2018

All The Right Noises: Causes And Consequences Of Stochastic Trimethylamine Oxide Reductase Expression In Escherichia Coli

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Abstract
Microbial populations can maximize fitness in dynamic environments through bet hedging, a process wherein a subpopulation assumes a phenotype not optimally adapted to the present environment but well adapted to an environment likely to be encountered. Here we show that oxygen induces fluctuating expression of the trimethylamine oxide (TMAO) respiratory system of Escherichia coli, diversifying the cell population and enabling a bet-hedging strategy that permits growth following oxygen loss. This regulation by oxygen affects the variance in gene expression but leaves the mean unchanged. We show that the oxygen-sensitive transcription factor IscR is the key regulator of variability. Oxygen causes IscR to repress expression of a TMAO-responsive signaling system, allowing stochastic effects to have a strong effect on the output of the system and resulting in heterogeneous expression of the TMAO reduction machinery. This work reveals a mechanism through which cells regulate molecular noise to enhance fitness. Further regulation of TMAO reductase expression is introduced during lysogenic infection by bacteriophage HK022. The HK022 prophage completely suppresses aerobic TMAO reductase expression, also by altering expression of the TMAO-responsive signaling system, and infected cells lose bet-hedging behavior. The prophage appears to control expression of the signaling system by disrupting a host promoter and replacing it with a prophage-encoded promoter. HK022-like prophages occur with some regularity in wild E. coli strains and may be important environmental regulators of TMAO respiration. These findings provide an unusual example of bacteriophage-host interaction in which a prophage reconfigures the regulation of a host metabolic process by rewiring the transcriptional control of a host gene.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Biochemistry & Molecular Biophysics

First Advisor
Mark Goulian

Keywords
anaerobic respiration, bacteriophage, bet hedging, gene expression variability, trimethylamine oxide, two-component signaling

Subject Categories
Biology | Microbiology | Molecular Biology

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ALL THE RIGHT NOISES: CAUSES AND CONSEQUENCES OF STOCHASTIC TRIMETHYLAMINE OXIDE REDUCTASE EXPRESSION IN ESCHERICHIA COLI

Jeffrey N. Carey

A DISSERTATION

in

Biochemistry and Molecular Biophysics

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2018

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For Michael
ACKNOWLEDGMENT

I don’t know how I managed to secure a mentor who ended up being the absolutely perfect match for me, but somehow I did in Mark Goulian. His ingenuity, rigor, and imagination have provided the template for the type of scientist I want to become, while his kindness, modesty, and patience have provided the template for the type of person I want to become. It has been a pleasurable challenge to attempt to keep up with his wit and sense of humor, and each new addition to the parade of contraptions he has invented for the lab has been a terrific surprise and a reminder that one can do anything one bothers to learn how to do. The depth of my gratitude and regard for Mark is profound, and I hope to keep him as a mentor even though I will no longer be his student.

I offer sincere thanks as well to my lab family: Manuela Roggiani, Sam Yadavalli, Jane Schulte, Annie Chen, and Daniel Fishman-Engel. Manuela’s research paved the way for all the work in this dissertation—in fact, I would venture that all the hardest parts were already done by her before I picked up the project—and her microbiological acumen plus her endless willingness to help others saved me from hundreds of wasted hours. Sam was a delightful baymate, her cheerful disposition the perfect foil to my more reticent mien, who provided many useful tips for navigating science and grad school and always knew how to talk me off the ledge when things weren’t going well. Jane, my fellow BMB grad student, was a model of determination in the face of stubborn scientific problems, an exemplary provider of honest feedback, and often exactly the right person to talk to about whatever happened to be on my mind (scientific or not). Annie, my fellow non-BMB grad student, possessed a work ethic that put mine to shame and was my number one concertgoing companion and orchestra compatriot, and her social and political engagement was an important inoculation against complacency. Jane and Annie’s sharp intellects and scientific integrity set a high standard in the lab that I only hope I was able to live up to. I am very lucky to have had them first as colleagues and then as friends. Daniel, my undergraduate trainee, taught me a great deal about teaching and was astonishingly patient with my fumbles and vague guidance as I learned how to be a mentor, and his eagerness and drive to
learn were irresistible. Finally, I would like to thank all the physicians, research staff, visiting scholars, rotation students, undergraduates, and middle and high school students and teachers who spent time in the Goulian lab during my tenure. I learned something from all of them.

I owe a very special acknowledgement to the members of the Philosophy of Science Club, particularly the core members Dirk Auman, Abby Cember, Joe Jordan, Hannah Richter, and the aforementioned Annie and Jane. I didn’t know how much I needed a forum for examining the meanings and implications of what we do as scientists—and humans—until joining this group. It has been immensely valuable to be able to share thoughts with this open-minded and insightful collection of friends, and I believe I have grown significantly as a thinker and as a person through our heartfelt and candid conversations.

I am grateful for the support I’ve received from the Biochemistry and Molecular Biophysics graduate group and for the wonderful students in my cohort. I will miss the good times we had the annual retreat—a definite highlight of every year—and wish we could have spent more time together before the inevitable diaspora. Thank you to my VMD-PhD program friends for helping me negotiate the challenges of endurance and culture shock endemic to the curriculum, and thanks as well to my VMD-PhD program advisor, Mike Atchison, for helping me stay on track through this long process.

There are many people to thank for their contributions to the scientific content of this dissertation, foremost among them the members of my dissertation committee: Arjun Raj, Fevzi Daldal, Jay Zhu, and Andy Binns. I came close to joining Arjun’s lab, and I almost wish I could do grad school a second time so that I could have a second mentor and have it be him. I truly appreciate his willingness to suffer through the minutiae of prokaryotic biology so that I could leverage his expertise on transcriptional noise and single-cell biology. His approach to the presentation of scientific information taught me a great deal about how to engage a broad audience, and I will never again have Figure 1 of a manuscript be a bar chart. Fevzi was very generous with his time (particularly in setting up and troubleshooting the anaerobic chamber) and his deep knowledge of bacterial respiration. He always brought fresh ideas to discussions of my
project, and I wish I had a fraction of his understanding of transition metal biochemistry. I suspect many of the remaining secrets of the Tor system are hidden away in its metal-binding enzymes. Jay never failed to be a voice of encouragement, and he always thought to ask the questions that I should have known to ask myself. I very much look forward to the road trip buddy movie starring Jay and Mark that, hopefully one day, will be coming soon to a theater near you. Finally, Andy’s encouragement, enthusiasm for science, friendly presence in lab, and expertise in two-component signaling were all of great value in completing my dissertation research, and his tales of the early days of transgenic plants and the working out of Agrobacterium tumefaciens virulence were consistently fascinating. Just as I couldn’t have wished for a better mentor than Mark, I couldn’t have dreamed up a better dissertation committee than these four expert scientists.

For further scientific contributions, I am much indebted to Tricia Kiley, Erin Mettert, Kevin Myers, Sergei Vinogradov, and Josie Clowney. Tricia, Erin, and Kevin made the entire IscR story possible, contributing essential ideas and data and performing key experiments. Without their involvement, this story would have gone nowhere. Sergei created and permitted us to use the technology that allowed for the measurement of oxygen concentration in experimental setups where the use of a conventional oxygen probe would have been impossible. He also generated the requisite calibration curves, spent many hours explaining to me the principles behind the technology (appropriately dumbed down to my level of understanding), and patiently fielded my endless barrage of questions. Finally, I owe a great deal to my college friend Josie, now a professor at the University of Michigan, for her encouragement, thoughtful reading of my manuscript, and invaluable advice on submitting for publication.

I thank my family, particularly my parents Brent and Valerie Carey, for the preparation they gave me for this long journey, the love and steadfast support they gave me during it, and for coming to terms with the fact that the answer to the question “so when are you going to graduate?” was always—until a few months ago—“I don’t know.” My non-human family deserves credit as well: Killian, Iris, Oscar, and above all Poth, whose memory I will always cherish.
My final acknowledgment goes to Michael O’Brien, my husband and the dedicatee of this work. We met in Chicago a decade ago this year. Michael uprooted his life for me, leaving his friends and home to move to Philadelphia when I decided to enter the VMD-PhD program here. He took care of me when I wouldn’t take care of myself, was patient with my many moods, reminded me to take pride in my accomplishments, and filled my life with boundless, undeserved love—all while earning three degrees of his own. Michael, I love you more every day and don’t know how I could have done any of this without you.
ABSTRACT

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Jeffrey N. Carey
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Microbial populations can maximize fitness in dynamic environments through bet hedging, a process wherein a subpopulation assumes a phenotype not optimally adapted to the present environment but well adapted to an environment likely to be encountered. Here we show that oxygen induces fluctuating expression of the trimethylamine oxide (TMAO) respiratory system of Escherichia coli, diversifying the cell population and enabling a bet-hedging strategy that permits growth following oxygen loss. This regulation by oxygen affects the variance in gene expression but leaves the mean unchanged. We show that the oxygen-sensitive transcription factor IscR is the key regulator of variability. Oxygen causes IscR to repress expression of a TMAO-responsive signaling system, allowing stochastic effects to have a strong effect on the output of the system and resulting in heterogeneous expression of the TMAO reduction machinery. This work reveals a mechanism through which cells regulate molecular noise to enhance fitness. Further regulation of TMAO reductase expression is introduced during lysogenic infection by bacteriophage HK022. The HK022 prophage completely suppresses aerobic TMAO reductase expression, also by altering expression of the TMAO-responsive signaling system, and infected cells lose bet-hedging behavior. The prophage appears to control expression of the signaling system by disrupting a host promoter and replacing it with a prophage-encoded promoter. HK022-like prophages occur with some regularity in wild E. coli strains and may be important environmental regulators of TMAO respiration. These findings provide an unusual example of bacteriophage-host interaction in which a prophage reconfigures the regulation of a host metabolic process by rewiring the transcriptional control of a host gene.
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The absolute change which threatens us from our birth until our death remains perpetually unpredictable and incomprehensible.

–Jean-Paul Sartre, *Being and Nothingness*
CHAPTER 1: Introduction

Survival in an uncertain world requires adaptability and anticipation, the complementary abilities to respond to and prepare for change. Bacteria, like all organisms, possess these capabilities. Bacterial adaptation has long been appreciated, and our understanding of how it occurs has greatly increased over the last several decades with the help of single-cell studies. The analysis of properties such as gene expression status, physiological state, and protein localization in individual cells in a population has revealed numerous examples of phenotypic diversification—the manifestation of more than one phenotype across a population of genetically identical cells. In many cases such cell-to-cell variability appears to function as a kind of bacterial “anticipatory behavior” in which a diverse population is more likely to contain individuals that can survive a sudden change in the environment than a homogeneous population (Ackermann, 2015; Freddolino and Tavazoie, 2012).

Anticipatory phenotypic diversification is commonly known as bet hedging. A population that hedges its bets protects itself against unpredictable future events by harboring individuals that are optimally adapted for life in an environment to which the population may be exposed in the future rather than the environment to which it is exposed at present (de Jong et al., 2011; Martins and Locke, 2015; Norman et al., 2015). The maladapted individuals suffer reduced fitness as long as the phenotype/environment mismatch persists. However, should the population experience a rapid environmental shift, those individuals that had been preadapted to the new environment can thrive even though the rest of the population suffers. This strategy allows the population to survive chance events that, because of their rapidity or severity, would be difficult to contend with by post hoc adaptation.

Bet hedging as described above is what is usually meant when the concept is applied to populations of microbes, but there are specific quantitative requirements that must be met for a behavior to conform to the formal mathematical definition of bet hedging. In the formal definition, bet hedging maximizes the geometric mean fitness of an isogenic population across different
environments by minimizing the variance in the mean number of offspring produced by individuals in the different environments (Grimbergen et al., 2015; Philippi and Seger, 1989; Simons, 2011). This concept of biological bet hedging was developed by evolutionary biologists studying macroscopic organisms and was later embraced by microbiologists seeking to understand phenotypic heterogeneity and population dynamics in microorganisms. Data have been accumulating that fit within the bet hedging framework as applied to microbes, but very few experimental studies in microbiology have included sufficient analysis to fulfill the formal definition (de Jong et al., 2011; Simons, 2011; Viney and Reece, 2013). This could be because the data required for such analyses are difficult to obtain or because making strong evolutionary claims is not the goal of most experimental studies of phenotypic diversification. In any case, the appellation “bet hedging” in experimental microbiology rarely functions as a rigorous mathematical evaluation of a phenomenon but rather as a qualitative descriptor. For convenience, we will be applying the term to behavior that appears to be consistent with the mathematical definition, given what is currently known about the system, even when (as in most cases) a complete quantitative analysis has not been performed.

Bet-hedging behaviors in bacteria can be roughly placed into two categories, bimodal or unimodal, by the pattern of phenotypic diversification exhibited by a population. In the bimodal pattern the population bifurcates into subpopulations with distinct phenotypes, whereas in the unimodal pattern the population exhibits a broad distribution of phenotypes that does not resolve into clearly demarcated subpopulations (Garcia-Bernardo and Dunlop, 2016). Examples of the bimodal pattern include sporulation (Veening et al., 2008b) and competence (Maamar and Dubnau, 2005; Smits et al., 2005) in Bacillus subtilis, persister cell formation in Escherichia coli (Balaban et al., 2004), and stringent response activation in Mycobacterium smegmatis (Sureka et al., 2008). Fewer examples of the unimodal pattern have been described, but these include transient antibiotic resistance (El Meouche et al., 2016) and antibiotic-induced acid resistance (Mitosch et al., 2017) in E. coli. It is worth noting that the distinction between these patterns is not
always clear, as categorization depends to some extent on what variables are used to assess phenotype.

Random switching between phenotypes can produce either a bimodal or unimodal distribution, depending on how long an individual and its offspring occupy each phenotype before switching to the other (Garcia-Bernardo and Dunlop, 2016). As an illustration, consider a bacterium that randomly switches between a state where high levels of some enzyme are produced and a state where no enzyme is produced. If cells remain in either state for a long time (many generation times), the states are inherited by the daughter cells and two distinct populations grow: a population with the enzyme and a population without. Since switching between the states is a rare event, the number of cells with an intermediate amount of enzyme is negligible. On the other hand, if cells remain in the enzyme-producing or non-enzyme-producing states for a short duration (one or few generation times), the phenotype is not stably inherited by the daughter cells: each daughter cell has a high likelihood of switching from producing enzyme to not producing enzyme or vice versa. If enzyme production, dilution, and degradation are assumed not to be instantaneous, the high switching frequency means that many cells contain some intermediate concentration of enzyme. In general, very low-frequency switching leads to a bimodal distribution in the population, and very high-frequency switching leads to a unimodal distribution.

Random switching between phenotypes, such as was invoked in the preceding example, is a common feature in bacterial bet-hedging strategies but is not required. Asymmetric cell division, for instance, can generate subpopulations that are differently fit in different environments. This is the strategy employed by *Sinorhizobium meliloti*, which can diversify into subpopulations of cells containing high or low levels of poly-3-hydroxybutyrate (a carbon- and energy-storage compound) through asymmetric cell division (Ratcliff and Denison, 2010). In this process, a single parent cell asymmetrically divides into a daughter cell with a high concentration of the compound and a low concentration of the compound, with different levels of the compound conferring greater fitness in different starvation conditions. Most bacterial bet-hedging strategies,
however, do not generate phenotypic diversity so deterministically but instead harness random (or seemingly random) phenotype switching.

Several excellent reviews have been published containing much more in-depth treatments of bet hedging in general (see, for instance, Childs et al., 2010, de Jong et al., 2011, Grimbergen et al., 2015, Philippi and Seger, 1989, and Simons, 2011). The focus of this thesis is on a specific system in E. coli that we will show can allow a population of aerobically growing cells to hedge its bets on a rapid transition to anoxic conditions. This system enables the use of trimethylamine oxide (TMAO) as a terminal electron acceptor for respiration and is encoded by the torCAD operon (McCrimble et al., 2005; Méjean et al., 1994). TMAO respiration has a much lower energetic yield than aerobic respiration, so the observation that torCAD is expressed in the presence of oxygen—and at roughly the same mean level as in the absence of oxygen—was surprising (Ansaldi et al., 2007), especially when considering that no other alternative respiratory system in E. coli is known to be significantly expressed during aerobic growth. Curiously, although oxygen does not affect mean torCAD expression, it does affect the variance around the mean (Roggiani and Goulian, 2015). In the absence of oxygen individual cells all express torCAD at approximately the same level, but in the presence of oxygen torCAD is expressed with exceptionally high cell-to-cell variability. This variability features a high switching frequency and follows a broad, unimodal distribution as described above. As will be seen in Chapter 2, when an aerobically growing population is shifted to an anaerobic environment, only those cells with high torCAD expression at the time of the shift continue to grow anaerobically—a behavior consistent with bet hedging.

A good deal is known about the signaling system that controls torCAD expression, and this has enabled us to address in some mechanistic detail the question of how E. coli cells generate and regulate the variability that permits bet hedging. In the course of our investigations we noticed several features of the system that, in conjunction, can account for how this system produces random behavior in one environment and uniform behavior in another. These features,
their pertinence to torCAD regulation, and examples of their occurrence in other biological contexts are briefly considered in the following sections.

Sensing and Signaling

TMAO activates transcription of the torCAD operon through a signaling system consisting of three proteins: TorT senses the presence of TMAO in the periplasm, TorS transmits this information across the cell membrane and phosphorylates TorR, and phosphorylated TorR activates transcription from the torCAD promoter (Baraquet et al., 2006; Jourlin et al., 1996a; Simon et al., 1994). In this system, signal sensing and transduction across the membrane are relegated to different proteins (TorT and TorS, respectively) that function as a complex. A cell’s responsiveness to TMAO depends on the abundance of TorT and TorS such that a cell with an excess of TorT relative to TorS fully activates torCAD transcription, while a cell with an excess of TorS relative to TorT responds weakly, if at all, to the presence of TMAO (Roggiani and Goulian, 2015). The sensitivity of the system output (torCAD transcription) to the relative amounts of TorT and TorS is significantly enhanced by the bifunctionality of TorS: in the absence of TorT-TMAO stimulation, TorS is not simply inert. Instead, it dephosphorylates TorR, shutting off torCAD transcription (Ansaldi et al., 2001; Jourlin et al., 1996b). Taken together, the features of this system allow for tunable responsiveness to the TMAO signal and form the basis for highly variable torCAD expression.

The TorT/TorS/TorR system belongs to a class of regulatory systems called two-component systems. As with TorT/TorS/TorR, many of these systems have more than two components, but the name stems from the shared core architecture of a histidine kinase (in this case, TorS) that phosphorylates a response regulator (TorR), which then goes on to effect some physiological change, usually by regulating gene expression. In the simplest cases, the histidine kinase is a single protein that both senses a signal and phosphorylates the response regulator. Examples of this arrangement can be found in the paralogous NarX/NarL and NarQ/NarP systems of E. coli, in which the sensor kinases NarX and NarQ directly bind nitrate and nitrite and
catalyze the phosphorylation of NarL and NarP (Mascher et al., 2006). Many systems are organized more like TorT/TorS/TorR, however, and involve one or more sensor proteins that interact with the histidine kinase. For example, the histidine kinase PhoR of the phosphate-sensing system PhoR/PhoB does not detect phosphate directly. Instead, its activity is regulated through its interactions with the phosphate transporter complex PstSCAB and the regulator protein PhoU (Hsieh and Wanner, 2010). We have already introduced the TMAO-sensing protein TorT that stimulates TorS, but there is at least one protein other than TorT that feeds information to TorS and regulates its activity. This secondary signal comes from TorC, the cytochrome component of TMAO reductase, which has a multistep maturation process that requires the insertion of multiple heme groups (Méjean et al., 1994; Sanders et al., 2010). TorC lacking its heme cofactors interacts with TorS and prevents it from phosphorylating TorR, thereby blocking further expression of torCAD and negatively regulating its own expression (Ansaldi et al., 1999; Gon et al., 2001). Finally, histidine kinases need not exclusively detect signals directly or through interactions with partner proteins. Some integrate both direct and indirect sensing, such as the histidine kinase VirA of the virulence-regulating VirA/VirG system in the opportunistic plant pathogen Agrobacterium tumefaciens. VirA detects attributes of the environment characteristic of plant wound sites, sensing phenolic compounds directly but interacting with a partner protein ChvE to sense monosaccharides (McCullen and Binns, 2006).

Bifunctionality of histidine kinases is extremely common in two-component systems, with the histidine kinase dephosphorylating its cognate response regulator in the absence of an inciting signal (Gao and Stock, 2009). Two opposing reactions, the phosphorylation and dephosphorylation of the response regulator, are thus carried out by a single enzyme, with the direction of the reaction being dictated by information received (or not received) by the sensor domain(s) of the enzyme. Because the TorT/TorS/TorR system assigns sensing to one protein subunit (TorT) and signaling to another (TorS), the ratio of TorT to TorS molecules in a cell sets the net direction and rate of TorR phosphorylation in a TMAO-replete environment. This sort of configuration, where the stoichiometry of two proteins coordinates two opposing reactions, is a
feature of some tunable systems. A particularly exquisite example can be found in the *E. coli* chemotaxis system, where the methylation state of methyl-accepting chemotaxis proteins determines the sensitivity of the chemotactic signaling network to a chemoattractant (Falke et al., 1997; Goy et al., 1977). CheR and phosphorylated CheB catalyze the methylation and demethylation, respectively, of the methyl-accepting chemotaxis proteins. An increase in the amount of phosphorylated CheB relative to CheR pushes the system toward methylation, and a decrease in the amount of phosphorylated CheB relative to CheR pushes the system toward demethylation (Dufour et al., 2016; Wadhams and Armitage, 2004). This is similar to how, in the TorT/TorS/TorR system, an increase in the amount of TorT-TMAO relative to TorS pushes the system toward phosphorylation of TorR, and a decrease in the amount of TorT-TMAO relative to TorS pushes the system toward dephosphorylation of TorR.

The example of chemotaxis is also useful in highlighting, by contrast, another key feature of the TorT/TorS/TorR system, which is the decoupled expression of proteins with coordinating functions. Most multiprotein complexes in bacteria are encoded in operons, which provide transcriptional coordination of the various subunits. In the chemotaxis system, the *cheR* and *cheB* genes described above are arranged in an operon and cotranscribed. Furthermore, translation of *cheR* and *cheB* has been shown to be coordinated, which maintains the stoichiometry of the two protein products. This coupling is thought to contribute to robustness in the chemotaxis system, buffering it against gene expression noise (Løvdok et al., 2009). The TorT/TorS/TorR system has a very different organization, with *torT* and *torS* independently transcribed from separate promoters. Rather than providing robustness, like the tight regulation of *cheR* and *cheB*, this decoupled transcription of *torT* and *torS* enables transcriptional noise to influence the ratio of TorT to TorS and is critical for the generation of highly variable expression from the *torCAD* promoter.

The architectures and diversity of two-component systems have been extensively studied, but we are far from a comprehensive understanding of how these pathways work and what they do. For instance, the signals that stimulate two-component system activities are still
undetermined or only partially characterized in most cases (Zschiedrich et al., 2016). Furthermore, complex signal processing can occur when two-component systems have overlapping regulons or are joined into larger networks through connector proteins or, rarely, cross-talk (Alvarez et al., 2016; Mitrophanov and Groisman, 2008). How these intricate networks evolved and what properties they confer on cells is largely unknown. Even TorT/TorS/TorR, which has a well-defined signal (TMAO) and does not appear to connect to other two-component systems, features one of the outstanding mysteries in two-component signaling: the hybrid histidine kinase (TorS). Hybrid histidine kinases have a curious architecture in which a single phosphoryl group is transferred along multiple domains in the kinase dimer before transfer to the response regulator. An understanding of why such a seemingly gratuitous scheme has evolved and been conserved continues to elude researchers. A leading hypothesis is that these phosphorelays offer additional points of control for integration of additional inputs into the system, but this explanation remains speculative (Alvarez et al., 2016; Appleby et al., 1996; Goulian, 2010). Hybrid histidine kinases are not uncommon, with *E. coli* alone featuring five of them (ArcB, BarA, EvgS, RcsC, and TorS) among its 30 two-component systems (Mizuno, 1997; Ortet et al., 2014). As with the other hybrid histidine kinases, we do not know what special role, if any, the phosphorelay plays in the function of TorS.

Harnessing Noise

Our research findings suggest a model of the TorT/TorS/TorR system wherein cells harness transcriptional and partitioning noise in TorT and TorS to generate and regulate the high cell-to-cell variability in *torCAD* expression that permits bet hedging. To generate variability during aerobic growth, cells express TorT and TorS at such exceptionally low levels that noise in the ratio of TorT to TorS leads to considerable fluctuations in the extent of TorR phosphorylation and results in noisy *torCAD* expression. To generate uniform *torCAD* expression when oxygen is absent, cells need only increase the expression of TorT and TorS to levels where gene expression noise and random partitioning have a negligible effect on the TorT-to-TorS ratio.
Before proceeding with the details of noise management in TorT/TorS/TorR signaling, there are a few relevant concepts in molecular noise that merit brief treatment.

Biological molecular noise can be loosely defined as random variability in a biological process originating in the intrinsic random behavior of molecules. Biomolecules abide by the same thermodynamic principles as all molecules, and accordingly the molecular interactions that subside biological processes are stochastic (Balázsi et al., 2011). Molecular noise, like bet hedging, has been of theoretical interest to biologists for longer than the experimental tools to investigate it have been available. Unlike for bet hedging, however, a large number of studies have been conducted and a huge amount of data generated on molecular noise as it pertains to all corners of molecular biology. Our concern is with two types of molecular noise that contribute to the observed distributions of proteins among populations of cells: transcriptional noise and partitioning noise. Transcriptional noise arises from the ensemble of random interactions that occur during RNA synthesis (Larson et al., 2009; Sanchez and Golding, 2013). These interactions include, among many others, the binding of transcription factors and RNA polymerase to the promoter region, the local unwinding and rewinding of the DNA strands, and transcriptional pausing and termination (Rajala et al., 2010; Sevier et al., 2016). Because of its dependence on random events, transcription is a stochastic process, and it is not possible to know with certainty whether a cell will produce a given transcript at a given time. Transcriptional noise, then, is a measure of the variability and unpredictability in RNA synthesis.

Partitioning noise is another measure of variability and unpredictability that is distinct from transcriptional noise, dealing instead with randomness in the allocation of biomolecules to daughter cells at cell division (Huh and Paulsson, 2011a; McAdams and Arkin, 1999; Rosenfeld et al., 2005). For molecules that are in high abundance and can diffuse freely, this type of noise is insignificant. For low-abundance molecules or those that are spatially restricted, partitioning noise can have a huge impact on the fates of the daughter cells. It is to control partitioning noise that chromosome segregation is so carefully regulated when the cell divides (Huh and Paulsson, 2011b). In bacteria that engage in differentiation through asymmetric cell division, the impact of
partitioning noise is limited by mechanisms that spatially restrict enzymatic activities, regulatory elements, or structural features to specific subcellular compartments (Jacobs and Shapiro, 1998). *Caulobacter crescentus*, for instance, directs various proteins to either the stalked or flagellar pole in order to preserve asymmetry (Curtis and Brun, 2010), and *Bacillus subtilis* activates different gene expression programs in a developing spore and its mother cell by confining certain sigma factors to one compartment or the other (Hilbert and Piggot, 2004). Most biomolecules, however, are not known to be subject to spatial control at cell division and are subject to partitioning noise. Membrane proteins are particularly impacted, as diffusion at the micrometer scale is slower in the membrane than in the cytoplasm (Chow et al., 2012; Kumar et al., 2010). The effect of partitioning noise is amplified for proteins that assemble into clusters or complexes in the membrane (Huh and Paulsson, 2011b). TorS functions as a dimer (Moore and Hendrickson, 2012), so the TorT/TorS complex may fall into this category.

In order for transcriptional and partitioning noise to play a significant role in the output of the TorT/TorS/TorR system, the average number of TorT and TorS proteins per cell must be very low. This assertion is supported by ribosome profiling and, for TorS, single-molecule imaging data that indicate that cells possess only a few copies of TorT and TorS during aerobic growth (Li et al., 2014; Taniguchi et al., 2010). We show in Chapter 2 that transcription of *torT* and *torS* increases during anaerobic growth, which suggests a correlative increase in the number of TorT and TorS proteins. The system appears to be organized such that this increased anaerobic expression sufficiently increases mean TorT and TorS levels to where transcriptional and/or partitioning noise no longer contribute much to the output, leading to uniform expression of *torCAD*.

That noise in mRNA and protein levels decreases as the mean increases is a phenomenon well supported by theory and experiment (Bar-Even et al., 2006; Elowitz et al., 2002; Ozbudak et al., 2002; Paulsson, 2004). Noise can therefore readily be modulated by changing mean expression. However, regulating noise independently from the mean requires more effort, and *E. coli* appears to have achieved this for *torCAD* by placing the important source
of noise upstream from torCAD in the signaling pathway. Cells can regulate noise in TorT and TorS levels by regulating mean torT and torS transcription, and our model is that this noise reaches the torCAD promoter via the TorT-to-TorS ratio without direct regulation of mean torCAD expression being necessary. In Chapter 2, we show that mean torT and torS expression is regulated by oxygen through the transcription factor IscR, which binds to a shared regulatory site between the genes and represses their transcription. IscR levels are oxygen-sensitive, with the concentration of IscR being higher in aerobic conditions than in anaerobic conditions (Giel et al., 2013; 2006; Mettert and Kiley, 2014). When IscR is more abundant, torT and torS are more repressed, which leads to increased noise in the relative levels of TorT and TorS. The presence of divergent promoters sharing a single regulatory site evokes the idea of correlated or anti-correlated transcription. Our data do not point to any kind of coordination between torT and torS transcription, although ruling it out would require further experiments.

This introduction has surveyed the major themes underpinning the experimental work to be presented in the following chapters. In Chapter 2, we offer the evidence for TMAO reductase expression being a bet-hedging strategy and for IscR being the regulator of phenotypic heterogeneity. In Chapter 3 we introduce a new character, bacteriophage HK022, and show that it commandeers the regulation of torS expression in the cells it infects, drastically altering the behavior of the TorT/TorS/TorR system. Finally, in Chapter 4 we discuss the broader implications of our findings and speculate on some outstanding questions unanswered by our work. All of the following research deals with a single regulatory system in a single species, but it is a system that provides a venue for the exploration of numerous biological phenomena, from molecular noise to signaling system design to population behavior.
CHAPTER 2: Regulated Stochasticity in a Bacterial Signaling Network Permits Tolerance to a Rapid Environmental Change

This chapter has been previously published in slightly altered form (Carey et al., 2018).

Introduction

Numerous studies have revealed that cell-to-cell variability in gene expression is a common phenomenon in bacteria, and indeed in all domains of life. Depending on context, this heterogeneity in cell behavior can be beneficial or harmful to an organism or population. Accordingly, it has been proposed that diverse gene network architectures have evolved either to generate or limit gene expression variability. In some cases, heterogeneity can benefit a population that experiences unpredictable switching between two or more environments if the different gene expression states confer fitness advantages in different conditions (Kussell and Leibler, 2005; Thattai and van Oudenaarden, 2004; Veening et al., 2008a). This evolutionary strategy, called bet hedging, enables a population to prepare for a potential switch to a new environmental condition by harboring a subpopulation that is pre-adapted to the new environment. Bet hedging has been characterized in a number of microbial systems and shown to play a role in many processes, including pathogenesis (Ackermann et al., 2008; Stewart and Cookson, 2012), antibiotic persistence (Balaban et al., 2004; Maisonneuve and Gerdes, 2014; Verstraeten et al., 2015; Wakamoto et al., 2013), cellular differentiation (Veening et al., 2008b), regulation of metabolism (Hassan et al., 2014; Kotte et al., 2014; Ratcliff and Denison, 2010), induction of stress responses (Sureka et al., 2008), and viral latency (Maslov and Sneppen, 2015; Rouzine et al., 2015). Stochasticity in gene expression is often responsible for the generation of phenotypic diversity in these systems and frequently results in a bimodal distribution of phenotypes (Kotte et al., 2014; Nielsen et al., 2010; Ozbudak et al., 2004; Veening et al., 2008b), although bimodality is not always observed (El Meouche et al., 2016; Levy et al., 2012). Here we describe a bet hedging mechanism in which an environmental cue induces high variance in gene expression...
expression—without changing the mean—and causes individual cells to rapidly traverse a broad distribution of gene expression levels.

*Escherichia coli* can carry out respiration using TMAO instead of oxygen as a terminal electron acceptor through a pathway that requires the gene products of the *torCAD* operon (McCrindle et al., 2005; Méjean et al., 1994; Pommier et al., 1998). Alternative respiratory pathways yield less energy for a cell than aerobic respiration, so most of these systems are repressed when oxygen is present (Unden and Bongaerts, 1997). Surprisingly, TMAO respiration is an exception; in fact, the mean *torCAD* expression across a cell population is independent of oxygen when TMAO is present (Ansaldi et al., 2007; Roggiani and Goulian, 2015). However, *torCAD* transcription is exceptionally noisy in the presence of oxygen but is relatively uniform in its absence, resulting in an oxygen-dependent variance about the mean (Roggiani and Goulian, 2015).

In this work, we show that cell-to-cell variability in aerobic *torCAD* expression enables cells to wager on the possibility of a rapid loss of oxygen by transiently pre-inducing the TMAO respiratory machinery encoded by *torCAD*. We further demonstrate that cells increase variability in *torCAD* transcription in response to oxygen by repressing expression of the TMAO receptor, TorT, and the sensor kinase, TorS, that are part of the signaling system regulating *torCAD*. Importantly, TorS can function as either a kinase or a phosphatase depending on whether or not the protein detects TMAO bound to TorT, making the system sensitive to the relative amounts of TorT and TorS. Stochasticity in the expression of TorT and TorS and/or randomness in their partitioning at cell division generates high variability in downstream expression of *torCAD* without changing the mean. This mechanism for generating regulated phenotypic diversity is a novel strategy for hedging against rapid changes in the environment.

Results

**Variability in aerobic *torCAD* expression permits growth after oxygen depletion**
Expression of *torCAD* in *E. coli* cells fluctuates rapidly during aerobic growth in the presence of TMAO, resulting in a broad distribution of expression across the population (Movie 2.1) (see also Roggiani and Goulian, 2015). To assess the plausibility of bet hedging as an explanation for this variability, we tested whether the ability of individual *E. coli* cells to switch to anaerobic TMAO respiration depends on the level of aerobic *torCAD* transcription at the time of oxygen depletion (Figure 2.1A). Because *E. coli* can grow by fermentation as well as anaerobic respiration, we carried out these experiments in media containing a single, non-fermentable carbon source (glycerol) to ensure that cell growth after oxygen depletion occurred solely by TMAO respiration. We hypothesized that following the transition to low oxygen, cells without a recent history of *torCAD* transcription would suffer a lag in growth, or fail to grow entirely, because they would be unable to produce enough ATP (or other metabolic resources) to synthesize the machinery required for TMAO respiration (TorC and TorA).

To perform these experiments, we developed a method for following single cells through an aerobic-to-anaerobic transition by microscopy (Figure 2.1B). Cells expressing fluorescent reporters are grown aerobically in liquid culture containing TMAO and a non-fermentable carbon source and then deposited on an agarose pad and sealed between a slide and coverslip. Embedded within the agarose pad are non-fluorescent cells, which scavenge oxygen and rapidly create an anaerobic environment. From measurements using a phosphorescence-based probe (Lebedev et al., 2009), we determined that the oxygen in the agarose pad reaches basal levels in under five minutes after the pads are sealed (data not shown).

We employed this microscopy technique to examine the relationship between *torCAD* expression in single cells at the time of oxygen depletion and the subsequent ability of the cells to grow anaerobically by TMAO respiration. To measure *torCAD* expression, we used a strain that contains a chromosomal copy of the *torCAD* promoter fused to the yellow fluorescent protein (YFP) gene. This P<sub>torCAD</sub>-yfp strain also constitutively expresses mCherry, which allows for the identification of cells of interest regardless of their YFP intensity. We grew this reporter strain aerobically, then deposited a sample on a cell-impregnated agarose pad and captured
fluorescence and phase contrast micrographs of randomly selected cells. For each cell, the YFP fluorescence at this starting time point (t = 0) provided a measure of torCAD transcription at the time of oxygen depletion. After five hours of incubation images were acquired again, and the ratio of microcolony size at five hours to the parent cell size at t = 0 was used as a metric of cell growth. A plot of cell growth against YFP fluorescence at the time of transition to anaerobiosis reveals a clear relationship between recent torCAD transcription and the ability of cells to thrive following oxygen depletion (Figures 2.1C and 2.2A). Interestingly, there appears to be a threshold level of torCAD expression below which cells are unable to grow. These results are consistent with bet-hedging behavior, with only the random subpopulation of aerobically growing cells that have synthesized sufficient TorC, TorA, and TorD able to grow after the switch to anaerobiosis. We verified anaerobiosis during these experiments by including a control ΔtorC strain that will not grow unless oxygen contamination is present (Figures 2.2B and 2.2C). We also verified that all cells, regardless of initial YFP fluorescence, are able to grow aerobically (Figure 2.2D). To further confirm that it is torCAD and not other co-regulated genes that allow growth, we conducted a similar experiment to that shown in Figure 2.1C in which all of the cells in the culture were ΔtorC and verified that there was no growth after oxygen depletion (Figure 2.2E).

Expression of torT and torS is elevated in anaerobic conditions

Having identified a physiologically significant consequence of cell-to-cell variability in torCAD expression, we next wanted to determine how oxygen regulates this variability. Transcription of the torCAD operon is regulated by the TorT/TorS/TorR signal transduction system (Baraquet et al., 2006; Jourlin et al., 1996a; 1996b; Simon et al., 1994), which is schematized in Figure 2.3A. TorT and TorS function as a unit, with TorT sensing periplasmic TMAO and causing TorS to autophosphorylate. TorS-P then transfers its phosphoryl group to TorR, and TorR-P activates torCAD transcription. In the absence of TMAO or TorT, TorS dephosphorylates TorR-P and torCAD is not transcribed (Ansaldi et al., 2001; Jourlin et al., 1996b). Protein abundance measurements indicate that on average TorT and TorS are present in only a few copies per cell.
during aerobic growth (Li et al., 2014; Taniguchi et al., 2010), and previous work demonstrated that even modest overexpression of TorT and TorS significantly decreases variability in torCAD transcription (Roggiani and Goulian, 2015). Taken together, these findings suggest a model in which aerobic variability in torCAD expression results from the high cell-to-cell variability in the relative amounts of TorT to TorS that would be expected to occur if the molecules are on average in very low abundance (Figures 2.3B and 2.4) (see also Roggiani and Goulian, 2015). Because torCAD variability is greatly diminished in anaerobic conditions, we predicted higher expression of torT and torS in the absence of oxygen than in the presence of oxygen. To compare aerobic and anaerobic expression of torT and torS, we constructed fluorescent reporters as operon (transcriptional) fusions at the native torT and torS loci, with torT fused to mcherry and torS fused to yfp (Figures 2.3C, 2.5A, and 2.5B). We grew the torT-mcherry torS-yfp reporter strain in aerobic or anaerobic conditions and used fluorescence microscopy to measure the very low cellular levels of mCherry and YFP fluorescence. Because mCherry and YFP fluorophores require oxygen for maturation (Shaner et al., 2005), early exponential phase cultures were treated with a translation inhibitor and aerated prior to fluorescence measurements. These measurements revealed that anaerobic expression of torT and torS is elevated compared to aerobic expression (Figure 2.3C), suggesting a higher abundance of TorT and TorS protein in anaerobically growing cells than in aerobically growing cells. We verified this result using lacZ reporters of torT and torS transcription, which do not require exposure to oxygen for reporter maturation (Figures 2.5C and 2.5D).

The transcription factor IscR binds to torT-torS intergenic sequence

The torT and torS genes are divergently transcribed from adjacent promoters in an 82-bp intergenic region. To our knowledge, no regulators of torT or torS expression have been either reported or predicted, so we sought to identify transcription factors that bind in the torT-torS promoter region and that could account for the oxygen regulation of these two genes. Previous studies have failed to detect binding of the canonical oxygen-dependent regulators ArcA or FNR.
in the torT-torS promoter region (Myers et al., 2013; Park et al., 2013). However, chromatin immunoprecipitation sequencing (ChIP-seq) with IscR, an oxygen-sensitive transcription factor that regulates iron-sulfur (Fe-S) cluster biosynthesis (Giel et al., 2006; 2013; Schwartz et al., 2001; Vinella et al., 2013) revealed binding of IscR to the torT-torS promoter region that was enriched in aerobic versus anaerobic culture (Figure 2.6A). This observation suggested that IscR might act directly at the promoters for torT and/or torS by repressing transcription in aerobically growing cells.

Consistent with this hypothesized role for IscR, transcriptional profiling of aerobically and anaerobically growing cells with or without iscR revealed that torT and torS transcript abundance depended on oxygen only in the wild-type strain (Figure 2.6A). During aerobic growth, the wild-type strain had fewer transcripts than during anaerobic growth (in agreement with the fluorescent reporter data shown in Figure 2.3C). Transcript abundance in the ΔiscR strain was unaffected by oxygen and was the same as in the anaerobically grown wild-type strain, supporting the premise that IscR is an oxygen-sensitive repressor of torT and torS expression. A comparison of ChIP-seq data for the σ70 component of RNA polymerase from (Myers et al., 2013) with the IscR data revealed an inverse relationship between σ70 binding and IscR binding (Figure 2.6A), further supporting the model that IscR represses transcription of torT and torS and suggests that it does so via steric inhibition of σ70 binding. IscR abundance is elevated during aerobic growth (Giel et al., 2013), which is consistent with the increased binding of IscR between torT and torS revealed by ChIP-seq and supports the mechanism of IscR regulation of torT and torS outlined in Figure 2.6B.

The IscR binding site between torT and torS, denoted bIscR, was identified as a type 2 site by sequence similarity with other sites of this class. Type 2 sites can bind both holo-IscR (IscR with its Fe-S cluster) and the apo (Fe-S clusterless) form, whereas type 1 sites bind only holo-IscR (Nesbit et al., 2009). To confirm that bIscR is a type 2 site and to assess the specificity and affinity of IscR binding to this sequence, we assayed IscR binding in vitro by fluorescence anisotropy. We performed DNA binding titration experiments using purified IscR-C92A, an IscR
mutant that cannot assemble with an Fe-S cluster and is therefore only in the apo form. We characterized IscR-C92A binding to $b_{\text{iscR}}$, $b_{\text{iscR}*}$ (a mutant $b_{\text{iscR}}$ sequence with a C-to-G substitution at a highly conserved cytosine in the binding site motif [Figures 2.6C and 2.7]), a known type 2 binding site sequence, and a shuffled DNA sequence (Figure 2.6D). As is evident in Figure 2.6D, IscR-C92A binds to $b_{\text{iscR}}$ and the known type 2 sequence but does not bind to $b_{\text{iscR}*}$ or the shuffled sequence. We also performed ChIP-qPCR to further confirm that, in vivo, IscR binds the promoter region between $torS$ and $torT$ and that binding is greatly diminished in a strain with the $b_{\text{iscR}*}$ mutation (Figure 2.6E).

IscR regulates $torT$ and $torS$ expression in an oxygen-dependent manner

To test whether IscR binding in the $torT$-$torS$ intergenic region regulates $torT$ and/or $torS$ expression, we first constructed an in-frame deletion of the $iscR$ gene and measured $torT$-$mcherry$ and $torS$-$yfp$ reporter fluorescence. Expression of both genes was elevated in the $\Delta iscR$ strain relative to the wild-type strain (Figures 2.8A and 2.8B), which is consistent with the transcriptional profiling results (Figure 2.6A) and indicates that IscR represses transcription of both $torT$ and $torS$.

Because IscR is a global regulator and its deletion might have an indirect effect on numerous cellular functions, we also tested the effect of the IscR binding site mutation $b_{\text{iscR}*}$. By measuring aerobic $torT$ and $torS$ transcription via the $torT$-$mcherry$ and $torS$-$yfp$ constructs, we found that strains containing $b_{\text{iscR}*}$ have elevated $torT$ and $torS$ expression relative to strains with wild-type $b_{\text{iscR}}$ (Figures 2.8C and 2.8D), and the fold increase of $torT$ and $torS$ expression is nearly the same in $b_{\text{iscR}*}$ and $\Delta iscR$ strains. Furthermore, the deletion of $iscR$ in $b_{\text{iscR}*}$ strains has little additional effect on $torT$ and $torS$ expression (Figure 2.9). Taken together, these results support the proposed role of IscR as a repressor of $torT$ and $torS$ expression. We also note that the results in Figure 2.9 indicate that the $b_{\text{iscR}*}$ mutation has little effect on the basal transcription from the $torT$ and $torS$ promoters.
To test whether IscR’s regulation of torT and torS expression is oxygen-dependent, we compared aerobic and anaerobic expression of torT and torS in wild-type and \textit{biscR}\textsuperscript{*} strains (Figures 2.8E and 2.8F). While transcription of torT and torS in wild-type strains is increased in anaerobic compared to aerobic conditions (see also Figure 2.3C), transcription of torT and torS in the \textit{biscR}\textsuperscript{*} strains is similar in both conditions. There may be some degree of IscR-independent, oxygen-dependent regulation of torT transcription (Figure 2.8E), but most of the oxygen-dependent regulation appears to occur through the action of IscR.

**IscR regulates variability in torCAD expression through oxygen-dependent repression of torS and torT transcription**

We next examined whether eliminating the repression of torT and torS transcription by IscR could suppress cell-to-cell variability in torCAD expression. Using a \textit{P}\textsubscript{torCAD-yfp} reporter strain, we found that variability in torCAD transcription is greatly diminished in a \textit{ΔiscR} background (Figure 2.10). Unexpectedly, we found that deleting \textit{iscR} also decreases mean torCAD expression (Figure 2.10, inset). We do not know the explanation for this effect on the mean, but we suspect that it may be due to inefficient maturation of the TorC cytochrome caused by deletion of \textit{iscR}, as apo-TorC lowers torCAD expression by inhibiting TorS kinase activity (Ansaldi et al., 1999; Gon et al., 2001). Consistent with this explanation, we found that deleting torC increases transcription from the torCAD promoter (Figure 2.11). However, we note that apo-TorC does not appear to play a role in regulating variable expression from the torCAD promoter, as deletion of torC has no effect on variability in either aerobic or anaerobic conditions (Figure 2.11).

We also found that introducing the IscR binding site mutation \textit{biscR}\textsuperscript{*} decreases cell-to-cell variability in torCAD transcription, conferring the same low variability phenotype found in wild-type cells under anaerobic conditions (Figure 2.12A). Thus, cells with the \textit{biscR}\textsuperscript{*} mutation are effectively blind to oxygen with respect to torCAD transcription. Furthermore, the mean torCAD expression level is relatively uniform between wild-type and \textit{biscR}\textsuperscript{*} strains (Figure 2.12B), which differs from the decreased torCAD expression seen in the \textit{ΔiscR} strains (Figure 2.10). We attribute this to the
fact that IscR protein is still present in the $b_{iscR}^*$ mutant strain and can fulfill its other regulatory roles.

To investigate the physiological impact of low variability of $torCAD$ transcription under aerobic conditions, we repeated the aerobic-to-anaerobic transition experiment shown in Figure 2.1C but this time using a $P_{torCAD}$-yfp reporter strain that has the $b_{iscR}^*$ mutation. A plot of cell growth against YFP fluorescence at the time of oxygen depletion shows that nearly every cell continues to grow after oxygen depletion (Figures 2.13 and 2.14). These results suggest that in the absence of IscR repression of $torS$ and $torT$, most of the cells in the population have sufficient TorC and TorA to effectively transition to anaerobic TMAO respiration.

Discussion

TMAO can be found in a number of natural environments (Barrett and Kwan, 1985; Stella et al., 2006; Wang et al., 2014). In the context of animal hosts, which are generally considered to be the primary habitat for *E. coli* (Tenaillon et al., 2010), TMAO may be directly ingested from the diet, particularly from seafood (Mitchell et al., 2002; Zhang et al., 1999), and may also be produced endogenously in the gut through host processes (Rivera-Chávez and Bäumler, 2015; Winter et al., 2013a; 2013b). Data on the concentration of TMAO in the intestine are generally lacking, but some human metabolomic studies have reported fecal TMAO concentrations well within the range that induces TMAO reductase expression in *E. coli* (Di Cagno et al., 2011; Francavilla et al., 2012; Roggiani and Goulian, 2015).

We hypothesize that the cell-to-cell variability in aerobic $torCAD$ expression has evolved to allow *E. coli* to thrive even if subjected to a sudden decrease in oxygen availability. Such rapid depletion of oxygen might occur through physical translocation of bacteria from an oxygenated to an anoxic environment (e.g., when moving from a free-living state to the mammalian gut) or through depletion of oxygen in a single environment (e.g., by consumption of oxygen by bacterial populations at high cell density). In the context of the gut environment, oxygen concentrations are extremely low in the bulk of the intestinal volume. However, spatially refined measurements have
revealed the existence of a radial oxygen gradient in the intestine, with relatively high dissolved oxygen at the intestinal epithelium, an aerotolerant microbiota in the epithelium-adjacent mucosa, and a steep gradient to anaerobiosis in the lumen (Albenberg et al., 2014). Because bacterial oxygen consumption is the likely cause of this gradient, at least some intestinal bacteria are exposed to oxygen and its effects. Variability in local dissolved oxygen concentrations may also occur as a direct or indirect result of variations in the longitudinal flux and composition of luminal contents in the intestine. Additionally, opportunities exist for *E. coli* to encounter TMAO and oxygen outside of a host. For example, urine often contains a significant amount of TMAO (Miller et al., 2014; Stubbs et al., 2015; Zhang et al., 1999), and growth to high cell densities in environments contaminated with urine would likely have a significant impact on the duration and magnitude of oxygen exposure.

An outstanding question regarding the regulatory mechanism described here concerns the cost of aerobic *torCAD* expression. Presumably, there is a metabolic cost associated with synthesizing and assembling the TMAO reductase complex, particularly considering its requirement for heme and molybdenum cofactors. Indeed, it is hard to imagine why *torCAD* expression would be regulated by TMAO if the cost were negligible. It is also possible that under some conditions there is a metabolic burden from shunting electrons to reduce TMAO instead of oxygen. We have been unable to demonstrate a fitness cost in competition experiments between strains that uniformly express *torCAD* aerobically (via the *b*<sub>iscR</sub>*<sup>-</sup> mutation) and wild-type strains (data not shown). However, laboratory growth conditions do not necessarily reflect the evolutionary pressures that shaped the architecture and behavior of this genetic circuit, and the disadvantage of uniform expression of *torCAD* could be unmasked in conditions we have not yet explored. Additionally, the sequence-level conservation of the *torT-torS* intergenic region, and of the IscR binding site sequence in particular, is high among many members of the *Enterobacteriaceae* (Figure 2.7). This strongly suggests that evolutionary pressures have favored retention of the particular regulatory mechanism described in this paper along with the regulation of TorCAD synthesis in general, and that in environments where *E. coli* encounters oxygen and
TMAO there is a fitness cost associated with uniform production of TorCAD that we have not yet been able to detect.

The regulation of *torCAD* variability in response to oxygen also invites consideration of several features of the TorT/TorS/TorR signaling system. First, the sensory and kinase/phosphotransfer roles belong to two separate proteins (TorT and TorS, respectively) (Baraquet et al., 2006), a feature that is also found in quorum sensing (Waters and Bassler, 2005), chemotaxis (Wadhams and Armitage, 2004), and various transport-associated signaling systems (Piepenbreier et al., 2017), among others. Second, TorT interaction with TorS determines whether TorS predominantly acts to phosphorylate or dephosphorylate TorR when TMAO is present. Third, the *torT* and *torS* genes are each expressed from their own promoter, suggesting their transcription is not tightly coupled. Finally, TorT and TorS are maintained at exceptionally low abundance in the presence of oxygen (Li et al., 2014; Taniguchi et al., 2010). Together, these features of the Tor system suggest that small differences in the number of TorT and TorS molecules between cells could result in large differences in intracellular TorR-P levels. Furthermore, at low numbers of TorT and TorS the variance in the output would be sensitive to relatively small changes in *torT* and *torS* transcription, as we have observed for aerobic versus anaerobic growth (Figure 2.12A). As a proof of principle, we have further demonstrated in a simple computational model that a small change in expression of these two signaling proteins can have a large effect on the output (Figure 2.4).

We have shown that the regulation of cell-to-cell variability by oxygen is mediated by the transcription factor IscR, which binds between *torT* and *torS* and represses transcription of both genes. IscR is more abundant during aerobic growth than during anaerobic growth (Giel et al., 2006; 2013; Mettert and Kiley, 2014), which suggests that the increased expression of *torT* and *torS* under anaerobic conditions results from decreased IscR concentration and lowers the cell-to-cell variability in *torCAD* transcription, consistent with our model and previous observations on the effects of plasmid-based expression of *torT* and *torS* (Roggiani and Goulian, 2015).
In our model of regulated cell-to-cell variability of \textit{torCAD} expression, the amount of TorT and TorS protein produced under IscR-repressed (aerobic) conditions is low enough that stochastic effects in protein production and partitioning at cell division are important. The inverse relationship between mean protein copy number and the significance of noise has long been recognized (Bar-Even et al., 2006; Elowitz et al., 2002; Ozbudak et al., 2002; Paulsson, 2004). Low abundance of TorT and TorS creates cell-to-cell variability in the TorT/TorS ratio, and because of the division of labor between TorT (sensing TMAO) and TorS (phosphorylating and dephosphorylating TorR), the cell's TorT/TorS ratio determines the concentration of phosphorylated TorR and the magnitude of the transcriptional response to the presence of TMAO (Figure 2.3B). In aerobic conditions, when \textit{torT} and \textit{torS} are repressed, the TorT/TorS ratio can differ markedly between cells in a population exposed to the same concentration of TMAO, which leads to high cell-to-cell variability in \textit{torCAD} expression. Anaerobically, \textit{torT} and \textit{torS} are derepressed, and at these higher TorT and TorS levels the TorT/TorS ratio becomes insensitive to stochasticity in gene expression and protein partitioning. Expression of \textit{torCAD} is therefore much more uniform across the population in anaerobic conditions. One particularly interesting consequence of this mechanism for generating heterogeneity in gene expression is that individual cell lineages frequently traverse the range of \textit{torCAD} induction: each TorT or TorS production burst and each cell division partitioning event significantly alters the concentration of TorR-P in the cell. A consequence of this behavior is that the cost associated with low fitness states is shared by all cell lineages in the population. This property contrasts with phenotypic variability based on bistability in which “on” and “off” states are Inherited over multiple generations (for reviews covering the subject, see Dubnau and Losick, 2006, Eldar and Elowitz, 2010, Losick and Desplan, 2008, and Smits et al., 2006), causing specific lineages to bear most of the fitness burden. To our knowledge, the Tor system is the first reported example of cells harnessing stochasticity in cellular protein levels to regulate the variability in the output of a signaling system without changing the mean. As evolution tends to reuse effective strategies, it is likely that other biological signaling systems have evolved to regulate variability through similar mechanisms.
Methods

Bacterial growth media and conditions

All bacterial strains were derived from *Escherichia coli* strain K-12 substrain MG1655. Routine liquid culture was performed in 2 mL LB (Miller) medium at 37°C with aeration on a roller drum, and growth on solid media was performed on LB agar plates at 37°C. When used, antibiotics were added to media at the following concentrations unless otherwise indicated: ampicillin, 100 μg/mL; kanamycin, 25 μg/mL; chloramphenicol 20 μg/mL; and streptomycin, 250 μg/mL.

Liquid cultures for fluorescence microscopy and β-galactosidase assays were grown in 2 mL minimal A medium (Miller, 1992) with 0.2% glucose except for microscopy experiments of anaerobically growing cells, which used minimal A medium or M9 medium with 0.2% glycerol, and for ChIP-seq experiments, which used MOPS medium as indicated below. M9 medium was supplemented with 0.5 mg/L FeSO$_4$$\cdot$7H$_2$O. All medium contained TMAO (Sigma-Aldrich Cat#T0514) at a final concentration of 10 mM except where indicated below. For the experiments that produced the data shown in Figure 2.10, Movie 2.1, and Figure 2.11, minimal A medium was also supplemented with 0.1% casamino acids. Cultures were grown with aeration on a roller drum unless otherwise specified. For anaerobic culture, screw-cap culture tubes were filled completely with culture media, inoculated, and sealed tightly with no gas headspace prior to standing incubation at 37°C.

Liquid cultures for ChIP-seq and transcriptomic analysis were grown in MOPS minimal medium supplemented with 0.2% glucose (Neidhardt et al., 1974) at 37°C and sparged with a gas mix of 95% N$_2$ and 5% CO$_2$ (anaerobic) or 70% N$_2$, 5% CO$_2$, and 25% O$_2$ (aerobic).

Strain construction

Strains, plasmids, and primers used in this study are listed in Table 2.1, Table 2.2, and Table 2.3, respectively. Transductions were performed with P1vir as described previously (Miller, 1992). To make the *torS-yfp* construct, the *yfp* gene with its own ribosome binding site and a linked
kanamycin resistance gene with flanking FRT sites was amplified by PCR from template pEB45 using primers torS-yfp-u2 and torS-yfp-l2. The PCR product was recombined into the MG1655 chromosome by recombineering using pKD46 (Datsenko and Wanner, 2000) and correct integration was verified by sequencing. The torS-yfp construct was transduced into a clean MG1655 background to create strain MMR149. To make the torT-mcherry construct, the mcherry gene with its own ribosome binding site and a linked kanamycin resistance gene with flanking FRT sites was amplified by PCR from template pMR5 using primers torT-mcherry-lred-u-v2 and torT-cfp-DtorR-lred-d-v2. The PCR product was recombined into the MG1655 chromosome by recombineering using pKD46 and correct integration was verified by sequencing. The torT-mcherry construct was transduced into a clean MG1655 background to create strain JNC92. A torT-cfp construct was also made by recombineering: the cfp gene with its own ribosome binding site and a linked kanamycin resistance gene with flanking FRT sites was amplified by PCR from template pEB47 using primers torT-cfp-lred-u-v2 and torT-cfp-lred-d-v2. The PCR product was recombined into the MG1655 chromosome using pKD46 and correct integration was verified by sequencing. The torT-cfp construct was transduced into a clean MG1655 background to create strain JNC105.

To construct the torS-yfp torT-mcherry dual reporter strain JNC100, the kanamycin resistance gene (kan) linked to torS-yfp in MMR149 was first excised by FLP recombinase using plasmid pCP20 (Cherepanov and Wackernagel, 1995) and then cured of pCP20 to create JNC73 following previously described protocols (Datsenko and Wanner, 2000). JNC73 was then transformed with a different FLP recombinase-expressing plasmid, pEL8, that lacks the cat gene. A FRT-cat-FRT-containing DNA fragment was amplified by PCR from pKD3 using primers psyn-u1 and oriR6kseqprim1 and introduced into JNC73/pEL8 by electroporation. Cells were allowed to recover with aeration at 37°C for 2.5 h before spreading a portion on LB plates with 6 μg/mL chloramphenicol. Colonies were purified on LB plates with 20 μg/mL chloramphenicol to create JNC99. JNC99 was tested for loss of pEL8 by confirmation of ampicillin sensitivity and for proper integration of the cat gene by PCR. The torT-mcherry construct was transduced from JNC92 into
JNC99 with selection on plates containing kanamycin and chloramphenicol to ensure maintenance of the torS-yfp fusion.

The Keio collection of single-gene knockouts (Baba et al., 2006) was used for construction of ΔiscR and ΔtorC strains. The ΔiscR construct was moved by transduction from JW2515 into JNC73 to create JNC74, into JNC148 (see below) to create JNC162, and into MG1655 to create MMR213. The kanamycin resistance gene of MMR213 was excised using pCP20 and the strain was then cured of pCP20 to create JNC102. The torS-yfp and torT-mcherry fusions were moved into JNC102 by transduction from JNC100 to create JNC104. The ΔiscR construct from JW2515 was moved into the PtorCAD-yfp ompA-cfp strain MMR8 (Roggiani and Goulian, 2015) by transduction to create MMR210. The ΔtorC construct was moved by transduction from JW0981 into EPB47 to create MMR11 and into MMR8 to create MMR12. The kanamycin resistance gene of MMR11 was excised using pCP20 and the strain cured of pCP20 to create MMR14. The same method was used to remove the kanamycin resistance gene from MMR12, creating MMR15.

The torT-lacZ reporter strain JNC163 was constructed by deleting lacZYA in JNC92 and replacing the mcherry gene with lacZ by recombineering. The kanamycin resistance gene of JNC92 was excised using pCP20 to create JNC148, and the lacZYA deletion was introduced from strain TIM89 by transduction to create JNC161. The lacZ construct for the torT-lacZ reporter was generated by overlap extension PCR: the first DNA segment was amplified from MG1655 genomic DNA using primers torT-lacZ-lred-u and lacZterm-l1, and the second DNA segment was amplified from the Keio ΔlacY strain JW0334 using primers lacY-Keio-u and torT-lacZ-lred-l. The assembled lacZ-kan construct was integrated downstream of torT in JNC161 by pKD46-mediated recombination, and correct integration was verified by sequencing. The construct was transduced into a clean JNC161 background to create strain JNC163. The torS-lacZ reporter strain JNC166 was constructed by amplifying lacZ-kan from JNC163 using primers torS-lacZ-lred-u2 and torS-lacZ-lred-l2 and integrating it downstream of torS in the ΔlacZYA strain TIM183 by pKD46-mediated recombination. Correct integration was verified by PCR.
The $P_{torCAD\text{-}yfp}$ transcriptional reporter integrated into the chromosome of MG1655 at the phage $\lambda$ attachment site in strains MMR7 and MMR8 is described in Roggiani and Goulian, 2015, as is the $P_{tetA\text{-}mcherry}$ fusion incorporated at the $xyl$ locus in strain MMR59. The $P_{tetA\text{-}mcherry}$ fusion was moved from MMR59 into MMR7 by transduction to create MMR64, and the kanamycin resistance gene marking $P_{tetA\text{-}mcherry}$ was eliminated by pCP20-mediated FLP recombination to create MMR65. JNC124, a strain containing the $P_{torCAD\text{-}yfp}$ transcriptional reporter and the $torT\text{-}cfp$ operon fusion, was constructed by transduction using MMR7 as the donor strain and JNC105 as the recipient strain.

Point mutations in the IscR binding site between $torS$ and $torT$ were constructed using a scarless mutagenesis technique (Blank et al., 2011). The point mutation is a C-to-G substitution at the -21 position relative to the $torT$ transcription start site (a sequence change from 5'-AAAGCCATTATT-3' to 5'-AAAGCGTTATT-3'), denoted $b_{iscR\ast}$. To introduce $b_{iscR\ast}$ into a $torS\text{-}yfp$ reporter strain, a DNA fragment containing an I-SceI cut site and chloramphenicol resistance gene was amplified from pWRG100 using primers PtorT-PtorS-pWRG100 and torT-pWRG100 and integrated at the $tor$ locus of JNC73 by recombineering using pKD46. Correct integration was confirmed by PCR, and the strain was cured of pKD46 to create JNC109. JNC109 was transformed with pWRG99, a derivative of pKD46 that contains a tetracycline-inducible I-SceI gene, and a DNA fragment containing the $b_{iscR\ast}$ mutation and homology to the chromosomal sequencing flanking the I-SceI cut site was incorporated into the chromosome. DNA incorporation was achieved by plating on 0.5 $\mu$g/mL anhydrotetracycline to induce expression of I-SceI so as to inhibit growth of cells containing a chromosomal I-SceI cut site (i.e. cells that failed to replace the I-SceI cut site with the introduced DNA harboring the $b_{iscR\ast}$ mutation). The resulting strain was JNC112. The DNA fragment containing $b_{iscR\ast}$ was generated by overlap extension PCR using MG1655 genomic DNA as a template with outer primers HK022-P1 and HK022-P4 and inner primers iscRbs-mut2-mid-r and iscRbs-mut2-mid-f. The chromosomal $b_{iscR\ast}$ mutation was verified by sequencing, and JNC112 was confirmed to be ampicillin and chloramphenicol sensitive. The $\DeltaiscR$ mutation was moved from JW2515 into JNC112 by transduction to create JNC158.
JNC115, the torT-mcherry reporter strain harboring the $b_{iscR}^*$ mutation, was constructed in a manner analogous to that of JNC112. The I-Scel cut site construct was first introduced into MG1655 by recombineering to create JNC108 and then transduced from JNC108 into JNC92 using P1vir to create JNC111. JNC111 was transformed with pWRG99, and $b_{iscR}^*$ was incorporated by electroporation of DNA with flanking homology and counterselection against I-Scel cut site-containing cells. JNC115 was verified by sequencing and confirmed to be ampicillin and chloramphenicol sensitive. This counterselection procedure was also used directly in JNC108 to create JNC122, which is identical to MG1655 but with the single point mutation $b_{iscR}^*$. All PCR primers and templates were the same as those used in the construction of JNC112. The kanamycin resistance gene of JNC115 was excised using pCP20 to create JNC157. The $\Delta iscR$ mutation was moved from JW2515 into JNC157 by transduction to create JNC159.

The $P_{torCAD}^{torCAD^*}$ transciptional reporter was amplified from pMR19 using primers lac-pMR10-U1 and lac-pMR10-L1 and integrated into the chromosome of MG1655 by recombineering to create MMR67. This construct was transduced into JNC73 to create the $P_{torCAD}^{torCAD^*}$ strain JNC117 and into JNC112 to create the $P_{torCAD}^{torCAD^*}$ strain JNC116. A different $P_{torCAD}$ reporter strain with the $b_{iscR}^*$ mutation, JNC123, was constructed by transduction of $P_{torCAD}^{torCAD^*}$ from MMR7 into JNC122.

**Phase contrast and fluorescence microscopy**

Liquid cultures in minimal medium without TMAO were inoculated from single colonies on LB agar plates and grown to saturation overnight. Cultures were diluted 1:1000 or 1:500 into fresh medium with TMAO and grown aerobically or anaerobically (as described above) to an optical density at 600 nm of 0.1 to 0.2. Cultures were then placed on ice and streptomycin was added to halt further protein synthesis. For experiments involving comparisons of aerobic and anaerobic growth of bacteria expressing fluorescent proteins, all of the cultures were aerated after streptomycin addition to allow for fluorophore maturation before imaging. For experiments employing mCherry and/or YFP, aeration occurred at room temperature overnight. For
experiments employing both CFP and YFP, cultures were held at 4°C overnight and then aerated at 4°C or room temperature for 2 – 3 h. For experiments involving only aerobic growth, cultures were held at 4°C overnight after streptomycin addition. Cultures were kept on ice until time of imaging. Cells were immobilized for imaging by placing 5 μL culture on a 75 μL 1% agarose LE (Dot Scientific Cat#AGLE) pad prepared in minimal A salts as described in Miyashiro and Goulian, 2007. Imaging was performed on an Olympus IX81 inverted microscope equipped with a 100 W mercury lamp and a UPlanApo 100× NA 1.35 oil immersion objective and filter sets from Chroma. Excitation filters, dichroic mirrors, and emission filters for CFP, YFP, and mCherry were D436/20x, 455dclp, D480/40m; HQ500/20x, Q515lp, HQ535/30m; and HQ575/50x, Q610lp, HQ640/50m, respectively. Images were acquired with a SensiCam QE CCD camera operated at -12°C or an Andor iXon EMCCD operated at -20°C. Electron-multiplying gain was employed when imaging strains containing the torS-yfp or torT-mcherry reporter constructs. IPLab v4.04 software (BD Biosciences) was used to record images.

**Aerobic-to-anaerobic transition microscopy**

Single colonies of fluorescent reporter strains and a non-fluorescent oxygen-scavenging strain (MG1655) were used to inoculate liquid cultures in minimal A medium plus glycerol without TMAO. Cultures were grown to saturation overnight and then diluted 1:100 (fluorescent reporter strains) or 1:50 (oxygen-scavenging strain) into M9 glycerol medium plus TMAO and ferrous sulfate and grown with aeration for 5 h. After 5 h, an agarose pad containing embedded oxygen-scavenging cells was prepared as follows. 3% Low-melting agarose (Fisher BioReagents Cat#BP165) in M9 salts plus ferrous sulfate was boiled and then cooled to 42°C. A 1:1 mixture of MG1655 from the saturated overnight culture and MG1655 from the growing culture was prepared, and this cell mixture was then mixed with the molten low-melt agarose in a 1:1 ratio (making the final agarose concentration 1.5%). Glycerol and TMAO were added to the cell/agarose mixture to final concentrations of 0.2% and 10 mM, respectively. The cell-embedded agarose pad was cast by depositing 1 mL of the agarose/cells mixture on a 22 × 22 mm cover
glass and floating another 22 × 22 mm cover glass on top. The pad was allowed to solidify for 45 min, and then one cover glass was removed and the pad transferred to a standard microscope slide (so that the pad is sandwiched between slide and cover glass) with care taken to avoid trapping air bubbles between the slide and the agarose pad.

To inoculate the agarose pad, the cover glass was removed and 5 μL fluorescent reporter strain culture was deposited on the pad surface. This 5 μL contained a 1:1 mixture of $P_{torCAD}$-$yfp$ reporter strain and, as a control for anaerobiosis, a CFP-fluorescent strain unable to grow anaerobically in M9 glycerol media plus TMAO (strain MMR14). A smaller (18 × 18 mm) cover glass was applied to the inoculated pad with care taken to avoid air bubbles. Any trapped air bubbles were removed by sliding the cover glass along the pad surface until the bubbles were liberated and then sliding the cover glass back to the center of the pad. The edges of the agarose pad were then trimmed with a razor blade to reduce the pad area to that of the 18 × 18 mm cover glass. The entire pad perimeter between cover glass and slide was then sealed with epoxy cement (Elmer’s Cat#E1009), which was allowed to set for 5 min. The prepared slide was moved to a microscope stage in a temperature-controlled chamber set to 34°C for time lapse imaging. Images were taken immediately after positioning the slide on the stage and within 30 minutes of sealing with epoxy, and this time was designated the start time (t = 0 min) of anaerobiosis.

The control strain for anaerobiosis, MMR14, is $\Delta torC$ and constitutively expresses CFP to allow its identification during microscopy. Because the growth medium contains only a non-fermentable carbon source (glycerol) and no respiratory electron acceptors other than TMAO, a $\Delta torC$ strain cannot grow within the time frame analyzed unless the system is contaminated with sufficient oxygen for aerobic respiration. During each experiment, at least 50 randomly selected $P_{torCAD}$-$yfp$ cells and at least 50 randomly selected $\Delta torC$ cells were monitored for growth, and lack of growth was confirmed for $\Delta torC$ cells (Figures 2.2B and 2.2C).

For the experiment shown in Figure 2.2D (mock aerobic-to-anaerobic transition), fluorescent reporter strains were grown exactly as described above. The agarose pad was prepared as above but without the embedded oxygen-scavenging cells. Bacterial culture (5 μL)
was deposited into the center of a cover-glass bottom cell culture dish (FluoroDish; World Precision Instruments Cat#FD35-100), and the agarose pad was placed on top of the culture. The inner perimeter of the dish was lined with tissue moistened with M9 salts plus ferrous sulfate to maintain hydration of the agarose pad during the course of the experiment. The lid of the dish was treated with anti-fogging agent (Cat Crap; EK Cat#10003B), sealed with plastic paraffin film, and transferred to a microscope stage in a temperature-controlled chamber for imaging. The chamber was maintained at 34°C for the duration of the experiment.

**Time-lapse microscopy**

To produce Movie 2.1, time-lapse microscopy of aerobically growing microcolonies was performed as previously described (Roggiani and Goulian, 2015) with slight modifications. Liquid cultures in minimal A medium plus casamino acids and glucose without TMAO were inoculated from single colonies on LB agar plates and grown to saturation overnight. Cultures were diluted 1:1000 into minimal A medium plus casamino acids, glucose, and TMAO and grown for 3.5 h. An agarose pad was prepared by dissolving 1% SeaKem GTG agarose (Lonza Rockland Cat#50070) in minimal A salts. When the agarose solution reached a temperature of 55°C, MgSO₄ (1 mM final), glucose (0.2% final), casamino acids (0.1% final), and TMAO (10 mM final) were added. The agarose solution was poured into a cover-glass bottom cell culture dish to a depth of approximately 5 mm and allowed to solidify. After solidification, the agarose pad was warmed to 37°C and inoculated by lifting the pad with a spatula, depositing 5 μL bacterial culture into the center of the dish, and lowering the pad. The lid of the dish was treated with an anti-fogging agent, sealed, and transferred to a microscope stage in a temperature-controlled chamber as described above. The chamber was maintained at 32°C for the duration of the experiment, and phase-contrast and fluorescence images were collected every 14 min.

**β-Galactosidase assays**
Liquid cultures in minimal medium without TMAO were inoculated from single colonies on LB agar plates and grown to saturation overnight. Cultures were diluted 1:1000 into fresh medium with TMAO and grown aerobically or anaerobically as described above. Cells were harvested at an optical density at 600 nm of 0.1 to 0.2, and the assay performed as in Miller, 1992.

**Sequence alignment**

Bacterial genomes containing DNA sequence similar to that of the *E. coli* torS-torT locus were identified using NCBI BLAST (Boratyn et al., 2013). Multiple sequence alignment was performed using T-Coffee (Notredame et al., 2000), and visualizations of alignments were produced using Jalview (Waterhouse et al., 2009).

**ChIP-seq and transcriptomics**

Strains MG1655 (wild type) and PK4854 (∆iscR) were grown in MOPS minimal medium without TMAO aerobically and anaerobically as described above. Cells were harvested for processing during mid-exponential phase (OD$_{600}$ = 0.3). ChIP-seq analysis for IscR was performed as previously described (Myers et al., 2013). The IscR antibody was affinity purified as described previously (Witte et al., 2011). ChIP-seq data for $\sigma^{70}$ was obtained from Myers et al., 2013.

Total RNA was isolated as previously described (Khodursky et al., 2003). The concentration of the purified RNA was determined using a NanoDrop 2100, while the integrity of the RNA was analyzed using gel electrophoresis. The RNA isolated from MG1655 or PK4854 cultures grown under either aerobic or anaerobic conditions was converted into strand specific, single-stranded cDNA as described previously (Cho et al., 2009). The cDNA was labeled with Cy3 and then hybridized to custom designed, high-density tiled microarrays containing 378,000 probes from alternate strands, spaced roughly every 12 bp through the genome as described previously (Cho et al., 2009). Microarray hybridization and scanning were performed as described previously (Myers et al., 2013) and the photomultiplier tube was adjusted until the median background value was approximately 100. All probe data were normalized using Robust
Multiarray Average in the NimbleScan software package, version 2.5 (NimbleGen Systems) (Irizarry et al., 2003). The median of the normalized probe signal within previously identified open reading frames was averaged between two biological replicates.

**DNA-binding fluorescence polarization assays**

DNA-binding isotherms were generated by measuring changes in fluorescence polarization when IscR-C92A, purified as previously described (Giel et al., 2006; Nesbit et al., 2009), bound a 30 bp dsDNA fragment, one strand of which was linked to a Texas Red fluorophore at the 5’ end. This fragment contained 25-bp of the predicted type 2 Isc binding site identified within the *torT* promoter region (5’-ATAAAGCCTTTATTATTGATGAGGCTATCAT-3’, denoted *b*\textsubscript{iScR}) based on similarity to the consensus type 2 motif (5’-AxxxCxxAxxTxxGxxxxTxAxGxxTxx-3’) (Nesbit et al., 2009). To demonstrate the specificity of binding, equilibrium-competition assays were used in which IscR-C92A (120nM) was incubated with the Texas Red-labeled *b*\textsubscript{iScR} fragment (5 nM) in the presence of unlabeled competitor DNA (8-1000 nM) in 40 mM Tris (pH 7.9), 150 mM KCl, 5% glycerol, and 10 μM DTT, and fluorescence anisotropy was measured upon addition of varying amounts of non-fluorescent competitor DNA. The non-fluorescent competitor DNA was either: 1) *b*\textsubscript{iScR} (i.e. the same sequence as the fluorescently labeled oligonucleotide); 2) *b*\textsubscript{iScR}\textsuperscript{*} (a C-to-G substitution at position 8 of *b*\textsubscript{iScR}); 3) the IscR binding site sequence from the well-characterized type 2 site in the *hyaA* promoter region (Giel et al., 2006; Nesbit et al., 2012); or 4) shuffled DNA sequence with no resemblance to *b*\textsubscript{iScR}. The annealing of complementary DNA strands, fluorescence measurements, fraction bound determination, and data fitting were performed as previously described (Rajagopalan et al., 2013); however, all manipulations were carried out under aerobic conditions. Assays were repeated on three independent occasions. The sequences of the labeled *b*\textsubscript{iScR} DNA and the unlabeled competitor DNA are provided in Table 2.3.

**ChIP-qPCR analysis**
ChIP assays for strains MG1655 and JNC122 were performed as previously described (Davis et al., 2011) except that cultures were grown under aerobic conditions in minimal A glucose medium containing 10 mM TMAO. Samples were immunoprecipitated using a polyclonal antibody raised against IscR that had been absorbed against a mutant strain lacking iscR. Promoter DNA from ChIP samples was quantified by real-time PCR using SYBR Green JumpStart (Sigma-Aldrich) and a BioRad CFX96 Real-Time PCR Detection System. Primer sequences for amplifying the torT, hyaA, and ydfZ promoters are provided in Table 2.3 (PtorT-forward and PtorT-reverse; PhyaA-forward and PhyaA-reverse; and PydfZ-forward and PydfZ-reverse).

**Computer simulations**

The computer simulation of $P_{torCAD-yfp}$ expression was implemented in Python v2.7.13 (Python Software Foundation). The simulation initializes a parent cell with a random number of TorS and TorT molecules (within the estimated range of the number of molecules produced in a generation, obtained from published values (Li et al., 2014; Taniguchi et al., 2010) and drawn from a Gamma distribution). The parent cell is then permitted to “divide” for ten generations with lineage information preserved. The computer simulation makes use of several simplifying assumptions regarding the behavior of the TorT/TorS/TorR signaling pathway and torCAD expression to isolate the key question of interest: whether a modest increase in population-wide distribution of TorT and TorS protein can have a significant effect on the behavior of torCAD expression. At each division, the amount of free TorS, free TorT, and TorST complex is computed assuming that every TorS and TorT molecule will form a complex if a partner is available (i.e. there is only free TorS or TorT if one is present in excess of the other). TMAO is assumed to be at saturated levels so that every TorT in a TorST complex has TMAO bound. Equilibration of the concentration of phosphorylated TorR is assumed to occur instantaneously, and the fraction of phosphorylated TorR is then calculated from the following equation (which includes the implicit assumption that the second-order rate constants for TorR phosphorylation by TorST and TorR-P dephosphorylation by TorS are equal):
\[
\frac{\text{TorR-P}}{\text{TorR}_{\text{tot}}} = \frac{\text{TorST}}{\text{TorST} + \text{TorS}_{\text{free}}}
\]

where TorR_{\text{tot}} is the total number of TorR molecules in the cell and is arbitrary for the purposes of this simulation.

In the interval between divisions, the cell synthesizes YFP, TorS, and TorT. The number of YFP molecules produced by the \( P_{\text{torCAD-ycfp}} \) reporter is taken to be directly proportional to the amount of TorR-P in the cell. The amounts of TorS and TorT produced are drawn from the Gamma distribution:

\[
p(x) = \frac{1}{b^a \Gamma(a)} x^{a-1} e^{-x/b}
\]

where \( p(x) \) is the distribution of a protein in a population of cells, \( x \) is the concentration of the protein in a cell, \( a \) is the mean number of protein production bursts per division cycle (assuming no active protein degradation), and \( b \) is the mean number of proteins produced per burst (Friedman et al., 2006). The product of \( a \) and \( b \) is the mean number of protein molecules per cell in the population, which we know to be very low under aerobic conditions. We assume that \( b \), the mean number of molecules produced per burst, is unaffected by the presence or absence of oxygen—i.e. that there is no post-transcriptional oxygen-dependent regulation of TorT and TorS production. This assumption allows us to fix the value of \( b \) for TorT and TorS and explore the effect of varying the value of \( a \) on downstream \( P_{\text{torCAD}} \) activity. For simplicity, we set \( b = 1 \) and only allowed \( a \) to vary when simulating different levels of repression of \( \text{torS} \) and \( \text{torT} \). At cell division, the simulation allows TorS, TorT, and TorST complex to partition randomly between the daughter cells and distributes YFP evenly between the daughters. The lifetime of YFP protein is assumed to be infinite, only decreasing by dilution through cell division.

The Python source code for the computer simulation of \( P_{\text{torCAD-ycfp}} \) expression is publicly available at https://github.com/GoulianLab/tor-simulation.

Quantification and statistical analysis
Analysis of fluorescent reporters of gene expression in single cells was performed using the MicrobeJ plugin v5.11y (Ducret et al., 2016) for ImageJ v1.51r (Schneider et al., 2012) or the MicrobeTracker Suite v0.937 (Sliusarenko et al., 2011) for MATLAB R2012a (MathWorks). Background subtraction of images was performed prior to fluorescence quantification using the ImageJ sliding paraboloid algorithm or by subtraction of the mean background as described in Miyashiro and Goulian, 2007. Single-cell fluorescence was quantified as mean fluorescence per cell (total cellular fluorescence divided by cell area), with cell area determined by particle detection and segmentation of the corresponding phase contrast images. The mean fluorescence of each data set was calculated by averaging the single-cell fluorescence of at least 100 cells. Cellular autofluorescence in each channel was determined by including in each experiment a control strain lacking the fluorescent reporter(s) of interest. The mean autofluorescence was subtracted from the mean fluorescence for each data set.

Microcolony areas for aerobic-to-anaerobic transition experiments were determined by summing the lengths of all cells in the microcolony and multiplying by a constant cell width. Parent cell areas were calculated using the same method. The limit of detection for cell growth in the aerobic-to-anaerobic transition experiments was determined by calculating the maximum apparent fold change in cell area for approximately 200 non-growing cells over 5 h. Apparent fold changes in cell area result from slight differences in image focus between the start and end of an experiment.

Statistical parameters for each experiment are reported in the relevant figure legends.
Figure 2.1. Cell-to-cell variability in \textit{torCAD} expression permits a subpopulation to continue growth through a transition to anaerobiosis. (A) For cells growing on a non-fermentable carbon source, only those cells that have recently expressed \textit{torCAD} at a high level (green shading) are expected to grow after \(	ext{O}_2\) depletion. Inset: Growth by respiration of TMAO requires the TorC and TorA proteins. (B) Experimental setup to observe the fates of individual cells undergoing an aerobic-to-anaerobic transition. Inset: Micrograph of aerobically growing \textit{E. coli} cells harboring a \(P_{torCAD}\)-\textit{yfp} transcriptional reporter. Scale bar, 5 μm. See Movie 2.1 for a time-lapse video showing \(P_{torCAD}\)-\textit{yfp} expression in aerobically growing microcolonies. (C) Cell growth after \(	ext{O}_2\) depletion correlates with \textit{torCAD} expression at the time of depletion. Each circle represents a cell or microcolony of the \(P_{torCAD}\)-\textit{yfp} \(P_{tetA}\)-\textit{mcherry} strain MMR65. Growth is
quantified as the ratio of microcolony area approximately 5 h after O$_2$ depletion to the area of the parent cell at the time of depletion. The blue line indicates the limit of detection for cell growth. Micrographs corresponding to the green-shaded circles are indicated by arrows. Data are shown from a single experiment, with data from a replicate experiment shown in Figure 2.2A. a.u., arbitrary units. Scale bars, 2 μm.
Figure 2.2. Cell-to-cell variability in torCAD expression permits a subpopulation to continue growth through a transition to anaerobiosis (supplemental data). (A) Each circle represents a cell or microcolony of the $P_{torCAD}^{yfp}$ $P_{tetA}^{mcherry}$ strain MMR65. Growth is quantified as the ratio of microcolony area approximately 5 h after $O_2$ depletion to the area of the parent cell at the time of depletion. The blue line indicates the limit of detection for cell growth. Data are shown from a single experiment in which at least 50 randomly selected cells were monitored for growth. The apparent smaller range of fluorescence values for dark (non-growing) cells in these data compared to the data shown in Figure 2.1C is due to differences in the background subtraction algorithms used to process the two data sets. For Figure 2.1C, background was subtracted as described in Miyashiro and Goulian, 2007. For this figure, the
ImageJ sliding paraboloid algorithm was used (Schneider et al., 2012). Fluorescence from the \( P_{torCAD-yfp} \) reporter is dim in some aerobically growing cells, so different algorithms were used to confirm that results were unaffected by the background subtraction method. a.u., arbitrary units. (B) For the experiment shown in Figure 2.1C, the \( \Delta torC \) CFP\(^+\) strain MMR14 was grown in identical culture conditions to those of the \( P_{torCAD-yfp} \) reporter strain MMR65. The two strains were mixed before inoculating the anaerobic agarose pad for microscopy. Randomly selected \( \Delta torC \) CFP\(^+\) cells were monitored for growth during the experiment to ensure that the agarose pad was free of \( O_2 \) contamination. A fold change in cell area of 1.0 indicates no growth. Small changes in focus over the 5 h experiment account for departures from a fold change in area of 1.0. (C) Same as (B), but for the experiment depicted in (A). (D) The \( P_{torCAD-yfp} P_{tetA-mcherry} \) strain MMR65 was transferred to an aerobic rather than an anaerobic agarose pad and monitored for growth for 5 h after transfer. (E) A strain carrying the \( P_{torCAD-yfp} \) reporter but lacking the \( torC \) gene (MMR15) was subjected to the same experimental procedure as in (A) and did not grow after the transition to anaerobiosis.
Figure 2.3. TorT/TorS/TorR signaling regulates torCAD transcription, and transcription of torT and torS is O₂-dependent. (A) TorR is either phosphorylated or dephosphorylated by TorS depending on whether or not TorS is interacting with TorT-TMAO. TorR-P activates transcription of the torCAD operon. (B) Illustration of our model as applied to the distribution of PtorCAD-yfp transcription in aerobically growing cells (data taken from Figure 2.10). Because aerobic TorT and TorS abundance is very low, small differences in the number of TorT and TorS molecules in an individual cell have a large effect on the concentration of TorR-P. This results in highly variable expression from the torCAD promoter. See Figure 2.4 for a simulation of the model. (C) Operon fusions of yfp and mcherry at the torS-torT locus (strain JNC100) were used to measure torS and torT expression. Bar heights represent the mean values for three independent experiments, and error bars represent standard deviations. a.u., arbitrary units. See also Figure 2.5.
**Figure 2.4.** Simulated distributions of $P_{torCAD-yfp}$ fluorescence for different rates of TorT and TorS protein production. Distributions were simulated for average protein production rates of one copy per generation (red) and two copies per generation (brown). The plotted distributions represent the cumulative results from 100 independent runs of the simulation for each condition. Inset: Simulated mean $P_{torCAD-yfp}$ fluorescence for the displayed distributions.
Figure 2.5. Schematics of \(torT\) and \(torS\) reporter constructs and behavior of \(lacZ\) transcriptional fusions. (A) Schematic of the wild-type \(E. coli\) tor locus. (B) Schematic of the tor locus of the \(torT\)-mcherry \(torS\)-yfp transcriptional reporter strain (JNC100). The \(torR\) gene was deleted from the reporter strain because of its overlap with \(torT\). TorR is not involved in the regulation of \(torT\) or \(torS\) expression. \(kan\), kanamycin resistance gene; \(cat\), chloramphenicol resistance gene. Transcriptional fusions of \(lacZ\) to (C) \(torT\) (JNC163) and (D) \(torS\) (JNC166) were used to corroborate the results from the fluorescent protein reporters shown in Figure 2.3C. Growth conditions were identical between the fluorescent protein and \(lacZ\) reporter experiments. Bar heights represent the mean values for five independent experiments, and error bars represent standard deviations.
Figure 2.6. IscR binds in the torS-torT intergenic region. (A) ChIP-seq for IscR in wild-type cells (MG1655) grown aerobically and anaerobically shows IscR binding between torT and torS that is elevated during aerobic growth. ChIP-seq data for σ^70 (Myers et al., 2013) are also displayed, showing an inverse relationship between IscR and σ^70 binding. The σ^70 binding within the torS coding sequence may indicate the presence of an uncharacterized promoter. Strand-specific transcription (ssRNA) data show increased transcription of torT and torS in anaerobically growing wild-type cells (MG1655) as compared to aerobically growing cells. A strain lacking iscR (PK4854) shows elevated torT and torS transcription during both anaerobic and aerobic growth. (B) Model of IscR repression of torT and torS transcription. IscR is more abundant during aerobic growth, leading to more binding between torT and torS and stronger repression than during
anaerobic growth. (C) Alignment of the sequence logo of the IscR binding site motif with the inferred IscR binding site sequence near the torT promoter (denoted b\textsubscript{IscR}). The point mutation designated b\textsubscript{IscR}* is a C-to-G mutation in the IscR binding site at position -21 relative to the start codon of torT. See Figure 2.7 for a sequence comparison among Enterobacteriaceae. (D) Fluorescence anisotropy measurements show that IscR-C92A binds \textit{in vitro} to the b\textsubscript{IscR} sequence but not to the b\textsubscript{IscR}* sequence. IscR-C92A was incubated with fluorescently labeled b\textsubscript{IscR} DNA, and increasing amounts of unlabeled competitor DNA were added. The hyaA promoter is a positive control for IscR-C92A binding, and shuffled DNA is a control for nonspecific binding. Error bars represent the standard deviations of three experiments. The concentration of unlabeled DNA required to decrease maximum anisotropy by a factor of 0.5 (IC\textsubscript{50}) is 145 ± 26 nM for the b\textsubscript{IscR} sequence and 106 ± 10 nM for the hyaA IscR binding site sequence (mean ± s.d. for 3 independent experiments). (E) ChIP-qPCR using anti-IscR antibody in a wild-type strain (MG1655, left) and a b\textsubscript{IscR}*-containing strain (JNC122, right). The DNA segments targeted for quantification were the promoter regions of torT (containing b\textsubscript{IscR} or b\textsubscript{IscR}*) hyaA (a positive control for IscR binding), and ydfZ (a negative control for IscR binding). Fold enrichment indicates the ratio of ChIP-qPCR signal to no-antibody control. Bar heights represent mean values for 3 independent experiments, and error bars represent standard deviations.
Figure 2.7. The torT-torS intergenic region shows a high degree of sequence conservation across members of the Enterobacteriaceae. Bases with greater than 70% identity conservation are shaded. The degree of conservation in the vicinity of the IscR binding site is particularly high.
Figure 2.8. IscR represses torT and torS transcription. Deletion of iscR increases aerobic transcription of (A) torT and (B) torS. Transcription of (C) torT and (D) torS is increased in strains harboring the $b_{iscR}^*$ IscR binding site mutation. Oxygen regulation of (E) torT and (F) torS expression is disrupted in $b_{iscR}^*$-containing strains. Bar heights represent the mean values for three independent experiments, and error bars represent standard deviations. a.u., arbitrary units. Reporter strains were JNC100, JNC104 (A and B) and JNC92, JNC115, JNC73, and JNC112 (C-F). See also Figure 2.9.
Figure 2.9. The \( \text{b}_{\text{iscR}}^* \) mutation has no significant effect on \textit{torS} transcription and a very small effect on \textit{torT} transcription in a \( \Delta\text{iscR} \) strain. Deletion of \textit{iscR} and/or introducing the \( \text{b}_{\text{iscR}}^* \) IscR binding site mutation increases aerobic transcription of \textit{torT} and \textit{torS}. (A) Fluorescent \textit{torT-mcherry} reporter strains with wild-type \textit{iscR} (JNC148), \( \Delta\text{iscR} \) (JNC162), \( \text{b}_{\text{iscR}}^* \) (JNC157) or \( \Delta\text{iscR} \text{b}_{\text{iscR}}^* \) (JNC159) were grown aerobically in minimal glucose medium with TMAO and analyzed by fluorescence microscopy. (B) Fluorescent \textit{torS-yfp} reporter strains with wild-type \textit{iscR} (JNC73), \( \Delta\text{iscR} \) (JNC74), \( \text{b}_{\text{iscR}}^* \) (JNC112) or \( \Delta\text{iscR} \text{b}_{\text{iscR}}^* \) (JNC158) were grown and analyzed as in (A). Bar heights represent the mean values for three independent experiments, and error bars represent standard deviations. a.u., arbitrary units.
**Figure 2.10. Deletion of iscR suppresses variability in aerobic torCAD transcription.** The plot shows the distributions of single-cell fluorescence for wild-type and ΔiscR strains (MMR8 and MMR210). Single-cell fluorescence was quantified as YFP fluorescence normalized by a CFP internal standard (Roggiani and Goulian, 2015). Values on the x-axis are expressed as fluorescence normalized by the mean value from the corresponding dataset. Each dataset consists of combined measurements from two independent experiments. Inset: mean fluorescence for the datasets shown in the main plot. Bar heights represent the mean values for two independent experiments, and error bars represent the ranges. a.u., arbitrary units. See also Figure 2.11.
Figure 2.11. Deletion of torC increases mean expression from P_{torCAD} but does not affect variability. Fluorescent P_{torCAD}-yfp reporter strains with wild-type torC (MMR8) or ΔtorC (MMR15) were grown aerobically or anaerobically in minimal glucose medium with casamino acids and TMAO and analyzed by fluorescence microscopy. The strains constitutively express cyan fluorescent protein (CFP) for fluorescence normalization (Roggiani and Goulian, 2015). (A) Mean single-cell fluorescence of wild-type and ΔtorC strains grown aerobically or anaerobically. Fluorescence for each cell was quantified as YFP fluorescence normalized by the CFP internal standard. Bar heights represent the mean values for two independent experiments, and error bars represent the ranges. a.u., arbitrary units. (B) Distributions of single-cell fluorescence for...
wild-type and ΔtorC strains grown aerobically or anaerobically. Values on the x-axis are expressed as fluorescence normalized by the mean value from the corresponding data set. Each data set consists of combined measurements from two independent experiments.
Figure 2.12. Variability in aerobic torCAD transcription requires IscR binding between torT and torS in aerobic conditions. (A) Distributions of aerobic and anaerobic single-cell fluorescence for wild-type and bIscR* strains (JNC117 and JNC116). Single-cell fluorescence was quantified as CFP fluorescence normalized by cell area. Values on the x-axis are expressed as fluorescence normalized by the mean value from the corresponding dataset. Each dataset consists of combined measurements from two independent experiments. (B) Mean fluorescence for the data shown in (A). Bar heights represent the mean values for two independent experiments, and error bars represent the ranges. a.u., arbitrary units.
Figure 2.13. Uniform expression of torCAD permits growth of nearly the entire population after O₂ depletion. Each circle represents a cell or microcolony of the P₉torCAD-yfp ΔiscR* strain JNC123. Growth is quantified as in Figure 2.1C. The blue line indicates the limit of detection for cell growth. Data are shown from a single experiment, with data from a replicate experiment shown in Figure 2.14. a.u., arbitrary units.
Figure 2.14. Uniform expression of torCAD permits growth of nearly the entire population after O$_2$ depletion (replicate experiment). Each circle represents a cell or microcolony of the $P_{torCAD}$-yfp $b_{iscR}^*$ strain JNC123. Growth is quantified as the ratio of microcolony area approximately 5 h after O$_2$ depletion to the area of the parent cell at the time of depletion. The blue line indicates the limit of detection for cell growth. Data are shown from a single experiment in which at least 50 randomly selected cells were monitored for growth. a.u., arbitrary units.
Movie 2.1 (digital supplement). Expression of *torCAD* fluctuates rapidly during aerobic growth in the presence of TMAO. A strain containing a fluorescent reporter of *torCAD* transcription (P*torCAD*-yfp, strain JNC124) was grown aerobically in in minimal medium supplemented with TMAO to early exponential phase. A sample of this culture was transferred to an agarose pad made using the same culture medium and subjected to time-lapse microscopy on a heated microscope stage. Images were acquired every 14 minutes for approximately 4 hours.
### Table 2.1. Strains used in Chapter 2.

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Table 2.2. Plasmids used in Chapter 2.

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Table 2.3. Oligonucleotides used in Chapter 2.
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CHAPTER 3: Control of a Bacterial Respiratory System by a Temperate Bacteriophage

Introduction

Bacteria, like all cellular organisms, can fall prey to viral infections. The bacteriophages that infect bacteria, like all viruses, have evolved exquisite mechanisms to achieve the protection and replication of their genomes. Some bacteriophages, the lytic phages, take the brute force approach of forcing their host cell to manufacture as many virions as possible before killing the host, allowing the release of a new batch of infectious particles. Other bacteriophages, the temperate phages, have a subtler survival strategy. These phages can delay reproduction and integrate their genome into the host’s genome (either by recombination into the host chromosome or by adopting the form of a plasmid). In this integrated state the phage does not express its replicative machinery; instead, it freeloads on the host as a prophage and until some signal (commonly host cell stress) induces its lytic cycle.

Bacteria and the phages that infect them have a generally antagonistic relationship, and evolution has armed each side with various schemes to outwit the other. Sometimes, though, a bacterium and a temperate phage can form an uneasy truce, wherein the phage confers some beneficial attribute to its host cell that provides a fitness advantage: after all, unless the host cell dies on the phage’s own terms, the phage dies too. Some of the boons prophages can confer on their hosts include the abilities to produce toxins, resist antibiotics, increase virulence, and repel further phage infections (Argov et al., 2017; Bondy-Denomy and Davidson, 2014; Canchaya et al., 2003; Chibani-Chennoufi et al., 2004). There are likely entire classes of phage-encoded proteins that impact host fitness, as the majority of phage genes have unknown function and no homology to any genes with known function.

Phages can also alter their host’s behavior in more subtle or indirect ways than, say, enabling toxin production. One study found that deleting all of the cryptic prophages in *E. coli* BW25113 increased the strain’s susceptibility to various exogenous stresses and decreased its growth rate through mechanisms yet to be worked out in detail (Wang et al., 2010). Lysogenic
infection of \textit{E. coli} AB257 \textit{met} by phage \( \lambda \) has been shown to increase growth rate in some conditions and decrease it in others (Edlin et al., 1975; Lin et al., 1977). In another study on phage \( \lambda \), the \( cI \) repressor expressed during lysogenic infection was discovered to act directly at the promoter of the metabolic gene \textit{pckA}, which encodes phosphoenolpyruvate carboxykinase, repressing its expression and producing a slow growth phenotype in some conditions (Chen et al., 2005). Some prophages can alter host gene expression because of the physical position in the host genome where they integrate. For instance, the \( \Phi13 \) phage of \textit{Staphylococcus aureus} integrates into the 5’ end of the \textit{hlb} gene, disabling \( \beta \)-toxin expression (Coleman et al., 1991). In most cases, however, the effects of lysogeny on host physiology are unknown.

We became interested in a particular temperate phage that infects \textit{E. coli}, called HK022 (Dhillon and Dhillon, 1972), because its attachment site lies precisely between the genes \textit{torT} and \textit{torS} (Yagil et al., 1989). These two genes are required for the activation of the \textit{torCAD} operon, which encodes TMAO reductase. The genes are independently transcribed but share a repressor binding site for the transcription factor \textit{IscR} (see Chapter 2). Curiously, the HK022 attachment site separates the \textit{torS} coding sequence from the \textit{IscR} binding site that regulates its expression. The insertion of a prophage between a gene’s coding sequence and operator would be expected to modify transcription and could represent another method by which a prophage alters the behavior of its host.

Results

\textbf{Lysogenic infection by HK022 disables aerobic expression of \textit{torCAD}}

The attachment site for bacteriophage HK022 sits in the 82-bp intergenic region between \textit{torT} and \textit{torS} and separates the \textit{torS} open reading frame from the \textit{IscR} binding site that regulates \textit{torS} expression. We suspected that the presence of a prophage at this attachment site would disrupt the regulation of \textit{torS} expression and, ultimately, \textit{torCAD} expression because of \textit{torCAD} expression’s dependence on TorS. To investigate the impact of HK022 lysogeny on \textit{torCAD} expression, we infected a strain carrying a fluorescent protein reporter of \textit{torCAD} transcription
with HK022 and selected for lysogens. We grew this lysogenized reporter strain aerobically in the presence of TMAO and measured torCAD transcription in single cells by fluorescence microscopy. Transcription of torCAD was abolished in the lysogen but occurred with its signature pattern of high cell-to-cell variability in the non-lysogen control strain (Figure 3.1).

The simplest explanation for the loss of aerobic torCAD expression in the lysogen is that the presence of the prophage destroys the torS promoter. Cells without TorS cannot phosphorylate TorR and activate torCAD transcription (Jourlin et al., 1996a). However, when we measured torCAD transcription in cells grown anaerobically in the presence of TMAO, we observed no difference between the lysogen and the non-lysogen (Figure 3.1). TorS, then, is still produced in the lysogen under some conditions, meaning there is a functional torS promoter in the lysogen even though the IscR-regulated promoter is now separated from the coding sequence by roughly 41 kbp.

We previously showed that high cell-to-cell variability in aerobic torCAD expression can function as a bet-hedging strategy that helps a population tolerate a rapid transition to anaerobiosis. Only cells with a recent history of high torCAD expression are able to continue growth after oxygen depletion when TMAO is present and no other respiratory electron acceptors or fermentative substrates are available. Because the HK022 lysogen does not express torCAD aerobically, we suspected that it would be unable to bet hedge and therefore unable to grow through an aerobic-to-anaerobic transition. We tested this hypothesis by growing aerobic liquid cultures of the HK022 lysogen and non-lysogen in media containing TMAO and then combining the cultures and transferring to an anaerobic agarose pad, where we could then observe the fates of single cells by time-lapse microscopy. Both strains contained the same fluorescent protein reporter of torCAD transcription so that growth after the transition to anaerobiosis could be correlated with the extent of recent torCAD expression at the time of transition, and each strain constitutively expressed a different fluorescent protein so that the strains could be distinguished from one another by microscopy. Both strains also carried deletion mutations of the HK022 receptor gene (fhuA) to prevent any infection of the non-lysogen by phage particles produced by spontaneous
prophage induction in the lysogen. The result of this experiment is given in Figure 3.2, which confirms that only the non-lysogen contained a subpopulation of cells that showed significant growth after oxygen depletion and that these cells had high torCAD expression at the time of transition. From this we conclude that lysogenic infection by HK022 deactivates TMAO bet hedging.

**The HK022 prophage increases torS transcription but not torT transcription**

The HK022 lysogen expresses torCAD in the absence of oxygen, which requires TorS and indicates that the prophage does not simply eradicate the torS promoter. To investigate the effect of the prophage on torS expression, we measured torS transcription by β-galactosidase assay in the HK022 lysogen and non-lysogen, both with and without oxygen (Figure 3.3). We found that torS transcription was substantially elevated in the HK022 lysogen and no longer regulated by oxygen. When we performed analogous experiments to measure torT transcription, we found no difference between the lysogen and non-lysogen (Figure 3.3). These results suggest that rather than shutting off aerobic torCAD transcription by preventing torS expression, the HK022 prophage has the opposite effect and shuts off aerobic torCAD transcription by significantly upregulating aerobic torS expression: cells with an excess of TorS relative to TorT would strongly favor TorR dephosphorylation and not express torCAD (Ansaldi et al., 2001). We note that the HK022 lysogen also shows elevated torS transcription in the absence of oxygen and yet torCAD is still expressed in these conditions. This likely indicates that anaerobic TorT levels are sufficiently high for any additional TorS not to have much impact on TorR phosphorylation and torCAD expression.

**Overexpression of torT in an HK022 lysogen restores aerobic torCAD transcription**

If our model of how HK022 prevents aerobic torCAD expression is correct, overexpressing torT in an HK022 lysogen should compensate for elevated TorS levels and restore aerobic expression of torCAD. To test this, we introduced a plasmid containing torT under control of a weakened trc
promoter into the lysogen carrying the fluorescent torCAD transcriptional reporter and quantified torCAD expression (Figure 3.4). The result of this experiment agreed with our prediction, with the lysogen carrying the torT overexpression plasmid now expressing torCAD in the presence of oxygen. We also observed a small increase in mean anaerobic torCAD expression when torT was overexpressed in the lysogen, which we interpret as meaning that the additional TorT produced allows more of the TorS that is present to engage in phosphorylation of TorR and activation of torCAD.

An element specific to the HK022 prophage drives torS expression

Transcription of torS in the HK022 lysogen is higher than we ever observe in a non-lysogen, even when the repressor IscR is deleted or the IscR binding site mutated so that the repressor cannot bind (see Chapter 2). This heightened torS expression suggests that the prophage either carries a promoter that reads outward toward torS or that it encodes an element that strengthens transcription from some secondary torS promoter normally overridden by the upstream IscR-regulated promoter. To test whether there is indeed something distinctive about the prophage that allows it to drive torS expression, we integrated a plasmid containing the HK022 attP sequence (but no other sequence similarity to HK022) into the HK022 attB site and measured torS transcription by β-galactosidase assay (Figure 3.5). The strain harboring the integrated plasmid showed decreased torS transcription relative to the HK022 lysogen both aerobically and anaerobically, indicating that there is some feature of the HK022 prophage that drives high torS expression. As expected, the analogous experiment to measure torT transcription revealed no difference between the plasmid-integrated strain and the HK022 lysogen.

E. coli strains carrying HK022-like prophages are widespread

The above results reveal that lysogenic HK022 infection has a strong effect on torCAD expression in E. coli K-12 substr. MG1655. This prompted us to explore the distribution of HK022-like prophages in wild E. coli strains. We searched for the torT and torS genes by BLAST
(Boratyn et al., 2013) against all of the *E. coli* genome sequences in the NCBI complete prokaryote RefSeq (O’Leary et al., 2016) and GenBank (Benson et al., 2005) databases and then calculated the *torT-torS* intergenic distance for each strain. For all strains with large insertions between *torT* and *torS* (relative to *E. coli* MG1655), we checked for the presence of phage genes to identify lysogens. Roughly 4% of the sequenced genomes carried prophages integrated at the HK022 *attB* site (Table 3.1). In most of these strains the *torT-torS* intergenic distance was roughly the same size as the HK022 genome, which is 40,751 bp long (Juhala et al., 2000). Prophages were in found in phylogenetically diverse *E. coli* strains, with the highest number of lysogens belonging to phylogenetic group B1.

A wild *E. coli* isolate containing an HK022-like prophage shows decreased aerobic expression of *torCAD*

We previously demonstrated that highly variable aerobic *torCAD* expression occurs in wild *E. coli* strains as it does in the laboratory strain MG1655, implying that the mechanism of *torCAD* regulation is widespread and not a quirk of the lab-adapted strain (Roggiani and Goulian, 2015). If the regulation of *torCAD* expression is similar across diverse strains, then the HK022-like prophages found in wild strains might be expected to block aerobic *torCAD* expression in a manner akin to how the HK022 prophage blocks aerobic *torCAD* expression in MG1655. To begin this investigation, we compared aerobic *torCAD* expression in MG1655 to expression in the prophage-carrying strain E2348/69, a well-characterized enteropathogenic *E. coli* (EPEC) strain (see Table 3.1). We integrated the same fluorescent reporter of *torCAD* transcription into E2348/69 as had been integrated into MG1655 and measured single-cell fluorescence of aerobically growing cells by microscopy (Figure 3.6). Mean expression of *torCAD* in E2348/69 was roughly half the mean expression in MG1655, which differs from the nearly total abolition of *torCAD* expression seen in an HK022 lysogen of MG1655 (Figure 3.1). However, E2348/69 cells appeared to be split into two subpopulations, with one subpopulation expressing *torCAD* with a similar mean level and variance as MG1655 and one subpopulation not expressing *torCAD* at all.
These preliminary results are insufficient for drawing any strong conclusions about the impact of the E2348/69 prophage on torCAD expression, but a possible role for the prophage is suggested by the observations that mean torCAD expression is lower in E2348/69 than in MG1655 and that E2348/69 harbors a large subpopulation of cells that do not express torCAD.

Discussion

In this work, we have shown that bacteriophage HK022 reconfigures the regulation of TMAO reductase expression in E. coli during lysogenic infection. Although other cases have been described wherein a prophage alters the expression of host metabolic genes, we are unaware of any other instances in which a prophage so dramatically modifies its host's response to the presence of a metabolite—in this case, by making expression of torCAD strictly oxygen-dependent.

HK022 reconfigures torCAD regulation by increasing expression of the regulatory protein TorS. The phage appears to achieve this by separating the torS coding sequence from its promoter and introducing a new promoter encoded within the prophage. This model is supported by the experiment in which we substituted arbitrary plasmid sequence for the HK022 prophage and observed a decrease in torS transcription from the high level seen in the lysogen. Although this outcome was suggestive of there being a phage-encoded promoter, the design of the experiment did not allow us to rule out the possibility that the prophage instead encodes some trans-acting regulator that enhances torS expression. Furthermore, the integrated plasmid did not decrease torS expression back down to the level of the non-lysogen, making the result of the experiment ambiguous: that the decrease in torS transcription was only partial implies that there is a plasmid-encoded element that can drive torS expression, albeit not to the same degree as whatever is encoded in the HK022 prophage. In all likelihood this plasmid-encoded element is its kanamycin resistance gene, which is oriented toward torS and lacks any associated transcriptional terminators. We are following up on this inconclusive experiment with ongoing work that will more directly address the question of whether or not torS transcription originates
from within the prophage. Our strategy is to integrate a strong transcriptional terminator into the prophage right at its junction with the host genome, thereby abolishing any torS transcription driven by a prophage-encoded promoter.

Interestingly, there is a computationally inferred secondary torS promoter that is closer to the torS coding sequence than is the IscR-regulated promoter (Huerta and Collado-Vides, 2003). In vitro transcription experiments indicate that this promoter may be real and may provide low-level constitutive expression of torS that is not responsive to IscR (P.J. Kiley, personal communication). This inferred secondary promoter straddles the HK022 attachment site and has a -10 site that remains intact during lysogenic infection but a -35 site that is replaced by prophage sequence. It is possible that the prophage provides a new, stronger -35 site, creating a chimeric promoter that drives torS expression higher than occurs with the unmodified bacterial promoter.

We are collaborating with Patricia Kiley at the University of Wisconsin on in vitro transcription experiments in an attempt to map the position of the torS transcription start site within the HK022 prophage sequence. This work complements the in vivo experiment outlined above (integration of a transcriptional terminator) and should reveal whether the transcript originates from a chimeric promoter or from fully within the prophage.

We can only speculate on why HK022 shuts off aerobic torCAD expression and, consequently, its associated bet-hedging behavior. We have argued previously that there must be a fitness cost to the expression of torCAD or its expression would not be regulated (see Chapter 2). It might be that the HK022 prophage prevents aerobic torCAD transcription to alleviate this fitness cost and thereby increase the rate of its own replication. If the primary function of aerobic torCAD expression is bet hedging on rapid oxygen depletion, shutting down aerobic expression could be a useful strategy if lysogenic HK022 infections occur most frequently in environments where E. coli is not likely to experience oxygen loss. (HK022 was isolated from a wastewater-receiving reservoir (Dhillon and Dhillon, 1972), but the spatiotemporal dissolved oxygen profile of this environment was not studied). This model can be extended to our preliminary findings in the prophage-carrying EPEC strain E2348/69, in which aerobic torCAD expression occurs in some
cells but is entirely shut off in a large fraction of the population. As with HK022-infected MG1655, these “off” cells do not experience the fitness cost of torCAD expression and would be predicted to have a competitive advantage over bet-hedging cells in an oxygenated environment. However, “off” cells would not be expected to grow through a rapid drop in oxygen, suggesting that E2348/69 is adapted to life in environments in which oxygen depletion is a gradual and/or infrequent process (in the presence of TMAO). Unlike in HK022-infected MG1655, aerobically growing E2348/69 still harbors a small subpopulation of torCAD-expressing cells: this strain might be able to hedge its bets, but with a large investment in remaining in an oxygenated environment.

The difference in behavior between HK022-infected MG1655 and E2348/69 is of great interest to us. Is one of these behaviors more common in the wild, and what other patterns of torCAD expression might exist in wild lysogens? We have yet to analyze the anaerobic behavior of E2348/69 to learn if and how torCAD expression differs in the presence and absence of oxygen. We are currently working toward to curing E2348/69 of its HK022-like prophage, which would enable a direct evaluation of the contribution of the prophage to the behavior of the strain. An informative experiment would be to infect MG1655 with the E2348/69 phage and to infect a cured E2348/69 with HK022 (a “prophage swap”), which would aid in distinguishing general effects of HK022-like infection from the specific effects associated with specific prophages and hosts. Relatedly, efforts are also underway to create HK022 lysogens in the other wild E. coli strains whose torCAD expression pattern we have previously studied (Nissle 1917 and HS, see Roggiani and Goulian, 2015) and to analyze torCAD expression in additional wild strains with naturally occurring HK022-like lysogenic infections. One such strain that we have recently obtained is NRG 857C, an adherent-invasive E. coli (AIEC) isolated from a patient with Crohn’s disease (Nash et al., 2010). Studying the diversity and distribution of prophage-mediated torCAD expression could provide insight into what evolutionary advantage there might be for the phage to reconfigure TMAO respiration regulation.

Prophage-mediated effects on host physiology are largely still enigmatic. Knowledge is mostly restricted to cases where the effects are readily apparent, as when the prophage encodes
a gene enabling some obvious trait such as toxin production, and cases where a prophage directly alters host metabolism have been described infrequently and generally with little mechanistic detail. To our knowledge, the phenomenon described in this study, where a prophage rewires the regulation of a metabolic pathway by modulating the expression of a signaling gene, has not been reported before. Because of the huge diversity of and abundance of phages, this phenomenon may exemplify a general class of mechanism phages can use to control host behavior. Phage infections certainly play a significant role in bacterial community dynamics, and much of our knowledge about the effects of phage infection is centered on lytic infection, horizontal gene transfer, and bacterial pathogenesis. A greater appreciation of the subtler effects of phage infection on host phenotype is a likely platform for developing enhanced understanding of the structure and behavior of microbial communities.

Methods

**Bacterial growth media and conditions**

Media and growth conditions were as described in Chapter 2 except that minimal A medium was supplemented with 0.1% casamino acids for all experiments.

**Strain construction**

Strains and plasmids used in this study are listed in Table 3.2 and Table 3.3, respectively. Standard methods were used for strain construction. P1vir transductions were performed as in Miller, 1992 to create strains JNC173 (JW0146 × DFE12) and JNC174 (JW0146 × MMR65). Plasmid integrations were performed as in Haldimann and Wanner, 2001 using helper plasmids pAH69 or pINT-ts to construct strains JNC171 (pAS41 integrated into the HK022 attB site of JNC163), JNC172a (pAS41 integrated into the HK022 attB site of JNC166), and EPEC-torC-yfp (pMR4 integrated into the λ attB site of E2348/69). HK022 lysogens were generated using a method adapted from protocols for making λ lysogens (Silhavy et al., 1984) and for making mycobacteriaphage lysogens (Sarkis and Hatfull, 1998). Briefly, the strain to be lysogenized was
grown to saturation in LB overnight culture and harvested by centrifugation. Cells were resuspended at 2x concentration in 10 mM MgSO₄, and 100 μL of cell suspension was added to 3 mL molten LB top agar at 45°C. The top agar was mixed, layered onto a prewarmed LB agar plate, and allowed to solidify. HK022 lysate (50 μL) was spotted onto the top agar and allowed to dry, and the plate was incubated at 37°C overnight. On the following day, an LB plate was spread with 100 μL HK022 lysate and allowed to dry. Selection for lysogens was carried out by streaking from the turbid zone of lysis formed on the top agar plate onto the HK022-spread LB plate and incubating at 37°C overnight. HK022-resistant colonies were patched onto LB agar, and the same colonies were tested for lysogenic infection by patching onto a top agar lawn containing an HK022-sensitive strain (MG1655). After overnight incubation at 37°C, candidate lysogens that produced a zone of lysis around the area of the patch (from spontaneous phage release) were nonselectively purified by streaking for single colonies from the LB plate patches and incubating at 37°C overnight. The entire patch test procedure was then repeated using the purified colonies. Candidate lysogen colonies that still produced a zone of lysis around the patch after purification were tested for the presence of the HK022 prophage by PCR. Strains produced by this method were DFE12 (HK022 lysogen of MMR8), JNC168 (HK022 lysogen of JNC163), and JNC169 (HK022 lysogen of JNC166). HK022 lysate was a generous gift from M.E. Gottesman (Columbia University).

**Phase contrast and fluorescence microscopy**

Microscopy was performed as described in Chapter 2 except that cultures were chilled on ice for 30 min at the time of streptomycin addition and then aerated on a roller drum at 37°C for 2 h before being held at 4°C overnight. Imaging was performed the next day with no additional aeration beforehand.

**Aerobic-to-anaerobic transition microscopy**
Aerobic-to-anaerobic transition microscopy was performed as described in Chapter 2 except that the ΔtorC control strain was omitted.

**β-Galactosidase assays**

β-Galactosidase assays were performed as in Chapter 2.

**Phylogenetic classification**

Phylogenetic group assignments of the prophage-carrying strains listed in Table 3.1 were made as described in (Clermont et al., 2013) using the strain sequences available from NCBI. Isolation source was identified from information in the NCBI sequence entry or linked BioSample entry (Barrett et al., 2012).
Figure 3.1. Bacteriophage HK022 shuts off aerobic expression of *torCAD* but leaves *anaerobic expression intact*. The distributions of single-cell fluorescence are shown for strains carrying a fluorescent reporter of *torCAD* transcription. The strains are an HK022 lysogen (DFE12) and a non-lysogen (MMR8) grown in the presence or absence of oxygen. Each circle represents a fluorescence measurement made in an individual cell. The single-cell measurements were used to generate the density curves shown in grey. Data are pooled from three independent experiments, with the vertical red lines marking the population mean fluorescence for each experiment. a.u., arbitrary units.
**Figure 3.2.** Most cells infected with HK022 fail to grow following rapid oxygen depletion.

Each circle represents an individual cell monitored for growth following an aerobic-to-anaerobic transition. The same data are presented on a linear scale (left) for easier comparison with Figure 3.1 and on a log scale (right) for clearer resolution of individual points. The HK022 lysogen (JNC173) constitutively expresses CFP to distinguish it from the non-lysogen (JNC174), which constitutively expresses mCherry. Both strains carry the YFP reporter of torCAD transcription and lack *fhuA*, the gene encoding the HK022 receptor. Growth is quantified as the ratio of microcolony area approximately 5 h after O₂ depletion to the area of the parent cell at the time of depletion. Data are shown for a single representative experiment.
Figure 3.3. The HK022 prophage increases torS transcription and has no effect on torT transcription. Aerobic and anaerobic transcription of torS and torT was measured by β-galactosidase assays in strains carrying torS-lacZ or torT-lacZ operon fusions with and without lysogenic HK022 infection (strains JNC166, JNC169, JNC163, and JNC168). Each circle represents a measurement obtained from an independent experiment, and the horizontal lines indicate average values.
Figure 3.4. Overexpression of torT restores aerobic torCAD expression in an HK022 lysogen. The distributions of single-cell fluorescence are shown for strains carrying a fluorescent reporter of torCAD transcription. The strains are an HK022 lysogen (DFE12) and a non-lysogen (MMR8) containing a plasmid for torT overexpression (pMR26) or an empty vector control (pDSW206) and grown in the presence or absence of oxygen. Expression of torT from the plasmid is driven by a weakened trc promoter without added inducer. Each circle represents a fluorescence measurement made in an individual cell. The single-cell measurements were used to generate the density curves shown in grey. Data are pooled from three independent experiments, with the vertical red lines marking the population mean fluorescence for each experiment. a.u., arbitrary units.
Figure 3.5. An arbitrary plasmid integrated at the HK022 attB site lowers torS transcription relative to the lysogen and has no effect on torT transcription. Aerobic and anaerobic transcription of torS and torT was measured by β-galactosidase assays in strains carrying torS-lacZ or torT-lacZ operon fusions with lysogenic HK022 infection or with a plasmid (pAS41) integrated at the HK022 attB site (strains JNC166, JNC172a, JNC163, and JNC171). Each circle represents a measurement obtained from an independent experiment, and the horizontal lines indicate average values. Data for the HK022 lysogens is reproduced from Figure 3.3.
Figure 3.6. A large subpopulation of cells in a wild *E. coli* strain carrying an HK022-like prophage does not express *torCAD*. The distributions of single-cell fluorescence are shown for strains carrying a fluorescent reporter of *torCAD* transcription. The reporter-containing MG1655 derivative is strain MMR8, and the reporter-containing E2348/69 derivative is strain EPEC-torC-yfp. Cells were grown in aerobic culture. Each circle represents a fluorescence measurement made in an individual cell. The single-cell measurements were used to generate the density curves shown in grey. E2348/69 data are pooled from three independent experiments, and the MG1655 data are from a single experiment. Vertical red lines mark the population mean fluorescence for each experiment. a.u., arbitrary units.
Table 3.1. Fully sequenced genomes of natural *E. coli* isolates carrying prophages at the HK022 *attB* site.

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<th>Isolation source</th>
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<td>NZ_CP012693</td>
<td>54,420</td>
<td>B1</td>
<td>Human feces</td>
</tr>
<tr>
<td>KSC64</td>
<td>NZ_CP018840</td>
<td>16,088</td>
<td>B1</td>
<td>Pig feces</td>
</tr>
<tr>
<td>LF82</td>
<td>NC_011993</td>
<td>38,764</td>
<td>B2</td>
<td>Human (Crohn’s disease patient)</td>
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<tr>
<td>M6</td>
<td>NZ_CP010186 / NZ_CP010196</td>
<td>40,124</td>
<td>B1</td>
<td>Mouse feces</td>
</tr>
<tr>
<td>NRG 857C</td>
<td>NC_017634</td>
<td>40,878</td>
<td>B2</td>
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<tr>
<td>VR50</td>
<td>NZ_CP011134</td>
<td>43,566</td>
<td>A</td>
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</tr>
<tr>
<td>09-00049</td>
<td>NZ_CP015228</td>
<td>38,832</td>
<td>B1</td>
<td>Lettuce</td>
</tr>
<tr>
<td>13E0767</td>
<td>NZ_CP020107</td>
<td>49,396</td>
<td>B1</td>
<td>Cattle</td>
</tr>
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</table>
Table 3.2. Strains used in Chapter 3.

<table>
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<th>Strain</th>
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<tr>
<td>MG1655</td>
<td></td>
<td>Coli Genetic Stock Center #7740</td>
</tr>
<tr>
<td>DFE12</td>
<td>MG1655 att(\lambda)::(cat (P_{torC-yfp}) ompA-cfp) HK022+</td>
<td>This work</td>
</tr>
<tr>
<td>EPB47</td>
<td>MG1655 ompA-cfp</td>
<td>Roggiani and Goulian, 2015</td>
</tr>
<tr>
<td>JNC163</td>
<td>MG1655 ΔlacZYA::FRT-cat-FRT torT-lacZ-FRT-kan-FRT ΔtorR</td>
<td>This work</td>
</tr>
<tr>
<td>JNC166</td>
<td>MG1655 ΔlacZYA::FRT torS-lacZ-FRT-kan-FRT</td>
<td>This work</td>
</tr>
<tr>
<td>JNC168</td>
<td>MG1655 ΔlacZYA::FRT-cat-FRT HK022+ torT-lacZ-FRT-kan-FRT ΔtorR</td>
<td>This work</td>
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<td>This work</td>
</tr>
<tr>
<td>JNC171</td>
<td>MG1655 ΔlacZYA::FRT-cat-FRT attHK022::(kan tetR tetA) torT-lacZ-FRT-kan-FRT ΔtorR</td>
<td>This work</td>
</tr>
<tr>
<td>JNC172a</td>
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<td>This work</td>
</tr>
<tr>
<td>JNC173</td>
<td>MG1655 ΔfhuA::FRT-kan-FRT att(\lambda)::(cat (P_{torC-yfp}) ompA-cfp) HK022+</td>
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<tr>
<td>JNC174</td>
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<td>JW0146</td>
<td>BW25113 ΔfhuA::FRT-kan-FRT</td>
<td>Baba et al., 2006</td>
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<td>MMR8</td>
<td>MG1655 att(\lambda)::(cat (P_{torC-yfp}) ompA-cfp)</td>
<td>Roggiani and Goulian, 2015</td>
</tr>
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<td>E2348/69</td>
<td></td>
<td>Iguchi et al., 2009</td>
</tr>
<tr>
<td>EPEC-torC-yfp</td>
<td>E2348/69 att(\lambda)::(cat (P_{torC-yfp}))</td>
<td>This work</td>
</tr>
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Table 3.3. Plasmids used in Chapter 3.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant genotype</th>
<th>Reference / source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAH69</td>
<td>oriR101 repA101(ts) λpr-intHK022 λcl857(ts) bla</td>
<td>Haldimann and Wanner, 2001</td>
</tr>
<tr>
<td>pAS41</td>
<td>oriRγ attPHK022 tetA tetR kan</td>
<td>Goulian lab stock</td>
</tr>
<tr>
<td>pDSW206</td>
<td>lacI β bla Pattenuated promoter</td>
<td>Weiss et al., 1999</td>
</tr>
<tr>
<td>pMR4</td>
<td>oriRγ attPλ PtorCAD-yfp cat</td>
<td>Roggiani and Goulian, 2015</td>
</tr>
<tr>
<td>pMR26</td>
<td>pDSW206 torT</td>
<td>Roggiani and Goulian, 2015</td>
</tr>
</tbody>
</table>
CHAPTER 4: Perspectives

This work has enriched our understanding of when, how, and to what end *E. coli* expresses its TMAO respiratory system. We have shown that highly variable aerobic expression can function as a bet-hedging strategy, that variability is regulated by the oxygen-dependent repression of genes in the TMAO sensing pathway, and that a temperate bacteriophage hijacks the host’s own regulation of TMAO reductase expression. These findings have enhanced our understanding of some basic biological phenomena, such as the impacts of molecular noise and the alteration of host gene regulation by bacteriophages, but this work has also raised many new questions about the biology of TMAO respiration and microbial community behavior.

The mechanism we have described can account for a decoupling of the control of variance from control of the mean, but it does not explain why mean *torCAD* expression does not significantly change between aerobic and anaerobic conditions. This phenomenon could emerge spontaneously from the properties of the known regulators, or there could be additional layers of regulation that are actively involved in holding the mean steady. This question has been outstanding since our initial description of oxygen-dependent variability in *torCAD* transcription (Roggiani and Goulian, 2015), but it has been made all the more intriguing by our observation that the threshold for growth upon a transition to anaerobiosis aligns very closely with the population mean (see Chapter 2). Is it simply a coincidence that the mean *torCAD* expression level corresponds to this threshold, or is there an as-yet-undescribed regulatory mechanism involved? The threshold for growth also closely matches the mean level of anaerobic *torCAD* expression, implying that in anaerobic conditions cells synthesize the minimal amount of TorCAD that they need for respiratory growth. If this is true, why do aerobic cells transiently express *torCAD* at levels much higher than the mean? Perhaps this extremely “bursty” expression pattern reduces the fitness cost of aerobic *torCAD* expression in some way we have not yet been able to detect.
We presume that there must be a fitness cost associated with aerobic \textit{torCAD} expression or else its expression would be unregulated (see Chapter 2). However, none of our efforts have revealed such a cost. Fitness is a measure of reproductive success, and in laboratory studies of bacteria growth rate is a convenient measure of fitness. Neither direct measurements of growth rate nor co-culture competition experiments revealed convincing evidence that fitness is negatively affected by aerobic \textit{torCAD} expression—even if we force the expression of \textit{torCAD} in the absence of TMAO. However, there are two major limitations of laboratory fitness experiments. First, the sensitivity to small fitness differences is poor. Small differences that are very important over long, evolutionary time scales may be undetectable over the short duration of laboratory experiments. Executing long-term fitness experiments in the lab also presents its own set of challenges, both practically in terms of the time required and, more significantly, as bacteria readily evolve adaptations for growth in the laboratory setting (Wiser et al., 2013), easily obscuring the phenotype that was originally under study. Second, and more important, there is no way to recreate in the laboratory the environments to which an organism is adapted. Our model that variable \textit{torCAD} expression could be a bet-hedging strategy can only ever be a proposition: we can never know what pressures shaped the evolution of the regulatory system that generates variable \textit{torCAD} expression, and we can only guess at what laboratory conditions would most closely approximate the conditions in which such behavior occurs in nature. In short, lack of laboratory evidence of a fitness cost is not evidence against the existence of a fitness cost.

Furthermore, bet hedging and its associated fitness costs may only be part of the story. Phenotypic diversity in microbial populations is associated with a kaleidoscope of social behaviors (West et al., 2006). It may be the case, for instance, that aerobic TMAO reduction is altruistic, meaning that it exerts a toll on the individual cell performing the reaction but benefits the population as a whole. It has been reported that aerobic TMAO reduction can protect a growing population against acidification of the growth medium (Ansaldi et al., 2007; Bordi et al., 2003). We have not observed this phenomenon in our experiments, but its occurrence would be consistent with altruistic behavior, wherein some cells in the population transiently take on the burden of
producing trimethylamine (the product of TMAO respiration) and, by doing so, help the entire population by counteracting acidification.

It may also be possible that TMAO reductase expression benefits individual cells in some as yet unforeseen way: torT has been computationally predicted to be regulated by σE and/or σF (Huerta and Collado-Vides, 2003), and we have generated preliminary data that suggest that torT does belong to the σE regulon. If this is true, we would expect torT expression to increase under conditions of envelope stress (Raivio and Silhavy, 2001). Can TMAO reductase perform some chemistry—possibly on a substrate other than TMAO—that helps alleviate envelope stress? Can TorT initiate signaling upon binding a ligand other than TMAO? We know that torT overexpression does little to change torCAD expression in a non-lysogen during aerobic growth but has a huge impact on torCAD expression in an HK022 lysogen (see Chapter 3). Perhaps envelope stress can increase TorT levels enough to activate aerobic torCAD expression in a lysogen. If so, does this benefit the phage or its host in some way?

Another curiosity about torCAD regulation is the fact that IscR serves as the oxygen sensor in this system. The catalog of IscR’s cellular functions is far from complete, but this protein is mostly understood in its role as a regulator of Fe-S cluster biogenesis (Mettert and Kiley, 2015). Why, then, is it involved in torCAD regulation, when TMAO reductase is the only anaerobic respiratory reductase in E. coli that does not use an Fe-S cofactor (Cammack and Weiner, 1990; Guigliarelli et al., 1996; Iverson et al., 1999; Jepson et al., 2007; Yamamoto et al., 1986)? It is tempting to speculate that IscR would act to increase expression of torCAD in conditions when Fe-S biogenesis is challenged, but this is not what appears to be occurring: IscR regulates the variance in torCAD expression, not the mean, and it does so indirectly through its repression of torT and torS transcription (see Chapter 2). (Neither TorT nor TorS, it should be mentioned, contains an Fe-S cluster.) We have not definitively ruled out the possibility that IscR can act directly at the torCAD promoter, but preliminary experiments have not lent any support to this proposition. The Fe-S question for now remains a mystery, and like all questions of evolutionary
teleology it may not have an answer: this regulation may have arisen by chance and persisted because it works.

There are undoubtedly still many specific aspects of TMAO respiration and its regulation that clamor for exploration. As in any field of inquiry, the answer to every question leads to another question. No biological system is isolated, and many of these questions are bound to stray outside the specific domain of TMAO respiration. For instance, we have already established links to iron metabolism and phage biology that warrant further treatment. It is important to consider that the complex phenotype explored in this work—oxygen-dependent heterogeneity in torCAD expression—could not have been predicted from a priori knowledge about respiration of either oxygen or TMAO and emerged only when cells were simultaneously provided with both species of terminal electron acceptor. What kinds of behaviors might we see if cells are presented with a third electron acceptor, or a fourth? When cells are grown in co-culture with other bacterial species, or in the gut of a mouse? There is no limit to the amount of complexity we can add to the experimental system, and there is no limit to the variety of biological phenomena we might observe by doing so. A maddening and marvelous thing about biology is the limited predictive power provided by a reductionist analysis. Maddening because we will never be capable of fully understanding life’s processes; marvelous because we will never run out of remarkable surprises and astonishing discoveries.
APPENDIX

Funding Sources

This work was funded by grants from the National Institute of General Medical Sciences of the NIH (R01-GM080279 to Mark Goulian and R01-GM115894 to Patricia J. Kiley) and by NIH training grants (T32-AI060516 and T32-GM007170 to Jeffrey N. Carey). This work was supported in part by the Penn Genome Frontiers Institute and funding from the Pennsylvania Department of Health. The Department of Health specifically disclaims responsibility for any analysis, interpretations, or conclusions. Additional funding was provided by the Armour-Lewis Foundation and the Dean's Office of the University of Pennsylvania School of Veterinary Medicine.


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