Two-Component Signal Transduction Pathways Regulating Growth and Cell Cycle Progression in a Bacterium: A System-Level Analysis

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation
Skerker, Jeffrey M., Melanie S. Prasol, Barrett S. Perchuk, Emanuele G. Biondi, and Michael T. Laub. 2005. Two-component signal transduction pathways regulating growth and cell cycle progression in a bacterium: A system-level analysis. PLoS Biology 3(10): e334.

Published Version
doi:10.1371/journal.pbio.0030334

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:4777437

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Two-component signal transduction systems, comprised of histidine kinases and their response regulator substrates, are the predominant means by which bacteria sense and respond to extracellular signals. These systems allow cells to adapt to prevailing conditions by modifying cellular physiology, including initiating programs of gene expression, catalyzing reactions, or modifying protein–protein interactions. These signaling pathways have also been demonstrated to play a role in coordinating bacterial cell cycle progression and development. Here we report a system-level investigation of two-component pathways in the model organism *Caulobacter crescentus*. First, by a comprehensive deletion analysis we show that at least 39 of the 106 two-component genes are required for cell cycle progression, growth, or morphogenesis. These include nine genes essential for growth or viability of the organism. We then use a systematic biochemical approach, called phosphotransfer profiling, to map the connectivity of histidine kinases and response regulators. Combining these genetic and biochemical approaches, we identify a new, highly conserved essential signaling pathway from the histidine kinase CenK to the response regulator CenR, which plays a critical role in controlling cell envelope biogenesis and structure. Depletion of either *cenK* or *cenR* leads to an unusual, severe blebbing of cell envelope material, whereas constitutive activation of the pathway compromises cell envelope integrity, resulting in cell lysis and death. We propose that the CenK–CenR pathway may be a suitable target for new antibiotic development, given previous successes in targeting the bacterial cell wall. Finally, the ability of our in vitro phosphotransfer profiling method to identify signaling pathways that operate in vivo takes advantage of an observation that histidine kinases are endowed with a global kinetic preference for their cognate response regulators. We propose that this system-wide selectivity insulates two-component pathways from one another, preventing unwanted cross-talk.

**Introduction**

Cells have the remarkable ability to sense, respond to, and adapt to their internal and external environments in order to maximize survival or accurately execute a developmental program. Such behavior requires the ability to process information, and cells have evolved complex regulatory and signaling systems capable of sophisticated information-processing tasks. It is ultimately the wiring of such systems and the relative quantitative strength of connections that confer on cells the ability to make decisions and regulate their behavior. Thus, there is a need to develop comprehensive, genome-wide maps of the complex signaling pathways operating inside cells. Although transcriptional networks in many organisms have recently been mapped on a global level using DNA microarrays, signaling pathways and networks can be considerably more difficult to study in a systematic, comprehensive fashion, requiring experimentally tractable systems amenable to a combination of genetic and biochemical methods.

Here we report the design and use of a suite of tools for the rapid, systematic mapping of signaling networks responsible for regulating growth, cell cycle progression, and differentiation in the Gram-negative bacterium *Caulobacter crescentus*. This organism has emerged as an excellent model system for studying regulation of cell cycle progression and development owing to its dimorphic lifestyle (Figure 1A) [1–3]. Each cell division produces two different daughter cells: a stalked cell and a swarmer cell. The motile, chemotactic swarmer cell is unable to initiate DNA replication. In response to poorly understood environmental and internal cues, a swarmer cell differentiates into a stalked cell by losing its polar flagellum, chemotaxis machinery, and polar pili, followed by growth of a stalk. This motile-to-sessile transition is accompanied by increased rates of growth and protein synthesis [4]. This transition also coincides with DNA replication initiation and is thus a G1–S cell cycle transition. A single round of DNA replication ensues, followed by

Received May 26, 2005; Accepted July 22, 2005; Published September 27, 2005
DOI: 10.1371/journal.pbio.0030334

Copyright: © 2005 Skerker et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abbreviations: kan<sup>R</sup>, kanamycin resistant/resistance; PYE, peptone yeast extract; sucrose<sup>o</sup>, sucrose-resistant/resistance; tet<sup>R</sup>, tetracycline resistant/resistance

Academic Editor: Adam Arkin, Lawrence Berkeley National Laboratory, United States of America

*To whom correspondence should be addressed. E-mail: laub@cgr.harvard.edu

Current address: Department of Molecular and Cell Biology, University of California, Berkeley, California, United States of America
segregation of the daughter chromosomes to opposite ends of the predivisional cell. The development of the predivisional cell includes construction of a new flagellum, chemotaxis machinery, and pil secretion apparatus at the pole opposite the stalk. Cell division is asymmetric, generating two distinct daughter cells. The stalked cell can immediately initiate DNA replication, whereas the swarmer cell must first differentiate into a stalked cell.

Swarmer cells can be easily isolated from a mixed population of cells by density centrifugation and followed as they proceed synchronously through the cell cycle.

The regulation of this complex life cycle centers on a single class of signaling molecules known as two-component signal transduction systems. These systems are one of the key signaling modalities in the bacterial kingdom, as well as being present in fungi, slime molds, and plants [5]. As they appear to be absent from metazoans, including humans, this class of molecules has been suggested as a major new target for antibacterial and antifungal drug development [6,7]. The canonical two-component signal transduction system is shown in Figure 1B. A histidine kinase, often in response to receipt of a signal or stimulus, autophosphorylates on a conserved histidine residue. The phosphoryl group is then transferred to a conserved aspartate residue of a cognate response regulator. Phosphorylation of the response regulator occurs within the receiver domain and typically leads to a change in cellular physiology by activating an output domain. In many cases, phosphorylation enables the response regulator to bind DNA and function as a transcription factor. However, many other types of output domains are found that endow their response regulators with the ability to mediate protein–protein interactions or to perform enzymatic functions [8]. Two-component signaling pathways have been shown to respond to a wide range of stimuli, including sugars, peptides, antibiotics, and quorum-sensing signals. These signals trigger major physiological changes by changing programs of gene expression, altering swimming behavior, regulating proteolysis, or triggering differentiation [9,10].

Both histidine kinases and their targets, the response regulators, are easily identified in bacterial genomes solely by sequence homology. C. crescentus encodes 106 such proteins: 62 histidine kinases and 44 response regulators [11]. Some bacterial genomes encode as many as 250 of these signaling proteins, often amounting to more than 5% of all genes in a genome [12]. In Escherichia coli, the vast majority of two-
component systems are encoded as operons of a histidine kinase and a response regulator that form an exclusive one-to-one phosphotransfer pair [13]. However, studies in *C. crescentus* and other bacteria reveal that two-component signaling pathways can often be highly branched, with many-to-one and one-to-many phosphotransfer relationships [5,14,15]. Such pathways are also often composed of kinases and regulators encoded in different operons scattered throughout a genome. In *C. crescentus* 41 histidine kinases and 19 response regulators, or 57% of all two-component genes, are orphans, not encoded in the same operon as another two-component gene. Identifying the connectivity of two-component signaling pathways is not possible by sequence analysis alone and is currently a major challenge. A recent report has attempted to map all such interactions in *E. coli* by systematically measuring phosphotransfer relationships between histidine kinases and response regulators [16].

Forward genetic screens in *C. crescentus* have identified 14 of the 106 two-component signaling genes as involved in cell cycle progression or differentiation (reviewed in [5,15]). The majority of these 14 are orphans and their connectivity remains poorly defined. Moreover, what role the other 92 two-component genes may play in regulating cell cycle progression and differentiation is largely unknown. Previous genetic screens may not have been saturated or may have had inherent biases, precluding identification of other important two-component regulators. To address these challenges, we undertook a systematic, comprehensive genetic and biochemical dissection of all 106 two-component signal transduction genes in the *C. crescentus* genome. Analysis of a complete set of deletion mutants identified 39 genes required for some aspect of growth or cell cycle progression, including nine essential genes. To identify phosphotransfer relationships, we developed a global in vitro biochemical approach that allows the identification of connections that are relevant in vivo. This technique takes advantage of data demonstrating that histidine kinases have an in vitro kinetic preference for their in vivo substrates. We demonstrate the utility of this integrated suite of systematic genetic and biochemical tools by identifying a previously unknown, but highly conserved, two-component pathway that is essential for growth of *C. crescentus* owing to a role in controlling cell envelope structure and integrity. The tools and approach presented can be applied to the study of two-component signaling proteins in other prokaryotes, including pathogens, and in any species having multiple two-component signaling systems, such as plants.

**Results**

**Systematic Deletion of Two-Component Signaling Genes**

We analyzed the *C. crescentus* genome and identified 106 genes that encode members of the two-component signal transduction family: 62 histidine kinases and 44 response regulators (for annotation procedures, see Materials and Methods). To begin comprehensive identification of two-component signaling pathways required for cell cycle progression, cell growth, or cell polarity in *C. crescentus*, we generated deletion strains for each of the histidine kinase and response regulator genes identified. Deletions were made using long-flanking homology constructs carried on suicide vectors and a two-step recombination process (Figure 2; Materials and Methods). Selection for tetracycline resistance ensures integration of the suicide vector, and growth on sucrose (*sacB* is lethal when sucrose is present in the medium) selects for plasmid excision and formation of a stable deletion strain (Figures 2A and S1). The two-step deletion procedure allows rapid identification of essential genes. If a gene is essential, the second recombination event always fails, and stable deletions (tetracycline-resistant [*tet*] sucrose-resistant [*sacB*] colonies) cannot be recovered (Figures 2A and S1). In such cases, all sucrose [*tet*] colonies recovered are a result of *sacB* mutation, not loss of *sacB*.

We successfully generated stable deletion strains in rich medium (peptone yeast extract [PYE]) for 97 of the 106 *C. crescentus* two-component signaling genes. For these 97 genes, stable deletions were found after screening 5–10 colonies. For the remaining nine genes we tested at least 100 colonies after the final sucrose counter-selection (Figures 2A and S1) and found that all still possessed the *sacB* gene, albeit inactivated. This suggests that each of these nine genes cannot be eliminated and hence each is essential for growth or viability (Table 1). This set includes all previously characterized essential two-component signal transduction genes in *C. crescentus*: *ctsA*, *ekkA*, *divK*, and *dwl* [17–21]. These results validate our method as a means to finding essential genes and strongly suggest that the five previously uncharacterized genes that could not be deleted (*CC0530*, *CC1743*, *CC2931*, *CC2932*, and *CC3743*) are also essential in *C. crescentus*.

*CC0530* and *CC3743* are both genes of unknown function. *CC0530* encodes a predicted histidine kinase with two transmembrane domains and a periplasmic loop of about 130 amino acids. The protein encoded by *CC3743* is a putative transcriptional regulator of the winged-helix OmpR subfamily (data not shown). *CC2932* and *CC2931* probably form an essential two-component pathway as orthologs of each are found in the same predicted operon, or adjacent open reading frames, in a wide range of bacterial genomes. *CC2931* encodes an ortholog of the response regulator PetR, which is essential in *Rhodobacter capsulatus* and required for oxidative respiration [22]. *CC1743* is an ortholog of the gene *ntrY*, which may control growth in the presence of nitrate [23].

**Figure 2. Systematic Deletion of Two-Component Signal Transduction Genes**

(A) Methodology used to generate chromosomal deletion strains. For each gene to be deleted, a suicide vector was constructed, with approximately 800-bp regions of homology upstream and downstream of the gene flanking a *tet* cassette. See Materials and Methods and Figure S1 for details of plasmid construction. In a two-step process, deletion strains are isolated by selecting first for tetracycline resistance and then by sucrose counter-selection utilizing the *sacB* gene carried on the vector. Cells harboring the *sacB* gene die in the presence of sucrose. Hence, a deletion strain is identified as *tet*/*sucrose*. For nonessential genes, stable deletions are easily identified by screening 5–10 colonies after the two-step recombination. For essential genes, no *tet*/*sucrose* strains can be recovered (see text and Figure S1 for additional details).

(B) and (C) Swarm plate analysis of 97 nonessential two-component deletion strains. (B) Map of strain positions in the swarm plates. Wild-type CB15N is in positions A1 and J10 for comparison to mutant strains. (C) PYE swarm plate after 3 d of growth at 30 °C. Swarm sizes and densities were scored visually and digital images analyzed in Matlab (MathWorks, Natick, Massachusetts, United States). Strains exhibiting swarm plate phenotypes are listed in Table 2, except for *ACC1221* in position E1, which is deleted for a kinase erroneously annotated as a histidine kinase.

DOI: 10.1371/journal.pbio.0030334.g002
Systematic Analysis of Two-Component Signaling

A

C. crescentus chromosome

\[ \text{deletion strain: } \text{tet}^R, \text{kan}^S, \text{sucrose}^R \]

B

| Column | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|--------|---|---|---|---|---|---|---|---|---|----|
| A      | CB15N 0026 0138 0237 0238 0247 0248 0248 0284 0285 0299 |
| B      | 0264 0432 0433 0436 0437 0440 0596 0598 0591 0594 |
| C      | 0656 0697 0612 0629 0630 0652 0723 0744 0758 0759 |
| D      | 0836 0909 0921 0934 0962 1063 1149 1150 1181 1182 |
| E      | 1221 1293 1294 1304 1305 1384 1594 1596 1683 1705 |
| F      | 1740 1741 1742 1767 1768 2249 2324 2462 2482 2501 |
| G      | 2521 2554 2576 2632 2670 2755 2757 2766 2768 2852 |
| H      | 2874 2894 2909 2971 2688 2993 3015 3048 3068 3075 |
| I      | 3100 3102 3155 3162 3170 3191 3198 3219 3225 3258 |
| J      | 3286 3315 3325 3327 3471 3474 3477 3560 3623 CB15N |

C

A B C D E F G H I J
thus suspected that CC1743 may be dispensable for growth in M2G minimal medium, where the sole nitrogen source is ammonium. Repeating the deletion procedure for CC1743 on minimal medium did in fact yield a stable deletion strain, so we classify CC1743 as a conditionally essential gene. We could not make any similar predictions for the other four new essential genes, and suggest that they are essential under most standard growth conditions.

Phenotypic Analysis of Nonessential Deletion Strains

We next examined the phenotypes of the 97 nonessential deletion strains using a swarm plate assay. Wild-type cells can swim through low-percentage agar, creating a large, circular colony, or swarm, via the combined effects of chemotaxis and growth. Defects in a number of processes, including cell motility, chemotaxis, growth, cell division, and cell cycle progression, can produce changes in swarm size or density. The swarm plate assay is thus a rapid, sensitive, and comprehensive method for initial phenotypic characterization. Each deletion mutant, as well as the wild-type CB15N, was inoculated into swarm plates made from rich (PYE) medium, and swarms were photographed after three days (Figure 2B and 2C). From digital images, swarm size and swarm density were quantified for each deletion strain relative to wild-type (Figure 2C). Of the 97 deletion strains, 30 exhibited a significantly altered swarm size or density (Table 2). Each of these genes was further characterized by measuring the log-phase generation time in rich medium and by examining cellular morphology for abnormalities in cell shape, cell length, motility, and stalk formation (Figure 3; Table 2).

Strong candidates for cell cycle or cell growth regulatory genes are those marked by deletion strains that show a decrease in swarm size and a longer generation time. Five strains matching these criteria were found, including dele-

Table 1. Essential Two-Component Signal Transduction Genes

| Gene | Name | Type | Functions | Reference |
|------|------|------|-----------|-----------|
| CC0530 | cenK | HK | Cell envelope biogenesis and integrity | This study |
| CC1678 | cckA | HK | DNA replication, cell division | [18] |
| CC1743 | RR | Homologous to nitrogen regulator ntrY | This study |
| CC22931 | RR | Homologous to oxidative respiration regulator petR | This study |
| CC2932 | RR | Putative kinase for PetR | This study |
| CC3035 | ctrA | RR | DNA replication, cell division | [17] |
| CC2463 | divK | RR | DNA replication, cell division | [20] |
| CC3484 | divE | RR | DNA replication, cell division | [19] |
| CC3743 | cemR | RR | Cell envelope biogenesis and integrity | This study |

In sum, the initial phenotypic characterization of our comprehensive library of two-component deletion strains has identified 39 genes (30 nonessential and nine essential)—or more than 35% of all two-component signaling genes—required for some aspect of growth, viability, morphogenesis, or cell cycle progression. This includes all 14 of the genes found by previous forward genetic screens for morphogenetic and cell cycle mutants (Tables 1 and 2), as well as 25 previously uncharacterized two-component signaling genes involved in regulating the C. crescentus life cycle. The uncharacterized genes are not simply those with subtle mutant phenotypes, as many have severe defects, including five that appear to be essential for growth or viability. Detailed characterization will be necessary to pinpoint the precise function of each of these uncharacterized genes.

Systematic Biochemical Analysis of Two-Component Signal Transduction

As a first step in further characterization of the two-component signaling genes involved in the cell cycle progression and development of C. crescentus, we sought to identify the response regulator targets of each histidine kinase. For orphan kinases and regulators, cognate pairs cannot easily be predicted based on sequence analysis alone. Of the 39 mutants showing phenotypes in the assays described above, 26 are orphans and their phosphotransfer pairings thus unknown. To systematically identify connectivity between two-component signaling proteins, we developed a global in vitro biochemical technique, which we term phosphotransfer profiling, to rapidly identify the targets of histidine kinases (Figure 4).

In a profiling experiment (Figure 4A and 4B), the purified cytoplasmic, soluble kinase domain of a histidine kinase is autophosphorylated with [$\gamma$-32P]ATP, and then split into separate reactions containing equimolar amounts of each purified, full-length response regulator (for details of protein purification, see Materials and Methods). Each phosphotransfer reaction is incubated for an identical period of time and then stopped by addition of sample buffer, separated by SDS-PAGE, and imaged on phosphor screens. A control of autophosphorylated kinase without any added response regulator is included for reference, and forms a single intense band. Efficient phosphotransfer to a response regulator can be manifested in two ways (Figure 4B). In the first case, a high-intensity band is seen at the appropriate molecular weight for phosphorylated response regulator. In the second case, efficient phosphotransfer can lead to depletion of radiolabel from the histidine kinase band. As some response regulators have high autophosphatase activity and some histidine kinases are bifunctional, also acting as specific phosphatases for their cognate response regulators, the net result of efficient phosphotransfer and phosphatase activities is the depletion of radiolabel from the autophosphorylated kinase (Figure 4A and 4B) [24]. Hence, to identify a phosphotransfer relationship, each reaction in a profile assay is inspected for (i) a band corresponding to the response regulator or (ii) a decrease in intensity of the kinase band relative to the kinase only control. Importantly, because our profile method relies on the comparison, in parallel, of all potential phosphotransfer substrates for a given kinase, it is independent of the specific activity of the kinase being tested.
Histidine Kinases Exhibit a System-Wide In Vitro Kinetic Preference for Their Cognate Response Regulators

We chose to test and validate our in vitro profiling technique using purified kinases and response regulators from *E. coli* as many of its in vivo phosphotransfer pairings are known. First, we characterized phosphotransfer to response regulators by the histidine kinase EnvZ, which responds in vivo to changes in osmolarity by controlling the phosphorylation state of the response regulator OmpR [25,26]. The profile of EnvZ after a 1-h reaction time with each of the 32 purified *E. coli* response regulators demonstrates phosphotransfer to 11 different response regulators, including OmpR (Figure 4C). However, with a shorter, 10-s reaction time the only efficient phosphotransfer is to OmpR (Figure 4D), demonstrating a clear kinetic preference of EnvZ for its cognate substrate OmpR. We next tested the CheA histidine kinase, which phosphorylates CheY and CheB in vivo to control chemotaxis [27,28]. At 1 h, CheA shows phosphotransfer to seven response regulators, including CheY and CheB (Figure 4E), but at 10 s we detect only phosphorylation of CheY and CheB (Figure 4F). We then tested a third kinase, CpxA, which is known to signal through CpxR in vivo [29]. With the long reaction time, CpxA phosphorylates CpxR as well as several other response regulators (Figure 4G). The short reaction time again reveals a kinetic preference of the kinase CpxA for its in vivo, cognate substrate, CpxR (Figure 4H). We have observed similar kinetic preferences of two other *E. coli* kinases, PhoQ and PhoR, for their respective phosphotransfer substrates, PhoP and PhoB (data not shown). We conclude that *E. coli* histidine kinases have a strong kinetic preference for their in vivo cognate response regulators, with promiscuity only observed after extended incubation times. We have estimated the kinetic preference of kinases to be at least 10³ in terms of relative $k_{cat}/K_m$ ratios (Figure S2).

Next, we tested *C. crescentus* histidine kinases to determine if kinetic preference for substrates extends to the two-component systems in this organism. We started by profiling a two-component pair of unknown function: CC1181/CC1182. Because the kinase and regulator are encoded in the same operon they likely form an exclusive phosphotransfer pair in vivo. As with *E. coli* histidine kinases, we found that multiple response regulators were phosphorylated by CC1181 at the 1-h time point, including CC1182 (Figure 5A). A shorter phosphotransfer incubation time of 10 s reveals a clear kinetic preference of CC1181 for CC1182 (Figure 5B). We then tested five other *C. crescentus* histidine kinases, CC0289 (PhoR), CC0759, CC1740, CC2765, and CC3327. In each case, the histidine kinase exhibited a strong kinetic preference for its cognate response regulator.
preference for its known substrate or the substrate encoded within its own operon, CC0294 (PhoB), CC0758, CC1741, CC2766, and CC3325, respectively (data not shown).

Next, we used profiling with orphan C. crescentus histidine kinases for which the cognate response regulators could not be predicted by sequence analysis alone. First, we tested the orphan kinases DivJ and PleC, which were identified in our deletion analysis, and in previous genetic screens [30,31], to be key regulators of cell cycle progression and morphogenesis. Both of these kinases have been shown previously to phosphorylate the essential response regulator DivK and the response regulator PleD, which are in the same operon together, but without an adjacent kinase [20,21,32]. Short, 10-s reaction time profiles of DivJ and PleC demonstrate a kinetic preference for DivK and PleD and suggest that these are the exclusive targets of DivJ and PleC (Figure 5C and 5D).

We conclude that, as in E. coli, C. crescentus histidine kinases have an in vitro kinetic preference for their in vivo cognate substrate. Kinetic preference of a kinase for its cognate response regulator has been noted before on a limited scale [25,33–35], but our data extend this observation to a genomewide level. Moreover, we suggest that this kinetic preference can be exploited to rapidly identify in vivo phosphotransfer relationships.

Identification of a New Essential Two-Component Pathway That Controls Cell Envelope Integrity

The systematic deletion analysis described above identified four histidine kinases that each appear to be essential for growth or viability: divL, cckA, CC2932, and CC0530 (see Table 1). divL and cckA have both been previously identified as essential regulators and are implicated in phosphorylation of the essential response regulator CtrA [18,19]. CC2932 is encoded in an operon with the essential response regulator CC2931, and these probably form a phosphotransfer pair. CC0530, however, is a previously uncharacterized, orphan kinase with no known or predicted substrate. Using phosphotransfer profiling, we demonstrated that CC0530 preferentially phosphorylates a single target, the orphan response regulator CC3743 (Figure 5E). As with CC0530, we had identified CC3743 as a previously uncharacterized orphan gene that is likely essential for growth or viability of C. crescentus (see Table 1). Together our genetic and biochemical observations strongly suggest that these two orphans comprise an essential two-component pathway in C. crescentus.

To test whether CC0530 and CC3743 are indeed essential, we generated strains in which the only copy of each gene is present on a low-copy plasmid under the control of the xylose-inducible, glucose-repressible promoter P\textsubscript{xy}X. For both genes, stable deletions were easily recovered when these complementing plasmids were present but not in the presence of an empty vector control (Table 3). This work produced strain ML521 (ΔCC0530 + P\textsubscript{xy}X-CC0530) and strain ML550 (ΔCC3743 + P\textsubscript{xy}X-CC3743). ML521 formed colonies only on plates supplemented with xylose, consistent with the CC0530 histidine kinase being essential for growth (data not shown). In contrast, ML550 formed colonies on PYE plates supplemented with xylose or glucose. We suspected that CC3743 may be a stable protein and hence difficult to deplete when expressed from a plasmid. We therefore made a destabilized version of CC3743 by adding a C-terminal ssrA tag, which targets proteins for degradation and decreases protein half-life inside the cell [36]. Using this destabilizing tag, we successfully created the strain ML591 (ΔCC3743 + P\textsubscript{xy}X-CC3743-ssrA), which forms colonies on PYE plates supplemented with xylose but not with glucose (data not shown). The ability of ML591 to grow on medium with xylose suggests that the ssrA tag does not interfere with the function of CC3743, but does allow the depletion of CC3743 during growth on glucose. The deletion strains ML521 and ML591 also grew only in minimal medium supplemented with xylose (M2X) and not
Figure 4. Phosphotransfer Profiling Method

(A) Phosphotransfer profile experiments involve three separate reactions: (1) autophosphorylation of the histidine kinase (HK) by radiolabeled ATP, (2) phosphotransfer to a response regulator (RR), and (3) dephosphorylation of the response regulator.

(B) Schematic of the phosphotransfer profiling technique. A single preparation of purified, autophosphorylated kinase (HK\(^{32}\)P) is mixed with each response regulator from a given organism and analyzed for phosphotransfer by SDS-PAGE and autoradiography. The first lane shows a single band corresponding to the autophosphorylated histidine kinase and is used as a comparison for every other lane. Lanes 2–4 illustrate the three possible outcomes of a phosphotransfer reaction. In lane 2, phosphotransfer from HK to RR1 leads to the appearance of a band corresponding to RR1. In lane 3, phosphotransfer from HK to RR2 also occurs, but owing to high phosphatase activity (either autophosphatase or catalyzed by a bifunctional HK), the net result is production of inorganic phosphate (Pi) and the depletion of radiolabel from both the HK and RR2. In lane 4, no phosphotransfer occurs, and the lane is indistinguishable from lane 1.

(C–H) Phosphotransfer profiling was performed for three *E. coli* kinases (EnvZ, CheA, and CpxA) against all 32 purified *E. coli* response regulators, with phosphotransfer incubation times of either 1 h (C, E, and G) or 10 s (D, F, and H). For these three histidine kinases, a comparison of the short and long time point profiles indicates a kinetic preference for only their in vivo cognate regulators: OmpR (C and D), CheY and CheB (E and F), and CpxR (G and H). After being examined for phosphotransfer, all gels are stained with Coomassie to verify equal loading of histidine kinase and response regulator in each lane (data not shown). For each kinase profiled, we purified only its soluble, cytoplasmic domain, either as a thioredoxin-His\(_6\) or a His\(_6\)-MBP fusion, using standard metal affinity chromatography (see Materials and Methods). When necessary, we made successive N-terminal truncations until we identified a construct that produced active kinase in vitro, always preserving the H-box and ATP binding domain (details on constructs used are in Table S3). All response regulators were purified as full-length fusions to a thioredoxin-His\(_6\) tag. Purity was assessed by Coomassie staining, with each purified kinase domain and response regulator, except for *E. coli* FimZ, yielding an intense band of the correct approximate molecular weight (see Figure S5; Table S3).

DOI: 10.1371/journal.pbio.0030334.g004

PLoS Biology | www.plosbiology.org October 2005 | Volume 3 | Issue 10 | e334
with glucose (M2G), supporting the general essential nature of these two genes (data not shown).

Next, we examined the phenotype of these strains in liquid medium after depleting each gene product. Cultures of each were grown in rich medium supplemented with xylose and then washed and resuspended at a low density in medium with xylose or glucose. We measured the growth rate and observed the cells by light microscopy (Figure 6A–6E). In the presence of xylose, growth of ML521 and ML591 was virtually indistinguishable from wild-type, suggesting that expression of either CC0530 or CC3743 under these conditions has no deleterious effect (Figure 6A). However, when shifted to glucose, the cultures of each depletion strain stopped growing and failed to accumulate significant optical density (Figure 6A). After 20 h of depletion by growth in glucose, we examined the morphological phenotype of each strain by light microscopy. Depletion of either gene product led to loss of motility, shorter stalks, and a dramatic, unusual membrane blebbing, resulting in bubble-like protrusions on the cell surface (Figure 6C and 6E). Cells were approximately wild-type in length and size, but had cell envelope blebs nearly covering the cell surface. We reasoned that the blebs were contiguous extrusions of the cell envelope that did not disrupt permeability as these cells did not lyse even after extended incubation in glucose-containing medium. Using high-resolution scanning electron microscopy, we examined cells from each depletion strain after extended growth in xylose and glucose. Consistent with the light microscopy results, we observed large, irregular protrusions across the surface of the cells grown in glucose and depleted of CC0530 or CC3743 (Figure 6F–6I). The growth and morphological phenotypes of the two depletion strains were nearly identical, further supporting the conclusion that CC0530 and CC3743 participate in the same signal transduction pathway. Based on our observations we have named

Figure 5. Phosphotransfer Profiling of C. crescentus Histidine Kinases

Profiles for four purified C. crescentus kinases versus 44 purified response regulators were obtained by the method described for E. coli in Figure 4. (A) One-hour time point profile of the C. crescentus kinase CC1181. (B) Ten-second time point profile. Only CC1182, encoded in the same operon as CC1181 and the likely in vivo target, is phosphorylated at the short time point. Kinetic preference of C. crescentus histidine kinases for their cognate substrates was similarly demonstrated for five other operon pairs (data not shown). (C and D) Ten-second time point profiles of the orphan kinases DivJ and PleC, demonstrating phosphorylation of only their shared in vivo targets, PleD and DivK. (E) Phosphotransfer profiling of the previously uncharacterized essential orphan kinase CC0530 (CenK) reveals a single preferred substrate, CC3743 (CenR).

DOI: 10.1371/journal.pbio.0030334.g005

Figure 6. CC0530 (cenK) and CC3743 (cenR) Are Essential for Growth and Required for Cell Envelope Integrity

Growth curves for the ML521 (ACC0530 + Pxyx-cenK) and ML591 (ACC3743 + pHXM-cenR-ssrA) depletion strains (A). Overnight cultures of each were grown in PYE plus xylose (PYE-X), washed with plain PYE, and diluted in PYE plus xylose or PYE plus glucose (PYE-G). After 12 h of growth in these conditions cells reached an optical density (OD_{600}) level that could be measured (this time is plotted as ‘’0 min’’). Morphology was observed by light microscopy for the cenK depletion (ML521) after a total of 20 h in PYE plus xylose (B) or PYE plus glucose (C) and for the cenR depletion (ML591) after 20 h in PYE plus xylose (D) or PYE plus glucose (E). Scanning electron micrographs under identical conditions are shown for ML521 in PYE plus xylose (F) and PYE plus glucose (G) and for ML591 in PYE plus xylose (H) and PYE plus glucose (I). For (F–I), scale bar represents 1 μm. Depletion of either gene product led to an unusual, irregular blebbing of the cell surface. Cells were not motile, and had reduced stalk length.

DOI: 10.1371/journal.pbio.0030334.g006
Systematic Analysis of Two-Component Signaling

A

- ML521, 0.03% xylose, (ΔOCD0530 + P$_{Y}$X-xcmK)
- ML521, 0.1% glucose, (ΔOCD0530 + P$_{Y}$X-xcmK)
- ML591, 0.3% xylose, (ΔCC3743 + pHXM-xcmR-srrA)
- ML591, 0.1% glucose, (ΔCC3743 + pHXM-xcmR-srrA)

B

PYE-X

C

PYE-G

ML521

D

E

ML591

F

G

PYE-X

PYE-G

ML521

H

I

ML591
CC0530 and CC3743 cenK (cell envelope kinase) and cenR (cell envelope regulator), respectively.

To understand the functions of the cenK–cenR pathway in more detail we examined the effects of overexpressing components of this pathway (Figure 7). First we examined the phenotype of strain ML603, which expresses a full-length copy of cenR under control of the P_{xyl} promoter on a low-copy plasmid (pMR20) in a wild-type background. In the presence of glucose, cells of this strain were virtually indistinguishable from wild-type cells (Figure 7A). However, in the presence of xylose, these cells showed significant cellular elongation, and many cells appeared to be losing their shape, exhibiting a bloated, enlarged morphology (Figure 7B). To increase expression further, we constructed a strain (ML675) with P_{xyl}cenR on pHXM- (a higher-copy-number vector than pMR20). In the presence of glucose, strain ML675 also appeared similar to wild-type (Figure 7C), but growth in xylose revealed a dramatic morphological phenotype, ranging from bloated, enlarged cells to pervasive cell lysis (Figure 7D). Measurements of optical density after shift to xylose indicated a rapid growth arrest (Figure 7K). Interestingly, we noted that in many predivisional cells, the cell was enlarged asymmetrically, always with the stalked half of the cell losing its rod-like appearance (indicated by white arrows in Figure 7D). These data, together with the depletion analysis, suggest that cenR is involved in maintaining proper cell envelope structure, and further suggest that peptidoglycan or cell membrane synthesis may proceed in an asymmetric fashion in wild-type C. crescentus cells.

For many response regulators, mutating the conserved phosphorylation site from aspartate to glutamate mimics constitutive phosphorylation [37,38]. We introduced such a mutation, D60E, into cenR, on a low-copy plasmid. In the presence of glucose, the resulting cells looked similar to wild-type (Figure 7E), but when shifted to xylose, they became severely enlarged, lost their usual rod shape, and within 5 h began to lyse and die (Figure 7F and 7K). Thus, the phenotype of overexpressing CenR(D60E) on a low-copy plasmid matched that of overexpressing wild-type CenR on a high-copy plasmid (compare Figure 7D and 7F). We conclude that the D60E mutation leads to phosphorylation-independent activity of CenR. We also attempted to generate strains expressing CenR(D60E) from the high-copy plasmid pJS71, but no colonies were recovered, even on glucose plates, suggesting that the D60E allele may be so active that even basal expression in glucose is lethal.

Unlike with CenR, overexpression of the full-length CenK (data not shown) or its cytoplasmic kinase domain had no effect on cell growth or cell morphology (Figure 7G and 7H). This may be because the amount of CenR is limiting in the cell, so that additional CenK expression may not alter the fraction of phosphorylated CenR. Alternatively, the cell may be robust to changes in kinase concentration, as suggested for the kinase EnvZ [39]. Regardless, we predicted that if CenK is the in vivo cognate kinase for CenR, then simultaneously overexpressing both CenK and CenR should phenocopy overexpression of CenR(D60E). As expected, the effect of co-overexpressing CenK_{cyto} and CenR (Figure 7J) was significantly more severe than that of expressing either protein alone (compare to Figure 7B and 7H), and phenocopied the overexpression of CenR(D60E) (Figure 7F). As a control to ensure that the effect was due to kinase activity of CenK, we mutated the active-site histidine to alanine (H273A) and showed that the growth rate of cells co-overexpressing CenK(H273A) and CenR was nearly indistinguishable from that of cells overexpressing CenR alone (data not shown). These results support the conclusion that CenK acts in vivo to phosphorylate, and hence activate, CenR, as suggested by the in vitro phosphotransfer profiling.

The CenK–CenR pathway appears to be widely conserved throughout the alpha subdivision of proteobacteria. Multiple sequence alignments indicate better than 60% identity (70% similarity) for CenR and better than 35% identity (50% similarity) for CenK (Figures S3 and S4). The similarity extends throughout the full length of each protein, including the putative periplasmic ligand-binding domain of CenK. We suggest that the CenK–CenR pathway may be essential and function similarly in a range of other bacteria.

### Table 3. CC0530 (cenK) and CC3743 (cenR) Are Essential Genes

| Plasmid* | Total Colonies Screened | Outcome for ΔcenK Deletion Attempts | Outcome for ΔcenR Deletion Attempts |
|----------|-------------------------|------------------------------------|------------------------------------|
| pMR20    | 96                      | 0                                  | 23                                 |
| pMR20-P_{xyl}cenK-cenR | 96                  | 36                                 | 36                                 |

*This column indicates the plasmid present when attempting to delete cenK or cenR as described in Materials and Methods. A two-step recombination procedure was used, similar to that shown in Figure 2A, except deletions were constructed to be in-frame and markerless. Hence, the second step of recombination (see Figure 2A) can produce three distinct outcomes, as tabulated: deletion, re-creation of the wild-type configuration, or sacB inactivation. In each case, 96 colonies were screened and scored.

DOI: 10.1371/journal.pbio.0030334.g007

Figure 7. Constitutive Activation of the CenK–CenR Pathway Leads to Dramatic Changes in Cell Morphology, Cell Lysis, and Death

Images are shown for strains grown overnight in PYE plus glucose and then diluted back to early log phase and grown for 5 h in PYE plus glucose (A, C, E, G, and I) or xylose (B, D, F, H, and J). In all panels, white arrows indicate cells with asymmetric bloating and black arrows indicate lysed cells. (A and B) ML603 (CB15N + pLXM-cenR + pJS71) expresses CenR alone from a low-copy vector. (C and D) ML675 (CB15N + pHXM-cenR) expresses CenR alone from a high-copy vector. (E and F) ML606 (CB15N + pLXM-cenR(D60E)) expresses CenR, from a low-copy vector, a mutant of CenR that mimics constitutive phosphorylation. (G and H) ML607 (CB15N + pMR20 + pHXM-cenR(D60E)) expresses CenR(D60E) alone from a high-copy vector. (I and J) ML604 (CB15N + pLXM-cenR + pHXM-cenK_{cyto}) expresses both CenR and CenK_{cyto} from low- and high-copy plasmids, respectively.

(K) Growth curve for all strains from (A–J) grown in PYE supplemented with xylose [64,65].

DOI: 10.1371/journal.pbio.0030334.g007
Systematic Analysis of Two-Component Signaling

ML603
(pLM-ccjR)

ML675
(pHXM-ccjR)

ML606
(pLM-ccjR(D85E))

ML607
(pHXM-ccjR)

ML604
(pLM-ccjR + pHXM-ccjR)

K

Time (min.)

OD600

A

B

C

D

E

F

G

H

I

J

K

Legend:
- ML603
- ML675
- ML606
- ML607
- ML604
Discussion

Systematic Deletion of Two-Component Signal Transduction Genes

By deleting each of the 106 two-component signal transduction genes encoded in the C. crescentus genome, we have identified 39 mutant strains with cell cycle or developmental phenotypes (see Tables 1 and 2). Previous forward genetic screens had identified 14 two-component signaling genes involved in cell cycle progression and morphogenesis in C. crescentus, including four essential for viability of the organism. However, forward genetic screens are typically designed to select for a particular phenotype or may not be screened to saturation. The comprehensive, unbiased nature of the reverse genetic approach taken here expands both the number and role of two-component signaling proteins in regulating the C. crescentus cell cycle. The newly identified mutants include many with severe phenotypes as well as four previously uncharacterized genes that appear to be essential for growth or viability in both rich and minimal media. The library of deletion strains created here will also serve as a resource for future explorations of two-component regulation in C. crescentus. The deletion strains can be individually characterized in more depth, in different conditions, or even in different strain backgrounds. In addition, the inclusion of unique molecular bar codes in each strain (see Materials and Methods) opens the possibility of parallel fitness studies similar to those used for the Saccharomyces cerevisiae whole-genome deletion collection [40,41].

Systematic Biochemical Analysis of Two-Component Phosphorylation

Similarity of mutant phenotypes can help to identify two-component genes acting in the same pathway, but ultimately, a biochemical demonstration of phosphorylation is required to define signal transduction pathways. Such a combination of genetics and biochemistry has successfully defined individual two-component signaling pathways in a number of organisms [10], but this report presents a global, integrated genetic and biochemical study of a bacterium’s complete set of two-component signal transduction systems.

Histidine kinases have been widely thought to function promiscuously in vitro, precluding correspondence with in vivo targets. However, a few studies have suggested that histidine kinases may have a kinetic preference in vitro for their in vivo cognate substrates. For example, in Bacillus subtilis, the kinase KinA can phosphorylate both Spo0A and Spo0F in vitro, but has a more than 50,000-fold preference, as measured by relative $k_{cat}/K_m$ ratios, for Spo0F, its in vivo cognate substrate [35]. A similar magnitude of kinetic preference was shown for the kinase VanS phosphorylating its cognate response regulator VanR relative to the noncognate substrate PhoB [34]. The phosphotransfer profiling data presented here extend these observations to a system-wide level and suggest that the apparent promiscuity of histidine kinases in vitro is attributable to excessive incubation times or a high concentration of reaction components, each of which acts to cross the kinetic barrier that enables a kinase to selectively phosphorylate to its cognate substrate. A recent comprehensive study of two-component signal transduction in E. coli examined phosphotransfer in vitro from each histidine kinase to each response regulator at a 30-s time point [16]. As with our data, all known cognate pairs showed significant phosphotransfer, but the study reported a small number of interactions between noncognate pairs [16]. However, the in vivo relevance of these interactions is not yet known, and because that study did not examine phosphotransfer at multiple time points, the strength of noncognate interactions relative to those of cognate pairs is also not yet clear.

Our profiling method examines, simultaneously and in parallel, the ability of a purified histidine kinase to phosphorylate each of the response regulators encoded in that organism’s genome. It would be impractical to determine $k_{cat}/K_m$ for each kinase–regulator combination, but kinetic preference can still easily be seen by conducting comprehensive profiles at multiple time points. Importantly, using a number of previously well-characterized E. coli histidine kinases, we demonstrated a direct correspondence between this kinetic preference and in-vivo-relevant response regulator substrates (see Figure 4). We were then able to use this kinetic preference to identify in vivo targets of uncharacterized histidine kinases such as the C. crescentus orphan CenK (see Figure 5E). Note, however, that phosphotransfer profiling is not used in isolation to identify phosphotransfer pairs, but is integrated with genetic data and in vivo experiments, as demonstrated here for CenK–CenR.

The phosphotransfer profiling technique is robust to a number of experimental variables. First, it is independent of the specific activity of the purified histidine kinase, because the method relies on a relative comparison of phosphotransfer kinetics from a single preparation of kinase to each possible substrate. Second, because the kinetic preference of kinases appears to be on the order of $10^3$ or even $10^4$, the method is not significantly affected by differences in response regulator concentration, even differences as great as 10-fold. Also, some histidine kinases are bifunctional, acting as both a kinase and a phosphatase for their cognate response regulators. In most cases, control of the relative ratio is not understood in vivo, making it difficult to predict the ratio of kinase to phosphatase activity of a particular purified construct in vitro. Any construct having net kinase activity can be probed by our method to identify the probable in vivo substrates, but determining whether the histidine kinase acts predominantly as a kinase or a phosphatase in vivo depends on integration with genetic and other in vivo observations. For example, our profiles of DivJ and PleC, as well as previous studies of these kinases, suggest that both target the regulators DivK and PleD [20,21,32]. In vivo, though, DivJ is thought to function primarily as a kinase for DivK and PleD, whereas the bifunctional kinase PleC appears to act as a phosphatase [42,43].

Identifying Novel Signal Transduction Systems

We demonstrated the integration of our genetic and biochemical methods to identify a novel, essential pathway from the histidine kinase CenK to the response regulator CenR, which appears to control critical aspects of cell envelope integrity. CenK is a predicted transmembrane protein with a periplasmic domain of $\sim$130 amino acids, although no periplasmic stimulus could be predicted based on sequence. CenR is a predicted DNA-binding protein of the OmpR subfamily, so defining the CenR regulon may help to unveil its role in controlling the cell envelope. Depletion of
### Table 4. Strains and Plasmids

| Strain or Plasmid | Organism or Plasmid Category | Strain or Plasmid Name | Description | Source or Reference |
|-------------------|-------------------------------|------------------------|-------------|---------------------|
| Strain            | C. crescentus                | CB15N                  | Synchronizable derivative of wild-type CB15         | [65] |
|                   |                               | ML521                  | CB15N Δcco030/pMR20-Parv-ccenK (tet)                 | This study |
|                   |                               | ML523                  | CB15N/pMR20-Parv-ccenK (tet)                         | This study |
|                   |                               | ML550                  | CB15N Δcc1743/pMR20-Parv-ccenR (tet)                | This study |
|                   |                               | ML591                  | CB15N Δcc1743/pHXM-ccenR-ssrA (spec)                | This study |
|                   |                               | ML592                  | CB15N/pHXM-ccenR-ssrA (spec)                        | This study |
|                   |                               | ML603                  | CB15N/pLXM-ccenR + pS71 (tet<sup>+</sup>, spec<sup>+</sup>) | This study |
|                   |                               | ML604                  | CB15N/pLXM-ccenR + pHXM-ccenK<sub>spec</sub> (H273A) (tet<sup>+</sup>, spec<sup>+</sup>) | This study |
|                   |                               | ML605                  | CB15N/pLXM-ccenR (D60E) + pS71 (tet<sup>+</sup>, spec<sup>+</sup>) | This study |
|                   |                               | ML606                  | CB15N/pLXM-ccenR(D60E) + pS71 (tet<sup>+</sup>, spec<sup>+</sup>) | This study |
|                   |                               | ML607                  | CB15N/pMR20 + pHXM-ccenK<sub>spec</sub> (tet<sup>+</sup>, spec<sup>+</sup>) | This study |
|                   |                               | ML608                  | CB15N/pMR20 + pHXM-ccenK<sub>spec</sub> (H273A) (tet<sup>+</sup>, spec<sup>+</sup>) | This study |
|                   |                               | ML675                  | CB15N/pHXM-ccenR (spec<sup>+</sup>)                  | This study |
| Plasmid           | General purpose vectors       | pMR20                  | Mini-RK2 derivative, low-copy replicon (tet<sup>+</sup>) | R. Roberts |
|                   |                               | pJ571                  | Derivative of pBBR1MCS, high-copy replicon (spec<sup>+</sup>) | J. Skerker |
|                   | Deletion plasmids             | pTet15b                | Used to make pHS-MBP-DEST (amp<sup>+</sup>)         | Novagen |
|                   |                               | pTET24a                | Used to make pTRX-HIS-DEST (amp<sup>+</sup>)         | Novagen |
|                   |                               | pBAD20-DM              | Source of His<sub>y</sub>-TEV tag (amp<sup>+</sup>)  | A. Geerlof |
|                   |                               | pETM-41                | Source of His<sub>y</sub>-MBP-TEV tag (amp<sup>+</sup>) | G. Stier |
|                   |                               | pENTR/D-TOPO           | ENTRY vector for Gateway cloning system (kan<sup>+</sup>) | Invitrogen |
|                   |                               | pHXM-DEST              | Source for propagation of destination vectors       | Invitrogen |
|                   |                               | TOP10                  | Strain for making pENTR/D-TOPO clones               | Invitrogen |
|                   | Plasmid                       | pMR20-P<sub>ssrA</sub> | yxI promoter in pMR20 (tet<sup>+</sup>)             | This study |
|                   |                               | pHPT5138               | srcF-containing suicide vector (kan<sup>+</sup>)     | D. Alley |
|                   |                               | pKOC3                  | Source of FRT-flanked tet<sup>+</sup> cassette (amp<sup>+</sup>, tet<sup>+</sup>) | This study |
|                   |                               | PCP20                  | Expresses FLP recombinase (amp<sup>+</sup>, chlor<sup>+</sup>) | [61] |
|                   |                               | pCenK<sub>spec</sub>   | In-frame deletion construct for ccenR (kan<sup>+</sup>) | This study |
|                   |                               | pCenK<sub>spec</sub>-R | In-frame deletion construct for ccenR (kan<sup>+</sup>) | This study |
|                   | Deletion plasmids             | pMR20-P<sub>ssrA</sub>-cenK<sub>spec</sub> | Full-length CC0300 in pMR20-P<sub>ssrA</sub> (kan<sup>+</sup>) | This study |
|                   |                               | pMR20-P<sub>ssrA</sub>-cenR<sub>spec</sub> | Full-length CC3743 in pMR20-P<sub>ssrA</sub> (kan<sup>+</sup>) | This study |
|                   | Entry clones                  | pENTR-cenR             | CC3743 in pENTR/D-TOPO (kan<sup>+</sup>)            | This study |
|                   |                               | pENTR-cenR(D60E)       | CC3743(D60E) in pENTR/D-TOPO (kan<sup>+</sup>)     | This study |
|                   |                               | pCenK<sub>spec</sub>-D61 | CC3743(D60E) in pENTR/D-TOPO (kan<sup>+</sup>) | This study |
|                   | Destination vectors          | pENTR-cenK<sub>spec</sub>-R<sub>ssrA</sub> | CC0300<sub>spec</sub> in pENTR/D-TOPO (kan<sup>+</sup>) | This study |
|                   |                               | pENTR-cenK<sub>spec</sub>-R<sub>ssrA</sub> (H273A) | CC0300<sub>spec</sub>(H273A) in pENTR/D-TOPO (kan<sup>+</sup>) | This study |
|                   |                               | pTRX-HIS-DEST          | pET-TRX-His-TEV (amp<sup>+</sup>, chlor<sup>+</sup>)  | This study |
|                   |                               | pHS-MBP-DEST           | pET-His-MBP-TEV (amp<sup>+</sup>, chlor<sup>+</sup>) | This study |
|                   |                               | pHXM-DEST              | pS71xM2: high-copy, Fur<sup>+</sup>, M2 tag (spec<sup>+</sup>, chlor<sup>+</sup>) | This study |
|                   |                               | pHXM-DEST              | pMR20xM2: low-copy, Fur<sup>+</sup>, M2 tag (tet<sup>+</sup>, chlor<sup>+</sup>) | This study |
|                   | Expression vectors            | pHXM-cenR              | pS71xM2-cenR (spec<sup>+</sup>)                      | This study |
|                   |                               | pHXM-cenK<sub>spec</sub>-R<sub>ssrA</sub> | pS71xM2-cenK<sub>spec</sub>-R<sub>ssrA</sub> (spec<sup>+</sup>) | This study |
|                   |                               | pHXM-cenK<sub>spec</sub>-R<sub>ssrA</sub> (H273A) | pS71xM2-cenK<sub>spec</sub>-R<sub>ssrA</sub>(H273A) (spec<sup>+</sup>) | This study |
|                   |                               | pHXM-DEST              | pMR20xM2-cenR (tet<sup>+</sup>)                      | This study |
|                   |                               | pHXM-DEST              | pMR20xM2-cenR(D60E) (tet<sup>+</sup>)               | This study |

amp<sup>+</sup>, ampicillin-resistant; chlor<sup>+</sup>, chloramphenicol-resistant; spec<sup>+</sup>, spectinomycin-resistant.

DOI: 10.1371/journal.pbio.0030334.t004

either gene product led to a severe membrane blebbing phenotype, which, to the best of our knowledge, has not been seen before in *C. crescentus*. A number of other *C. crescentus* genes are involved in maintaining cell wall integrity and cell shape, including *mreB, rodA*, and *cicA*, but the relationship, if any, of these genes to *ccenK* and *ccenR* is not yet clear [44–46].

CenK–CenR is, to our knowledge, the first essential two-component pathway discovered in Gram-negative bacteria controlling cell envelope processes. In some Gram-positive bacteria, an essential two-component pathway, YycG–YycF, also plays a role in cell envelope biogenesis [47–49], but does not appear to be orthologous to the CenK–CenR system. However, the CenK–CenR pathway does appear to be highly conserved throughout the alpha subdivision of proteobacteria, including a number of important plant, animal, and human pathogens. Two-component systems have been highlighted as a possible new antibiotic target given their absence in humans and other animals [6,7,50,51]. Furthermore, as the physical construction of the cell wall has long been a major target of antibiotics, the CenK–CenR regulatory pathway may be a particularly suitable target for novel antibiotic development.

### Signaling Pathway Specificity and Insulation

All organisms use a relatively small number of signaling modalities. For bacteria such as *C. crescentus* two-component
signaling systems are widely employed, whereas eukaryotes have large families of other signaling systems, such as MAP kinase cascades, TGF-β pathways, and receptor tyrosine kinases. By definition, cross-talk between pathways must be minimal, otherwise an organism would be unable to trigger specific responses to specific stimuli. However, the mechanisms and strategies employed by cells to insulate highly related pathways are poorly understood and have been a recent focus of attention in many organisms [32–55].

We propose that the system-wide kinetic preference of histidine kinases for their cognate response regulators is a fundamental mechanism by which bacterial cells maintain the insulation of two-component signaling pathways. The large kinetic preference of kinases for their cognate substrates suggests that cross-talk observed in vitro likely arises from excesses in reaction time or reaction components and does not occur in vivo. Importantly, we distinguish deleterious cross-talk from cross-regulation in which a single kinase has multiple bona fide targets or multiple kinases regulate the same response regulator. There are several well-studied examples of cross-regulation, such as the E. coli kinase CheA, which phosphorylates both CheY and CheB as part of its role in regulating chemotaxis [27], and some of the noncognate interactions found in a systematic study of E. coli two-component systems may represent additional cases of cross-regulation [16]. In C. crescentus, cross-regulation occurs between the orphan kinases DivJ and PleC, and the two response regulators DivK and PleD. Our profile data for CheA, DivJ, and PleC demonstrated that kinases involved in cross-regulation have approximately equal kinetic preference for their multiple response regulator targets (see Figure 5C and 5D).

There are, of course, many additional means by which cells ensure signaling specificity. For example, subcellular localization of interacting components, scaffolding, and mutual inhibition can all act to ensure specificity [53]. However, our in vitro results point to biochemical selectivity as a fundamental mechanism, on which other layers of regulation and insulation may be built. Recent results with the cyclic-dependent kinases suggest that biochemical selectivity may also play a fundamental role in this process in X. censecessus [54]. It remains a major challenge to understand in complete detail how organisms robustly and accurately ensure signal fidelity within a cell [55].

Concluding Remarks

The techniques and approach described here can be directly extended to any organism containing two-component signal transduction systems, and are particularly useful for species with large sets of these molecules. This includes most bacteria, which typically encode at least 20 or 30 two-component genes and sometimes more than 100. Many plant species, including the model system Arabidopsis thaliana and the agriculturally and economically important rice plant Oryza sativa, also contain large sets of two-component signaling genes.

Finally, all cells, even relatively simple bacteria, are capable of complex information-processing tasks, such as converting continuous signals to discrete outputs, signal amplification, coincidence detection, and cellular-level memory. The successful implementation of these tasks is not carried out by individual proteins, but rather by multiple proteins, arranged into complex, highly connected circuits. For example, MAP kinase pathways are capable of converting continuous signals to an all-or-none output owing to a precise connectivity, a three-tiered MAPK cascade, and positive feedback [56]. Mapping the structure of signaling pathways and networks, as initiated here for C. crescentus, will thus be critical to our understanding of how cells process information and make decisions in order to regulate their behavior.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. E. coli strains were routinely grown in Luria Broth (BD Biosciences, Franklin Lakes, New Jersey, United States) at 37 °C, supplemented with carbenicillin (100 μg ml⁻¹ or 50 μg ml⁻¹), chloramphenicol (30 μg ml⁻¹ or 20 μg ml⁻¹), ampicillin (50 μg ml⁻¹ or 30 μg ml⁻¹), and spectinomycin (50 μg ml⁻¹) as needed for solid and liquid media. C. crescentus strains were grown in PYE (complex medium) or M2G (minimal medium) at 30 °C [57]. PYE medium was supplemented with 0.5% sucrose, oxytetracycline (2 μg ml⁻¹ or 1 μg ml⁻¹), 25 μg ml⁻¹ or 5 μg ml⁻¹), or spectinomycin (100 μg ml⁻¹ or 25 μg ml⁻¹), as required. PYE swarm plates contained 0.5% agar. Site-directed mutagenesis of cekK and cekρ was carried out using the primers CenKHK2734dw, CenKHK2734rev, CenKRD60Erev and the QuickChange protocol (Stratagene, La Jolla, California, United States). pKO3C was constructed by PCR amplification of the tetE cassette from pMR20 using the primers tet-fw and tet-rev, digestion with EcoRI, and ligation into the EcoRI site of pBluescript. Strains, plasmids, and primers used in this study are listed in Tables 4 and S1–S3.

Deletion of C. crescentus two-component genes. Response regulators and histidine kinases were identified by BLAST analysis of the C. crescentus genome sequence using known two-component protein sequences as input. For response regulators, sequences with BLAST E-values less than 0.01 were inspected for presence of the conserved residues D12, D13, D57, 187, and K109, where numbering is for E. coli CheY [10]. In sum, 44 response regulators were identified; these include two which may not be phosphorylated owing to mutation of one of the five highly conserved residues: CC3100 and CC0612. For histidine kinases, sequences with BLAST E-values less than 0.01 were inspected for presence of the conserved H-, N-, D-, F-, and G-boxes [10]. Two histidine kinases, CC0433 and CC0594, are CheA-like and have a P1 domain instead of the usual H-box. Nine histidine kinases are members of the newly identified HWE group [58] and lack the F-box.[57]. CC3100 and CC0612, which phosphorylate both CheY and CheB as part of its role in regulating chemotaxis [27], and some of the noncognate interactions found in a systematic study of E. coli two-component systems may represent additional cases of cross-regulation [16]. In C. crescentus, cross-regulation occurs between the orphan kinases DivJ and PleC, and the two response regulators DivK and PleD. Our profile data for CheA, DivJ, and PleC demonstrated that kinases involved in cross-regulation have approximately equal kinetic preference for their multiple response regulator targets (see Figure 5C and 5D).

There are, of course, many additional means by which cells ensure signaling specificity. For example, subcellular localization of interacting components, scaffolding, and mutual inhibition can all act to ensure specificity [53]. However, our in vitro results point to biochemical selectivity as a fundamental mechanism, on which other layers of regulation and insulation may be built. Recent results with the cyclic-dependent kinases suggest that biochemical selectivity may also play a fundamental role in this process in X. censecessus [54]. It remains a major challenge to understand in complete detail how organisms robustly and accurately ensure signal fidelity within a cell [55].

Concluding Remarks

The techniques and approach described here can be directly extended to any organism containing two-component signal transduction systems, and are particularly useful for species with large sets of these molecules. This includes most bacteria, which typically encode at least 20 or 30 two-component genes and sometimes more than 100. Many plant species, including the model system Arabidopsis thaliana and the agriculturally and economically important rice plant Oryza sativa, also contain large sets of two-component signaling genes.

Finally, all cells, even relatively simple bacteria, are capable of complex information-processing tasks, such as converting continuous signals to discrete outputs, signal amplification, coincidence detection, and cellular-level memory. The successful implementation of these tasks is not carried out by individual proteins, but rather by multiple proteins, arranged into complex, highly connected circuits. For example, MAP kinase pathways are capable of converting continuous signals to an all-or-none output owing to a precise connectivity, a three-tiered MAPK cascade, and positive feedback [56]. Mapping the structure of signaling pathways and networks, as initiated here for C. crescentus, will thus be critical to our understanding of how cells process information and make decisions in order to regulate their behavior.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. E. coli strains were routinely grown in Luria Broth (BD Biosciences, Franklin Lakes, New Jersey, United States) at 37 °C, supplemented with carbenicillin (100 μg ml⁻¹ or 50 μg ml⁻¹), chloramphenicol (30 μg ml⁻¹ or 20 μg ml⁻¹), ampicillin (50 μg ml⁻¹ or 30 μg ml⁻¹), and spectinomycin (50 μg ml⁻¹) as needed for solid and liquid media. C. crescentus strains were grown in PYE (complex medium) or M2G (minimal medium) at 30 °C [57]. PYE medium was supplemented with 0.5% sucrose, oxytetracycline (2 μg ml⁻¹ or 1 μg ml⁻¹), 25 μg ml⁻¹ or 5 μg ml⁻¹), or spectinomycin (100 μg ml⁻¹ or 25 μg ml⁻¹), as required. PYE swarm plates contained 0.5% agar. Site-directed mutagenesis of cekK and cekρ was carried out using the primers CenKHK2734dw, CenKHK2734rev, CenKRD60Erev and the QuickChange protocol (Stratagene, La Jolla, California, United States). pKO3C was constructed by PCR amplification of the tetE cassette from pMR20 using the primers tet-fw and tet-rev, digestion with EcoRI, and ligation into the EcoRI site of pBluescript. Strains, plasmids, and primers used in this study are listed in Tables 4 and S1–S3.

Deletion of C. crescentus two-component genes. Response regulators and histidine kinases were identified by BLAST analysis of the C. crescentus genome sequence using known two-component protein sequences as input. For response regulators, sequences with BLAST E-values less than 0.01 were inspected for presence of the conserved residues D12, D13, D57, 187, and K109, where numbering is for E. coli CheY [10]. In sum, 44 response regulators were identified; these include two which may not be phosphorylated owing to mutation of one of the five highly conserved residues: CC3100 and CC0612. For histidine kinases, sequences with BLAST E-values less than 0.01 were inspected for presence of the conserved H-, N-, D-, F-, and G-boxes [10]. Two histidine kinases, CC0433 and CC0594, are CheA-like and have a P1 domain instead of the usual H-box. Nine histidine kinases are members of the newly identified HWE group [58] and lack the F-box.
plasmids “knockout plasmids” and name each according to the nomenclature pKO-CCXXXX, where CCXXXX is the unique GenBank identifier. To determine the deletion cassette, plasmids were digested with NheI and SacI and transformed into CnpC5N by electroporation, and first integrants selected by plating on PYE containing oxytetracycline. Colonies were inoculated into liquid PYE medium with oxytetracycline and grown for 12–16 h. Five microliters of each culture was then plated on PYE plates containing oxytetracycline and sucrose. Colonies were screened for tetracycline resistance and kanamycin sensitivity to identify deletion strains. Proper construction of the gene deletion was also engineered by PCR. The cassette was digested with two direct repeats (FRT sites) such that expression of the FLP recombinase catalyzes removal of the tet cassette, leaving behind an in-frame deletion construct [61]. Each deletion strain was also engineered to incorporate two unique 20mer bar codes which is not found elsewhere in the C. crescentus genome. The bar code sequences were adapted from the S. cerevisiae deletion project [40,41], enabling similar high-throughput phenotypic characterization of deletion mutants. Fluorescence-activated cell sorting (FACS) was employed to verify the correct insert size. Positive clones were sequenced-verified using M13F and M13R primers. In total, 76 Gateway adapted response regulator pENTR clones were generated for this study (32 for E. coli and 44 for C. crescentus). Each clone contained the 5′ and 3′ untranslated regions of the gene of interest, except for 5′ or 3′ untranslated regions predicted by the SMART database (http://smart.embl-heidelberg.de). For complete primer lists, see Table S5.  

**Destination vectors and recombinational cloning.** Expression vectors were constructed and adapted for recombinational cloning using the Gateway vector conversion system (Invitrogen). Two plasmids (pTRX-HIS-DEST and pHIS-MBP-DEST) were derived from the IPTG-inducible pET32a and pET15b vectors (Novagen, Madison, Wisconsin, United States). To construct pHXM-DEST, a MscI-NheI fragment of pBADM-20 (EMBL protein purification and storage system) was used to replace the same region of pHIS-DEST (see Figure S5). 

**Protein expression and purification.** Expression plasmid DNA was transformed into E. coli BL21 (DE3) cells, grown in 500 ml of LB to OD600 ~0.6 and fusion proteins induced by addition of 300 μg IPTG. Cells were grown at 37 °C until needed. Native purifications of His6-tagged proteins were performed using affinity chromatography with Ni-NTA agarose beads (Qiagen). All steps of the purification (except for elution) were performed in bulk using 50-mL conical tubes. The following buffers were used for purification: lysis buffer (20 mM Tris-HCl [pH 7.9], 0.5 M NaCl, 10% glycerol, 0.1 mM PMSF, 1 mg/ml lysozyme, 125 units benzonase nuclease [Novagen]), wash buffer (20 mM HEPES-KOH [pH 8.0], 0.5 M NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM PMSF), elution buffer (20 mM HEPES-KOH [pH 8.0], 0.5 M NaCl, 10% glycerol, 250 mM imidazole), and storage buffer (10 mM HEPES-KOH [pH 8.0], 50 mM KCl, 10% glycerol, 0.1 mM EDTA, 1 mM DTT). Cell pellets were resuspended in 10 ml of lysis buffer, incubated at room temperature for 20 min, sonicated, and then centrifuged for 60 min at 30,000 g to generate a cleared lysate. His6-tagged proteins were bound to 1 ml of Ni-NTA beads with 50 ml of 50 mM HEPES-KOH buffer and then loaded onto an Econo-column (Bio-Rad, Hercules, California, United States) for elution. Purified protein was eluted using 2.5 ml of elution buffer and loaded directly onto a PD-10 column (Amersham Biosciences, Piscataway, New Jersey, United States) that had been pre-equilibrated with storage buffer. If necessary, samples were filtered with a 0.2-μm HT Tuffryn filter ( Pall Gelman Sciences, East Hills, New York, United States), and then concentrated to approximately 1–10 mg/ml using Centricon YM-10 or YM-30 columns (Millipore, Billerica, Massachusetts, United States). All samples were filtered through a Ultrafree-MC (0.22 μm) spin filter (Millipore) and then aliquoted for storage at −80 °C. Protein concentrations were measured using Coomassie Plus Protein Assay Reagent and a BSA standard (Pierce Biotechnology, Rockford, Illinois, United States). An equal amount (500 ng) of each protein sample was analyzed by 12% SDS-PAGE to verify molecular weight and purity. Prior to phosphotransfer profiling, all response regulator concentrations were normalized against a 500-ng BSA standard using a ChemiImager 5500 and densitometry (Alpha Innotech, San Leandro, California, United States) (see Figure S5).  

**Phosphotransfer profiling.** Each purified kinase was autophosphorylated in storage buffer supplemented with 2 mM DTT, 5 mM MgCl2, 500 μM ATP, and 5 μCi [γ-32P]ATP (~6,000 Ci/mmol, Amersham Biosciences). Reactions were allowed to proceed until equilibrium at 30 °C (15 min). Depending on the kinetics of the response regulator, phosphorylases to a final concentration of 5 μM in storage buffer plus 5 mM MgCl2. Phosphotransfer reactions contained 5 μl of phosphorylated kinase and 5 μl of response regulator (20 μg/l). Reaction volume was 10 μl, and reactions were incubated at 30 °C. Reactions were stopped with 3.5 μl of 4X sample buffer (500 mM Tris [pH 6.8], 8% SDS, 40% glycerol, 400 mM β-mercaptoethanol) and stored on ice until loaded. The entire sample was loaded, without heating, on 10% Tris-HCl polyacrylamide gels and run at room temperature for 50 min at 150 V. The dye front and
unincorporated ATP was removed with a razor blade and the wet gel (still on the back glass plate) placed in a Ziploc bag and exposed to a phosphor imager at room temperature. The gel was scanned with a Storm 860 imaging system (Amsersham Biosciences) at 50 μm resolution. E. coli profiles consisted of three protein gels, which were scanned separately and the images stitched together for analysis. E. coli profiles consisted of four protein gels, and were analyzed in the same fashion.

Estimation of kinetic preference. To estimate kinetic preference, we purified radiolabeled kinase by repeated washing with a NanoSep-30K column. A 30K column was packed with glass beads (East Hills, New York, United States). Autophosphorylation and phosphotransfer reactions were as described for phosphotransfer profiling, except that response regulators were diluted in storage buffer plus 5 mM MgCl₂ plus 0.5 mg/ml bovine serum albumin. The final concentrations of kinase and regulator were 3 μM and 10 μM, respectively. Kinetics of phosphotransfer were determined by quantifying bands using ImageQuant software (Amsersham Biosciences). The fraction of phosphorylated response regulator was calculated by normalizing to the intensity of the band corresponding to kinase alone. These normalized values were plotted versus reaction time and used to estimate initial reaction velocities for cognate versus noncognate substrates.

Depletion, overexpression, and coexpression strains. A xylose-inducible low-copy plasmid was generated by amplifying the xylR promoter region with XLYSCw and XLYNCw rev and cloning into pMR20, to generate pMR20-P. This plasmid contains a NcoI site engineered at the start codon of the xylR gene. We then amplified, by PCR, full-length versions of CC0530 (xynK) and CC3743 (ssrA) flanked by NcoI and HindIII sites using the primers CentKfw and CenXylXrev. This full-length xynK and ssrA PCR products were cloned into pMR20-P to generate pMR20-P-xynK and pMR20-P-ssrA.

Next, we produced in-frame derivatives of pKO-CC0530 and pKO-CC3743 (see Table S2). Each of these plasmids was cotransformed into E. coli with pCP20, which contains an arabinose-inducible FLP recombinase gene [61]. Expression of the FLP recombinase, according to the methods of Datsenko and Wanner [61], led to recombination between the direct repeat FRT sites flanking the tet cassette. The resulting plasmids, pΔxynK-1F and pΔssrA-1F, were sequenced to verify formation of an in-frame, markerless deletion construct. CB15N was then electroporated with pΔxynK-1F and pΔssrA-1F to generate kan⁺ΔxynK and kan⁺ΔssrA, respectively. Colonies that were kanamycin sensitive and yielded a single PCR band of the expected size were determined by measuring the slope (counts/second) for OmpR between 0 and 5 s, and for CpxR between 0 and 1000 s. This method also allows the identification of putative essential genes (tetcat⁻, kan⁺, sucrose⁻). From a quantification of our time course data, (A) Time courses for phosphorylation of OmpR and CpxR by EnvZ. In our phosphotransfer profiling (Figure 4C), OmpR and CpxR were both phosphorylated at the 10-s time point, but only OmpR was phosphorylated at the 10-s time point. (C) Plot of normalized PhosphorImager counts for OmpR and CpxR phosphorylation based on a quantification of the gels shown in (A) and (B). Initial velocities (v0) were determined by measuring the slope (counts/second) for OmpR between 0 and 5 s, and for CpxR between 0 and 4000 s. (D and E) Time courses for phosphorylation of CC1182 and CC2951 by CC1181. In our profiling, both CC1182 and CC2951 were phosphorylated by CC1181 at 60 min, but only CC1182 was phosphorylated at 10 s.
Figure S3. Multiple Sequence Alignment of CenR Orthologs
Putative CenR orthologs were identified by reciprocal best BLAST analysis. CenR proteins are highly conserved in the alpha subdivision of proteobacteria (C. crescentus CB15, Agrobacterium tumefaciens C58, Sinorhizobium meliloti 1021, Mesorhizobium loti MAFF303099, Brucella melitensis 16M, Rhodopseudomonas palustris CGA009, Bradyrhizobium japonicum USDA 110, Rhodobacter sphaeroides 2.4.1, Silicibacter pomeroyi DSS-3).

Found at DOI: 10.1371/journal.pbio.0030334.sg001 (5.4 MB TIF).

Figure S4. Multiple Sequence Alignment of CenK Orthologs
Putative CenK orthologs were identified by reciprocal best BLAST analysis. CenK proteins are highly conserved in the alpha subdivision of proteobacteria (C. crescentus CB15, Agrobacterium tumefaciens C58, Sinorhizobium meliloti 1021, Mesorhizobium loti MAFF303099, Brucella melitensis 16M, Rhodopseudomonas palustris CGA009, Bradyrhizobium japonicum USDA 110, Rhodobacter sphaeroides 2.4.1, Silicibacter pomeroyi DSS-3).

Found at DOI: 10.1371/journal.pbio.0030334.sg002 (3.2 MB TIF).

Figure S5. Purified C. crescentus and E. coli Response Regulators
(A) Thirty-two E. coli response regulators were purified as thioredoxin-In-His, fusion proteins. (B) Forty-four C. crescentus response regulators were purified as thioredoxin-In-His, fusion proteins. Approximately 500 ng of purified protein was analyzed by SDS-PAGE. The predicted molecular weights can be found in Table S3. Only one response regulator, E. coli FimZ, was not purified in a soluble form (no band of the correct weight is found in this lane). A molecular weight ladder is labeled in kilodaltons.

Found at DOI: 10.1371/journal.pbio.0030334.sg003 (2.3 MB TIF).

Table S1. Primer Names and Sequences Used for Plasmids Constructed in This Study

| Primer Name | Primer Sequence |
|-------------|-----------------|
| P1 | 5’-TCGTCAGCAGGACCTACGCT-3’ |
| P2a, P2b | 5’-AACACACACATCCACACTACGCT-3’ |
| P3a, P3b, P4 | 5’-GAGTTGCGGTTAGGCTGCTGCTGCT-3’ |

For each gene to be deleted, six primers were required (P1, P2a, P2b, P3a, P3b, and P4) plus one gene-specific confirmation primer (Pconf) (see Figure S1). The resulting deletion constructs are called “pKO-CCXXXX” where CCXXXX is the unique GenBank identifier number.

Found at DOI: 10.1371/journal.pbio.0030334.s001 (66 KB XLS).

Table S3. Primers for pENTR Clones of Histidine Kinases and Response Regulators

| Primer Name | Primer Sequence |
|-------------|-----------------|
| pENTR-CCXXXX | 5’-TCGTCAGCAGGACCTACGCT-3’ |
| pENTR-bXXXX | 5’-AACACACACATCCACACTACGCT-3’ |

List of primers used to clone 44 C. crescentus response regulators, and 32 E. coli response regulators. Each resulting pENTR clone is called pENTR-CCXXXX or pENTR-bXXXX for C. crescentus and E. coli genes, respectively. Three E. coli histidine kinases and four C. crescentus histidine kinases were also cloned, and the primers used are listed.

Found at DOI: 10.1371/journal.pbio.0030334.s003 (35 KB XLS).

Accession Numbers
The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for the CenK orthologs discussed in this paper are Agrobacterium tumefaciens C58 (Atu1988), Bradyrhizobium japonicum USDA 110 (bll8095), Brucella melitensis 16M (BME1168), C. crescentus CB15 (CC0530), Mesorhizobium loti MAFF303099 (MNB03011306), Rhodobacter sphaeroides 2.4.1 (Rhosp0300729), Rhodopseudomonas palustris CGA009 (RPA09283), Silicibacter pomeroyi DSS-3 (STM1w01002705), and Sinorhizobium meliloti 1021 (SM03820). GenBank accession numbers for the CenK orthologs are Agrobacterium tumefaciens C58 (Atu1988), Bradyrhizobium japonicum USDA 110 (bll8095), Brucella melitensis 16M (BME1168), C. crescentus CB15 (CC0530), Mesorhizobium loti MAFF303099 (MNB03011306), Rhodobacter sphaeroides 2.4.1 (Rhosp0300729), Rhodopseudomonas palustris CGA009 (RPA09283), Silicibacter pomeroyi DSS-3 (STM1w01002705), and Sinorhizobium meliloti 1021 (SM03820).

Acknowledgments
We thank Kathleen Ryan, Hartley McAdams, Kurt Thorn, Laura Garwin, and Andrew Murray for helpful discussions and comments on the manuscript. We also thank Richard Schalek at the Center for Nanoscale Systems at Harvard University for assistance in scanning electron microscopy.

We gratefully acknowledge support from the Office of Science (BER), U.S. Department of Energy, grant numbers DE-FG03-01ER63219 and DE-FG02-01ER63922. Support was also provided in part by a National Institute of Health grant to MTL at the Bauer Center for Genomics Research.

Competing interests. The authors have declared that no competing interests exist.

Author contributions. JMS, MSP, EGB, and MTL conceived, designed, performed, and analyzed the experiments. JMS and MTL wrote the paper.

References
1. Ryan KR, Shapiro L (2003) Temporal and spatial regulation in prokaryotic cell cycle progression and development. Annu Rev Biochem 72: 367–394.
2. McAdams HH, Shapiro L (2003) A bacterial cell-cycle regulatory network operating in time and space. Science 301: 1874–1877.
3. Skerker JM, Laub MT (2004) Cell-cycle progression and the generation of asymmetry in Caulobacter crescentus. Nat Rev Microbiol 2: 325–337.
4. Iba H, Fukuda A, Okada Y (1978) Rate of major protein synthesis during the cell division cycle in Caulobacter crescentus. J Bacteriol 135: 647–655.
5. Stock AM, Robinson VL, Goulet FE (2000) Two-component signal transduction. Annu Rev Biochem 69: 183–215.
6. Barrett JF, Hoch JA (1998) Two-component signal transduction as a target for microbial anti- infective therapy. Antimicrob Agents Chemother 42: 1292–1296.
7. Stephenson K, Hoch JA (2002) Two-component and phosphoryl relay signal transduction systems as therapeutic targets. Curr Opin Pharmacol 2: 507–512.
8. Galperin MY, Nikolskaya AN, Koonin EV (2001) Novel domains of the prokaryotic two-component signal transduction systems. FEMS Microbiol Lett 205: 11–21.
9. Jouve M, Dutta R, editors (2003) Histidine kinases in signal transduction. San Diego: Academic Press. p 507.
10. Galperin MY, Nikolskaya AN, Koonin EV (2001) Novel domains of the prokaryotic two-component signal transduction systems. FEMS Microbiol Lett 205: 11–21.
11. Jouve M, Dutta R, editors (2003) Histidine kinases in signal transduction. San Diego: Academic Press. p 507.
12. Galperin MY, Nikolskaya AN, Koonin EV (2001) Novel domains of the prokaryotic two-component signal transduction systems.
13. Jouve M, Dutta R, editors (2003) Histidine kinases in signal transduction. San Diego: Academic Press. p 507.
14. Galperin MY, Nikolskaya AN, Koonin EV (2001) Novel domains of the prokaryotic two-component signal transduction systems. FEMS Microbiol Lett 205: 11–21.
15. Schwarzer R, Goutsas J, Wehland J (1992) Two-component signal transduction systems and the bacterial cell cycle. Curr Opin Microbiol 5: 297–303.
Thr247 residue in the functioning of the osmosensor EnrZ, a histidine kinase phosphatase, in *Escherichia coli*. J Biol Chem 275: 38643–38653.

25. Igo MM, Nifia AJ, Stock J, Silhavy TJ (1989) Phosphorylation and dephosphorylation of a bacterial transcriptional activator by a transmembrane receptor. Genes Dev 3: 1725–1734.

26. Igo MM, Nifia AJ, Silhavy TJ (1989) A bacterial environmental sensor that functions as a protein kinase and stimulates transcriptional activation. Genes Dev 3: 598–605.

27. Hess JF, Ooscze K, Kaplan N, Simon MJ (1988) Phosphorylation of three proteins in the signaling pathway of bacterial chemotaxis. Cell 53: 79–87.

28. Nifia EG, Stock A, Mowbray S, Stock J (1991) Reconstitution of the bacterial chemotaxis signal transduction system from purified components. J Biol Chem 266: 9764–9770.

29. Raivio TL, Silhavy TJ (1997) Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. J Bacteriol 179: 7724–7733.

30. Sommer JM, Newton A (1989) Turning off flagellum rotation requires the pleiotropic gene pdeD, pdeA, pdeC, and pdeD define two morphogenic pathways in *Caulobacter crescentus*. J Bacteriol 171: 392–401.

31. Sommer JM, Newton A (1991) Pseudoreversion analysis indicates a direct role of cell division genes in polar morphogenesis and differentiation in *Caulobacter crescentus*. Genetics 129: 623–630.

32. Paul R, Wenner S, Amiot NC, Ghun C, Schirmer T, et al. (2004) Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanulate cyclase output domain. Genes Dev 18: 715–727.

33. Burbulya D, Trach KA, Hoch JA (1991) Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell 64: 1575–1585.

34. Fisher SL, Kim SK, Wanner BL, Walsh CT (1996) Kinetic comparison of the VanR and PhoB. Biochemistry 35: 4732–4740.

35. Grimsbaw CE, Huang S, Hanstein CG, Strauch MA, Burbulya D, et al. (1998) Synergistic kinetic interactions between components of the phosphorelay controlling sporulation in *Bacillus subtilis*. Biochemistry 37: 1563–1575.

36. Keller KL, Ninfa AJ, Silhavy TJ (1993) Phosphorylation and dephosphorylation in a two-component regulatory system. Proc Natl Acad Sci U S A 90: 691–696.

37. Smith JG, Latiasla JIA, Guanga GP, Pennington JD, Silversmith RF, et al. (2004) A search for amino acid substitutions that universally activate response regulators. Mol Microbiol 53: 887–901.

38. Kloke KE, Weiss DS, Kustu S (1993) Glutamate at the site of phosphor-ylation of nitrogen-regulatory protein NTRC mimics aspartyl-phosphate homology regions for gene disruptions in *S. cerevisiae*. J Biol Chem 268: 9764–9770.

39. Batchelor E, Goulain M (2003) Robustness and the cycle of phosphorylation and dephosphorylation in a two-component regulatory system. Proc Natl Acad Sci U S A 100: 691–696.

40. Gaever G, Chu AM, Ni L, Connelly C, Riles L, et al. (2002) Functional profiling of the *Saccharomyces cerevisiae* genome. Nature 418: 387–391.

41. Shoemaker DD, Lashkari DA, Morris D, Mittmann M, Davis RW (1996) Quantitative phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar-coding strategy. Nat Genet 14: 450–456.

42. Aldridge P, Paul R, Goymer P, Rainey P, Jenal U (2003) Role of the GGDEF regulator PdeD in polar development of *Caulobacter crescentus*. Mol Microbiol 47: 1605–1708.

43. Wheeler RT, Shapiro L (1999) Differential localization of two histidine kinases controlling bacterial cell differentiation. Mol Cell 4: 685–694.

44. Fuchs T, Wiger P, Osteras M, Jenal U (2001) Precise amounts of a novel member of a phosphotransferase superfamily are essential for growth and normal morphology in *Caulobacter crescentus*. Mol Microbiol 39: 679–692.

45. Figge RM, Divakaruni AV, Gober JW (2004) MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in *Caulobacter crescentus*. Mol Microbiol 51: 1321–1332.

46. Wagner JK, Galvani CD, Braun VV (2005) *Caulobacter crescentus* requires RodA and MreB for stalk synthesis and prevention of ectopic pole formation. J Bacteriol 187: 544–553.

47. Ng WL, Karmirmatz K, Winkler ME (2004) Defective cell wall synthesis in *Streptococcus pneumoniae* R6 depleted for the essential Pcb putative murein hydrolase or the VicR (YycF) response regulator. Mol Microbiol 53: 1161–1175.

48. Martin PK, Li T, Sun D, Biek DP, Schmid MB (1999) Role in cell permeability of an essential two-component system in *Staphylococcus aureus*. J Bacteriol 181: 3666–3673.

49. Faber C, Hoch JA (1998) A two-component signal transduction system essential for growth of *Bacillus subtilis*: Implications for anti-infective therapy. J Bacteriol 180: 6373–6383.

50. Furuta E, Yamamoto K, Tatebe D, Watabe K, Kitayama T, et al. (2005) Targeting protein homodimerization: a novel drug discovery system. FEBS Lett 579: 2063–2070.

51. Watanabe T, Hashimoto Y, Yamamoto K, Hirao K, Ishihama A, et al. (2003) Isolation and characterization of inhibitors of the essential histidine kinase, YycG in *Bacillus subtilis* and *Staphylococcus aureus*. J Antibiot (Tokyo) 56: 1045–1052.

52. Zarrimpour A, Park SH, Lim WA (2003) Optimization of specificity in a cellular protein interaction network by negative selection. Nature 426: 676–680.

53. Schwartz MA, Madhani HD (2004) Principles of MAP kinase signaling specificity in *Saccharomyces cerevisiae*. Annu Rev Genet 38: 725–749.

54. Loog M, Morgan DO (2005) Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. Nature 434: 104–108.

55. Elion EA, Qi M, Chen W (2005) Signal transduction. Signaling specificity in yeast. Science 307: 687–688.

56. Ferrell JE Jr, Machleder EM (1998) The biochemical basis of an all-or-none cell fate switch in *Xenopus* embryos. Science 280: 895–898.

57. Elly B (1991) Genetics of *Caulobacter crescentus*. Methods Enzymol 204: 372–384.

58. Karniol B, Vierstra RD (2004) The HWE histidine kinases, a new family of bacterial two-component sensor kinases with potentially diverse roles in environmental signaling. J Bacteriol 186: 145–153.

59. Wach A (1996) PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. Yeast 12: 259–265.

60. Schweizer HP, Huang TT (1995) An improved system for gene replacement and xIe fusion analysis in *Pseudomonas aeruginosa*. Gene 158: 13–22.

61. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97: 6640–6645.

62. Meszenzah AC, Shapiro L, Jenal U (1997) Isolation and characterization of a xylose-dependent promoter from *Caulobacter crescentus*. J Bacteriol 179: 592–600.

63. Yoshida T, Cai S, Inouye M (2002) Interaction of EnrZ, a sensory histidine kinase, with phosphorylated OmpK, the cognate response regulator. Mol Microbiol 46: 1293–1294.

64. Laub MT, McAdams HH, Feldblum T, Fraser CM, Shapiro L (2000) Global analysis of the genetic network controlling a bacterial cell cycle. Science 290: 214–2148.

65. Einiger M, Agabian N (1977) Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. J Bacteriol 132: 294–301.