein segments to facilitate their co-translational folding[16]. This hypothesis is difficult to prove *in vivo* because the dynamics of individual ribosomes are stochastic and nonsynchronized, and re-initiation of translation cannot be efficiently prevented. So far, experimental evidence supporting this hypothesis has been scarce[17,18], and the precise effect of the nonlinear translation rate on the early steps of folding of nascent chains remains a fundamentally unresolved issue.

Taking advantage of the vast amount of data (proteomic, transcriptomic, tRNA and protein abundance) available for *E. coli*, we have used a sensitive system that allows us to map the folding status of translation intermediates to determine whether the local discontinuous translation at certain regions in the mRNA sequence is needed to efficiently coordinate the rate of elongation of the peptide chain and its co-translational folding.

RESULTS

Algorithm to predict translational attenuation sites

To determine whether the rate of translation could be part of a control loop for co-translational folding, we first performed a transcriptome-wide search to identify putative regions for translational attenuation in

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nature genetics

Intrinsic variability of gene expression encoded in nucleosome positioning sequences

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Variation in gene expression is an essential material for biological diversity among single cells[1–3], individuals[4–6] and populations or species[7–9]. Here we show that expression variability is an intrinsic property that persists at those different levels. Each promoter seems to have a unique capacity to respond to external signals that can be environmental, genetic or even stochastic. Our investigation into nucleosome organization of variably responding promoters revealed a commonly positioned nucleosome at a critical regulatory region where most transcription start sites and TATA elements are located, a deviation from typical nucleosome-free status. The nucleotide sequences in this region of variable promoters showed a high propensity for DNA bending and a periodic distribution of particular dinucleotides, encoding preferences for DNA–nucleosome interaction. Variable expression is likely to occur during removal of this nucleosome for gene activation. This is a unique example of how promoter sequences intrinsically encode regulatory flexibility, which is vital for biological processes such as adaptation, development and evolution.

It has recently been suggested that there is a common genetic signature that is associated with genetic expression variations at different levels[7,10]. Moreover, genetically variable genes are also sensitive to changing internal and external conditions, regardless of whether they are specific environmental cues or stochastic fluctuations. The presence of a TATA box has been suggested to enhance the sensitivity of gene expression to both genetic and environmental perturbations[7,10], but the underlying mechanism remains to be elucidated.

Here we collected a variety of genome-wide studies in yeast that reported expression variation arising from various origins as measured among single cells, different conditions, individuals, strains and species (summarized in **Table 1** and given in **Supplementary Table 1** online). Comparison of the seven variability measures showed a highly significant correspondence among them (**Fig. 1a**). For example, as a general variability measure with a large sample size (>1,500 experiments[7]), responsivenessis intimately related to stress response, *trans* variability and expression divergence (Spearman's rank correlation, $R > 0.50$ and $P \approx 0$).

We have previously shown that *trans* variability explains about 70% of individual variation observed among segregants of a cross between two strains[11]. Notably, *trans* variability was mostly accounted for by chromatin regulation. Chromatin regulation effect was measured by deletion effects of chromatin regulators on the expression of each gene. Transcription-factor regulation effect, as measured by deletion effects of transcription factors, was not significant compared to chromatin regulation effect.

Given these findings, we measured the impact of chromatin regulation effect on the various variability measures. As expected, chromatin regulation effect was closely associated with all the measures, especially with responsiveness, *trans* variability and expression divergence ($R \geq 0.40$ and $P < 10^{-220}$; **Fig. 1a**). When considered simultaneously, the influence of chromatin regulation effect was markedly higher than that of transcription-factor regulation effect

Table 1 Genome-wide studies on gene expression variation in yeast

Source of variation	Observation level	Datasets and descriptions
Stochasticity	Single cells	Stochastic noise: variation measured among genetically, environmentally identical single cells[3]
Environmental perturbations	Conditions	Responsiveness: variability measured from curated datasets representing various conditions[7]
		Stress response: differences in response to diverse defined stress conditions[25]
Genetic perturbations	Individuals or lines	*trans* variability: variability caused by different *trans*-acting genetic loci among individuals[11]
		Mutational variance: variance among mutation accumulation lines[10]
Evolution	Strains or species	Interstrain variation: variation among four natural isolates[8]
		Expression divergence: variation between yeast species under controlled environmental conditions[7]

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nature genetics

Gene expression divergence in yeast is coupled to evolution of DNA-encoded nucleosome organization

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Eukaryotic transcription occurs within a chromatin environment, whose organization has an important regulatory function and is partly encoded in *cis* by the DNA sequence itself. Here, we examine whether evolutionary changes in gene expression are linked to changes in the DNA-encoded nucleosome organization of promoters. We find that in aerobic yeast species, where cellular respiration genes are active under typical growth conditions, the promoter sequences of these genes encode a relatively open (nucleosome-depleted) chromatin organization. This nucleosome-depleted organization requires only DNA sequence information, is independent of any cofactors and of transcription, and is a general property of growth-related genes. In contrast, in anaerobic yeast species, where cellular respiration genes are relatively inactive under typical growth conditions, respiration gene promoters encode relatively closed (nucleosome-occupied) chromatin organizations. Our results suggest a previously unidentified genetic mechanism underlying phenotypic diversity, consisting of DNA sequence changes that directly alter the DNA-encoded nucleosome organization of promoters.

Changes in transcriptional regulation are important for generating phenotypic diversity among species, but the mechanisms underlying these regulatory changes are not well understood. Consistent with the centrality of transcription factors to transcriptional control, some phenotypic changes have been associated with changes in the binding-site content of promoters[1] or with changes in the targets bound by transcription factors[2]. However, modulation of other processes key to transcriptional regulation may also lead to phenotypic diversity. Recent studies that measured nucleosome occupancy genome-wide have revealed strong associations between chromatin organization and gene expression[3–9], and other studies have shown that the organization of nucleosomes is partly encoded in the genome through the sequence preferences of nucleosomes[3,10–13]. However, the relationship between evolutionary changes in DNA-encoded nucleosome organization and expression divergence has not been examined.

Here, we study the relationship between gene expression and the DNA-encoded nucleosome organization of promoters across two yeast species, the budding yeast *Saccharomyces cerevisiae* and the human pathogen *Candida albicans*, for which large compendia of gene expression data are available. These species show several phenotypic differences. Most notably, in high glucose, *C. albicans* grows by respiration and correspondingly activates transcription of genes required for the TCA cycle and oxidative phosphorylation, whereas *S. cerevisiae* grows primarily by fermentation and correspondingly reduces transcription of respiration genes. We henceforth term the respiratory growth 'aerobic' and the fermentative growth 'anaerobic'. Our approach consists of three steps. First, we quantify the extent to which the gene expression patterns of biologically meaningful gene sets are conserved across the two species. Next, we examine the DNA-encoded nucleosome organization of these gene sets using both a computational model and *in vitro* reconstitutions of nucleosome on purified DNA from each species. Finally, we test whether orthologous gene sets with divergent expression patterns between the two species show corresponding changes in their DNA-encoded nucleosome organization.

RESULTS

Expression changes linked to aerobic versus anaerobic growth

We downloaded two large collections of microarray-based gene expression data from ~1,000 and ~200 different cellular states and environmental conditions in *S. cerevisiae* and *C. albicans*, respectively, compiled in ref. 1. To compare the expression patterns of orthologous genes, we downloaded a yeast orthology map[14] and quantified the degree to which the co-expression relationships of a gene in one species are similar to the co-expression relationships of its orthologous counterpart in the other species. Such an approach has been successfully used to compare expression patterns across distant species[15,16].

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Human mutation rate associated with DNA replication timing

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Eukaryotic DNA replication is highly stratified, with different genomic regions shown to replicate at characteristic times during S phase. Here we observe that mutation rate, as reflected in recent evolutionary divergence and human nucleotide diversity, is markedly increased in later-replicating regions of the human genome. All classes of substitutions are affected, suggesting a generalized mechanism involving replication time-dependent DNA damage. This correlation between mutation rate and regionally stratified replication timing may have substantial evolutionary implications.

Evolutionary divergence and inferred mutation rates are known to vary across the human genome[1–3], and it has long been speculated that this is a consequence of covariance with an epigenetic feature[1,2]. In human cells, the temporal order of DNA replication during an S phase lasting approximately 10 h shows marked regional variability across the genome[4,5]. To parallel the conventional division of S phase into four sequential temporal states (S1–S4), we used a hidden Markov model[6] to perform unbiased four-state partitioning of continuous, high-resolution replication timing measurements across 1% of the human genome[7]. We then determined human-chimpanzee sequence divergence rates and the density of well-ascertained SNPs[8] at putatively neutrally evolving sites within each temporal state, excluding any bases within annotated exons, repetitive elements, CpG islands, transcriptional start sites (± 2 kb), intronic splice sites and conserved noncoding sequences[9] (**Supplementary Tables 1** and **2** online).

We observed a pronounced trend relating the rate of evolutionary divergence and the density of human SNPs to the progress of DNA replication (**Fig. 1**). Human-chimpanzee substitutions and human SNP density increase 22% and 53%, respectively, during the temporal course of replication, both of which are highly statistically significant ($P < 8.43 \times 10^{-26}$, Cochran-Armitage; **Fig. 1a–c,g–i**). To rule out potential confounding by the overall low genome-wide rate of human-chimpanzee divergence, we also analyzed human-macaque divergence, with similar results ($P < 2.7 \times 10^{-54}$; **Fig. 1d–f**). We confirmed the absence of bias due to a sampling or stratification effect across different genomic regions by testing (Cochran-Mantel-Haenszel) for three-way interactions, treating region assignment as controlling variable ($P < 7.2 \times 10^{-12}$, $P < 0.00026$ for human-chimpanzee divergence and human SNPs, respectively). Additionally, we repeated all analyses with an independent set of randomly ascertained SNPs (Celera individual A versus NCBI build 35), with nearly identical effect ($P < 9.69 \times 10^{-22}$).

Next we examined whether the observed correlation between mutation rate and replication time could be explained by variation in another genomic feature for which replication timing might be acting as a surrogate. Regional variation in G+C content[2,3] and, independently, recombination rate[2,3] have been invoked as potential causes of human mutation rate variation. We therefore obtained the distribution of G+C content, CpGs, recombination hot spots[9], and gene, exon and conserved noncoding sequence[9] densities in sliding nonoverlapping 50-kb windows (approximating the size of chromosomal domains linked to replicons) across each temporal replication state (**Supplementary Fig. 1** online). We binned each distribution into three classes (low, medium and high content), with an equal number of windows at each level and carried out separate tests for three-way interactions using each factor as a controlling variable (total 12 tests). All were highly significant, with P values not exceeding 3.0×10^{-12} (**Table 1**), as were repeated tests with the additional permutation resampling of temporal states ($P < 5.0 \times 10^{-6}$ for divergence; $P < 2.2 \times 10^{-4}$ for SNPs; **Table 1**).

To address potential interplay between more than one variable, we developed multiple regression models of both divergence and diversity, confirming the independent effect of replication timing (**Supplementary Tables 1** and **2** and **Supplementary Fig. 2** online). These models suggest that replication time alone may explain 40–70% of the variability explained by the full model, and ~8% of overall variability in diversity and divergence. The observed correlation between rates of nucleotide change and replication timing is therefore highly unlikely to be caused by variation in G+C content or by a mutagenic effect of recombination. To rule out any hidden dependence on window size, we repeated all analyses conditioned on smaller (30 kb) and larger (100 kb) windows, with equivalent results (**Supplementary Fig. 3** online). The direction of effect of replication timing on evolutionary divergence and SNP density is highly similar when other genomic features are controlled. These findings are compatible with a process that influences mutation rate, which should affect both diversity and divergence in a stable fashion over evolutionary time. Furthermore, the findings persist across the spectrum of selected sites, from ancestral repeats and fourfold degenerate sites to conserved noncoding sequences and nondegenerate coding sites (**Supplementary Fig. 4** online), and across the human and chimpanzee lineages following the split from macaque (**Supplementary Fig. 5** online). We note, however, that the effect on SNP density appears larger than that on evolutionary divergence.

We next considered whether the relationship with mutation rate might be due to a consequence of transcription such as

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