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Known Bioactive Small Molecules Probe the Function of a Widely Conserved but Enigmatic Bacterial ATPase, YjeE

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SUMMARY

Escherichia coli YjeE is a broadly conserved bacterial ATPase of unknown function that has been widely characterized as essential. Here, the transcriptional regulation of the promoter of *yjeE* (P_{yjeE}) was probed using a luciferase reporter and 172 antibiotics of diverse mechanisms. Norfloxacin and other fluorquinolones were found to be the most potent activator of P_{yjeE} through binding to DNA gyrase. The stimulation of P_{yjeE} by norfloxacin was most impacted by lesions in two-component signal transduction systems with roles in respiration, central metabolism, and oxidative stress responses. This suggested that YjeE may have a critical role in aerobic metabolism. Remarkably, YjeE was found to be dispensable when cells were grown in the absence of oxygen. To the best of our knowledge, these findings represent the first definitive phenotypes for this enigmatic protein.

INTRODUCTION

Despite accelerating efforts in genome sequencing, progress in understanding gene function continues to be hampered by a lack of success in systematically probing the roles of individual genes. Approximately one-third of the genome of the model microbe *Escherichia coli* encodes proteins of poorly characterized function (Tatusov et al., 2003). Many of these have been found to be indispensable for growth, and represent uncharted aspects of core bacterial physiology (Freiberg et al., 2001). Here we have tackled one such bacterial protein, YjeE, using a library of well-characterized small molecules to probe the transcriptional activity of its promoter (P_{yjeE}).

E. coli yjeE encodes a broadly conserved and indispensable bacterial protein whose function has remained elusive (Gerdes et al., 2003; Kobayashi et al., 2003; Salama et al., 2004; Tatusov et al., 1997; Winterberg et al., 2005). Its widespread essentiality and lack of a human ortholog have made this protein an attractive antibacterial drug target. YjeE was the subject of a recent drug discovery effort to identify small molecules that have an affinity to the recombinant protein (Lerner et al., 2007). The structure of the YjeE ortholog from *Haemophilus influenzae* was solved as part of a genomics initiative and showed conserved features similar to that of the TRAFAC class of P-loop NTPases (Koretke et al., 2001; Leipe et al., 2002; Teplyakov et al., 2002). The YjeE protein has a very slow intrinsic ATPase activity (k_{cat} = 1 h^{-1}, K_m = 0.8 mM) and binds stoichiometric amounts of ADP with high affinity (Allali-Hassani et al., 2004). Though depletion of YjeE in *E. coli* leads to cell death, no signature phenotype has been identified that points to a cellular function (Allali-Hassani et al., 2004). Nevertheless, we have recently identified *rstA* as a multicopy suppressor of YjeE depletion (Brown, 2005; Campbell et al., 2007). RstA is the response regulator of the RstAB two-component system that controls the expression of many genes. Insights into the function of RstA have come from recent studies that implicate it in acid shock response, biofilm formation, and control of the degradation of the stationary phase sigma factor, RpoS (Cabeza et al., 2007; Ogasawara et al., 2007; Yamamoto et al., 2005). The only other observations concerning YjeE come from the cyanobacterium *Anabaena sp.*, where a *yjeE* homolog, *hetY*, was found to be involved in the physiological changes required for cell differentiation into heterocysts (Yoon et al., 2003). In total, the disparate descriptions of YjeE have failed to contribute significantly to assignment of even a broad functional role for this essential protein.

To better probe the function of YjeE, we took a nontraditional approach of using bioactive molecules of known mechanism to search for chemical-genetic interactions using a YjeE-responsive promoter. Chemical-genetic refers to the use of small molecules to inhibit biological process, in much the same way that genetics can perturb biology. By measuring the expression of *yjeE* in the presence of these chemical-induced perturbations, we aimed to uncover a phenotype for *yjeE* that is more informative than its known lethal phenotype. Recent chemical-genetic approaches have been successful in elucidating new components of the DNA damage response and in discovering new machinery in outer membrane assembly in bacteria (Ruiz et al., 2005; Van Dyk et al., 2001).

Herein, we have used chemical probes in a directed manner against a protein of unknown function to ascribe a broad functional role. This work serves as a proof of principle of the potential of chemicals in discovery of novel biological activities. We constructed a luminescence-based reporter that is upregulated in response to depletion of YjeE. This promoter-reporter system was used to screen a well-characterized chemical library for compounds that could induce expression of *yjeE*. Norfloxacin, a fluoroquinolone inhibitor of DNA metabolism, was found to be the most profound inducer. Noting the recently uncovered

role of norfloxacin in oxidative stress (Dwyer et al., 2007; Kohanski et al., 2007), we further characterized the norfloxacin-dependent response of P_{yjeE} in deletion backgrounds of two-component regulatory systems. The latter encompassed some 37 genes responsible for adaptation of *E. coli* to various stresses by controlling diverse aspects of physiology (Oshima et al., 2002). This experiment uncovered links to central metabolism, stress responses, and metal homeostasis. These interactions, along with observations from literature, led us to the hypothesis that YjeE may be involved in respiration. Remarkably, we found that *E. coli yjeE*, a well-known essential gene, is dispensable under anaerobic conditions.

RESULTS

Promoter Validation

Wild-type *E. coli* MG1655 contains two promoters upstream of *yjeE* that may influence its expression (Kitagawa et al., 1996). The promoter proximal to *yjeE* (P_{yjeE}) is located within the upstream gene, *yjeF*, whereas the promoter distal to *yjeE* (P_{yjeF}) is located upstream of *yjeF* (Figure 1A). The activity of each of these promoters was monitored using the reporter plasmid pCS26-*bla*, which encodes the luciferase operon, *luxCDABE*. This promoterless plasmid is an ampicillin-resistant derivative of pCS26-pac (Bjarnason et al., 2003). The 750 bp regions upstream of the start sites of *yjeE* and *yjeF* were cloned into pCS26-*bla* to generate pCS26-*bla*-P_{yjeE} and pCS26-*bla*-P_{yjeF}, respectively.

To determine whether expression from either P_{yjeE} or P_{yjeF} is regulated by the intracellular concentration of YjeE, the activities of the promoters were tested in a strain where the level of YjeE could be modulated. In this conditional null, native *yjeE* was replaced by a chloramphenicol resistance cassette, and an ectoptic copy of *yjeE* was placed at the *araBAD* locus under the control of the arabinose-inducible P_{BAD} promoter (Allali-Hassani et al., 2004). Thus, depletion of cellular YjeE can be achieved by growth in the absence of arabinose. To counter leaky expression of *yjeE* from the P_{BAD} promoter, cells were subjected to three rounds of growth and dilution into media lacking arabinose. This resulted in almost no growth during the fourth round of growth in the absence of arabinose (Figure 1B, closed symbols), whereas cells grown in the presence of arabinose grew robustly (Figure 1B, open symbols). The presence of plasmids pCS26-*bla*-P_{yjeE} (Figure 1B, triangles) or pCS26-*bla*-P_{yjeF} (Figure 1B, circles) did not affect the growth of cells. Under these conditions, pCS26-*bla*-P_{yjeF} showed a modest 3-fold induction of expression upon depletion of the YjeE protein (Figure 1C, circles), whereas, pCS26-*bla*-P_{yjeE} exhibited a 73-fold induction (Figure 1C, triangles). This large increase in the activity of P_{yjeE} in response to YjeE depletion suggested that this promoter is subject to regulation in the cell by the level of YjeE and is thus likely to be the physiologically relevant promoter for *yjeE*.

To test if the promoter activity of P_{yjeE} was only influenced by the addition of arabinose by some trivial mechanism, expression from the promoter was monitored in the presence or absence of arabinose in a strain diploid for *yjeE*. The diploid strain contains a copy of *yjeE* at its native locus as well as a copy at the *araBAD* locus. In this strain, addition of arabinose only caused a small 3-fold change in the activity of P_{yjeE} (see Figure S1 available online). Furthermore, promoter activity was similar in the *yjeE* conditional null grown in the presence of arabinose as it was in wild-type *E. coli* MG1655 (Figure S1). These data suggest that induction of P_{yjeE} activity was not an artifact of arabinose treatment or the consequence of polar effects on genes downstream of *yjeE*.

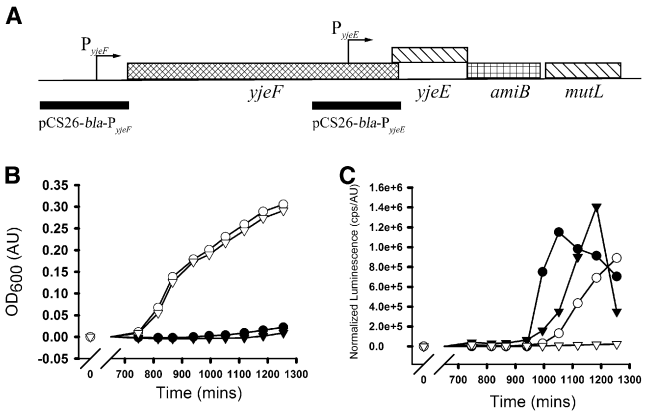

Figure 1. Identification of a Promoter that Responds to the Intracellular Level of YjeE

(A) The *yjeE* chromosomal locus of *E. coli* MG1655. Black bars represent the upstream regions, termed P_{yjeF} and P_{yjeE}, which were cloned into the luminescence reporter plasmid to produce either pCS26-*bla*-P_{yjeF} or pCS26-*bla*-P_{yjeE}, respectively.

(B and C) Growth and luminescence produced by an arabinose-inducible *yjeE* conditional null harboring either pCS26-*bla*-P_{yjeE} (triangles) or pCS26-*bla*-P_{yjeF} (circles). The conditional null was grown under YjeE-deplete (closed symbols) or YjeE-replete (open symbols) conditions. OD_{600} (B) and density-normalized luminescence (C) are shown. Values represent the average of duplicates.

Screen for Small Molecules with Known Biological Activity that Influence the Expression of P_{yjeE}

Wild-type *E. coli* MG1655 harboring pCS26-*bla*-P_{yjeE} was used to query the Prestwick Chemical Library. This library contains a variety of FDA approved drugs and bioactive compounds, including 172 antibiotics. Preliminary tests revealed that spectinomycin and tetracycline were both able to induce expression from P_{yjeE}. These two antibiotics were used to develop a sensitive and robust assay for screening. It was found that addition of compound at OD_{600} of ~0.1 produced an optimal promoter response (data not shown). For both compounds, induction was maximal near their published minimum inhibitory concentrations (MIC), but significant induction was observed within a range of 4-fold above or below the MIC (data not shown). To reduce false negatives, the screen was carried out at two concentrations of compound, 1 μM and 10 μM, which is within 4-fold of the MIC of the majority of antibiotics in the library. The screen was performed in duplicate. Cell density (OD_{600}) and luminescence were measured hourly for 8 hr after addition of compound, and for each time point, luminescence was normalized by cell density (see Table S1).

The maximum fold-increase in normalized luminescence between treated and untreated cells was plotted for each compound (Figures 2A and 2B). The hit zone was defined as those compounds that caused an increase in normalized

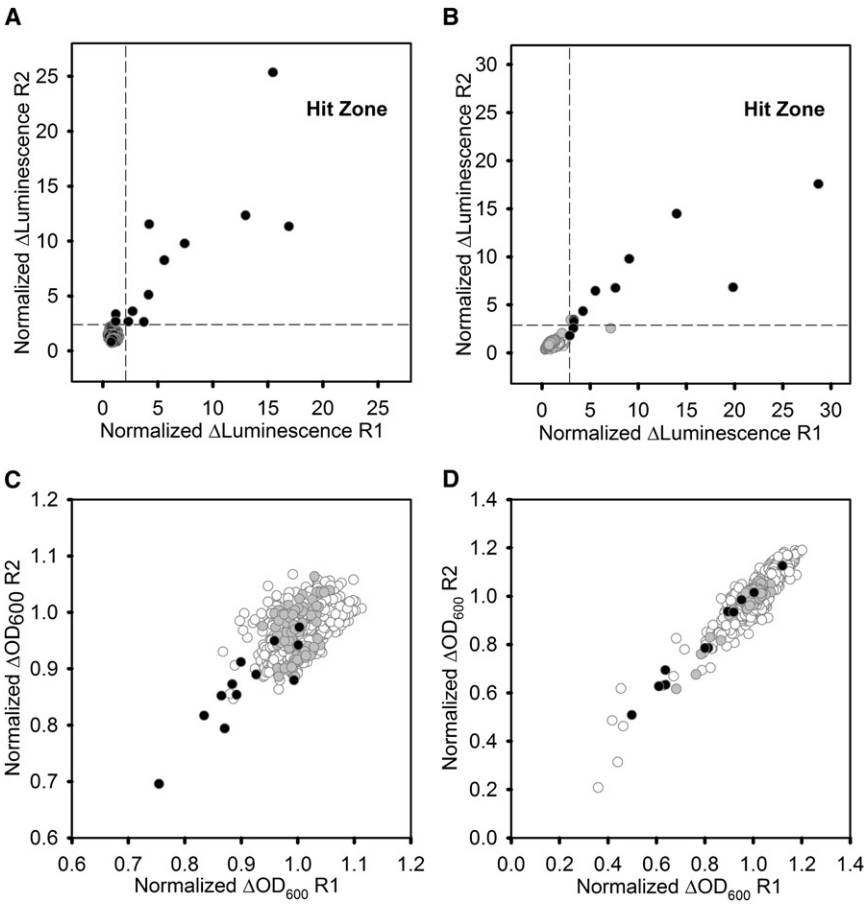

Figure 2. Screen of 1121 Bioactive Molecules with Known Mechanism of Action for Stimulation of P_{yjeE}

E. coli MG1655 harboring pCS26-*bla*-P_{yjeE} were treated with 1 μM (A and C) or 10 μM (B and D) of compound at an OD_{600} ~0.1, and luminescence and OD_{600} was monitored for 8 hr.

(A and B) Plots of replicate 1 (R1) versus replicate 2 (R2) showing the maximal fold change in OD_{600}-normalized luminescence of treated versus untreated cells.

(C and D) Maximal OD_{600} of treated cells normalized by the OD_{600} of the untreated controls. Hashed lines represent six standard deviations away from the mean activity of untreated cells. Compounds outside of this area were defined as hits. Shown are the hits (black circles), nonhits (open gray circles), and untreated controls (grey circles). Values represent the average of duplicates.

luminescence that was more than six standard deviations above the untreated controls. Black circles represent the compounds that were defined as hits, open circles represent the compounds that did not affect the activity of P_{yjeE}, and grey circles represent untreated controls. Figures 2C and 2D show the densities of compound-treated cultures, normalized by the density of the untreated controls. These data revealed that compounds that had an impact on growth also caused stimulation of P_{yjeE}. For example, at 1 μM, all of the compounds that decreased growth rate also induced P_{yjeE}.

Dose Dependence of Six Representative Hit Compounds

Antibiotics with well-characterized mechanisms of action that caused stimulation of P_{yjeE} were chosen for further study of the function of YjeE. Of 26 hits, 20 were known antibiotics, and these fell into six different chemical classes (Table 1). Six compounds had no known target in *E. coli* and were not further characterized. Experiments were carried out with a representative probe from each class of antibiotics—namely, chloramphenicol, dirithromycin, norfloxacin, spectinomycin, streptozotocin, and tetracycline. Norfloxacin was found to cause the strongest stimulation of promoter P_{yjeE}.

The concentration dependence of the response of P_{yjeE} to these six representative antibiotics was tested. The response of the constitutively expressed sigma70-16 synthetic promoter (Kim and Surette, 2006) was also tested to determine if the observed change in luminescence was specific to P_{yjeE} (Figure 3). The maximal induction of P_{yjeE} caused by dirithromycin, chloramphenicol, spectinomycin, streptozotocin, or tetracycline was less than 5-fold more than the induction observed for the control sigma70-16 promoter (Figures 3A, 3B, 3D, and 3F). Norfloxacin, however, generated a massive 50-fold induction of P_{yjeE} compared with the sigma70-16 control (Figure 3C). The ability of norfloxacin to stimulate a transcriptional response from P_{yjeE} suggested that this molecule could inform on the physiological role of YjeE.

Growth and luminescence profiles of wild-type *E. coli* harboring pCS26-bla-P_{yjeE} was measured (Figure 4C). The activity of P_{yjeE} increased continuously over the growth cycle (Figure 4C, solid line, closed circles). Treatment with norfloxacin at an OD_{600} of 0.15 resulted in similar activity of P_{yjeE} through log phase growth compared with untreated cells; however, activity of P_{yjeE} was greatly increased by norfloxacin treatment as cells progressed into stationary phase (Figure 4C, solid line, open circles).

Determination of the Mechanism of Stimulation of P_{yjeE} by Norfloxacin

Norfloxacin has four known cellular targets in *E. coli*, which are ParC, ParE, GyrA, and GyrB. The Par proteins are responsible for the active partitioning of sister chromosomes and plasmids (Adams et al., 1992; Kato et al., 1990; Zechiedrich and Cozzarelli, 1995), whereas the Gyr proteins encode DNA gyrase, which introduces negative supercoils into DNA (Gellert et al., 1976; Nollmann et al., 2007). In *E. coli*, the principal target of norfloxacin is thought to be *gyrA*, because mutations in *gyrA* alone can give rise to a high level of quinolone resistance (Everett et al., 1996; Khodursky et al., 1995). To determine if the observed stimulation of P_{yjeE} by norfloxacin occurred through its target, GyrA, or by some other mechanism, mutations in *gyrA* (Ser83Leu, Asp87Tyr)

Table 1. Compounds that Induce P_{yjeE}

Drug and Class	Compound
Antibiotics	
Chloramphenicols	Chloramphenicol
	Thiamphenicol
	Florefenicol
Macrolides	Dirithromycin
Tetracyclines	Doxycycline
	Oxytetracycline
	Meclocycline
	Methacycline
	Demeclocycline
Aminoglycosides	Spectinomycin
Fluoroquinolones	Norfloxacin
	Ofloxacin
	Lomefloxacin
	Enoxacin
	Cinoxacin
Glucosamine nitrosoureas	Streptozotocin
No known bacterial target	
Testosterone analog	Danazol
Acetylcholine analog	Methacholine
Dihydroindolone	Molindone
Morphinan	Naloxone
Arylalkanoic acid	Zomepirac

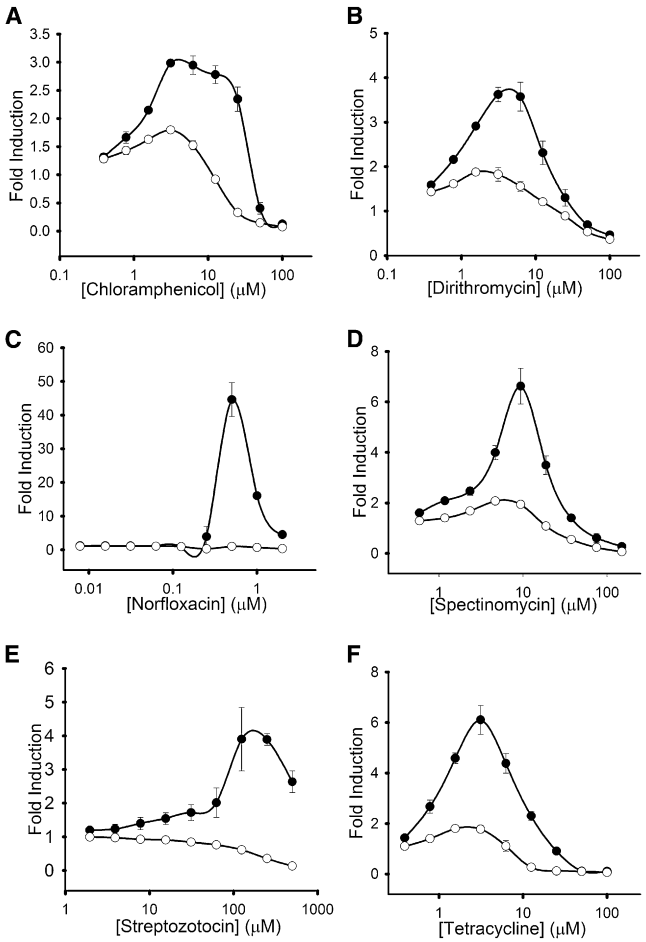

Figure 3. Dose Response of Stimulation of P_{yjeE} by Representative Antibiotics

Wild-type *E. coli* harboring the reporter plasmid containing P_{yjeE} (closed circles) or the synthetic constitutive promoter sigma70-16 (open circles) were treated with various concentrations of chloramphenicol (A), dirithromycin (B), norfloxacin (C), spectinomycin (D), streptozotocin (E), or tetracycline (F). Fold induction, which is the ratio of OD_{600}-normalized luminescence of treated compared with untreated cells, 8 hr post addition of drug is shown. Error bars represent the standard deviation of triplicates.

that give rise to norfloxacin resistance were created (Vila et al., 1994). When this double mutation was introduced into *gyrA* on the chromosome, norfloxacin was no longer able to stimulate P_{yjeE} (Figure 4A). The DNA-alkylating agent streptozotocin, which is known to damage DNA directly, (Fram et al., 1989), was still able to induce P_{yjeE} in the *gyrA* mutant strain, confirming that induction of luminescence in this strain is still possible (Figure 4B). These data suggested that the mechanism of norfloxacin stimulation of P_{yjeE} involves its cellular target, GyrA.

Recently, norfloxacin and other bactericidal antibiotics were found to generate toxic oxygen radicals in vivo, which was important for their bactericidal activity (Kohanski et al., 2007). Thus, we tested if such a mechanism could explain the chemical-genetic interaction between norfloxacin and P_{yjeE}. The sensitivity of YjeE-depleted and YjeE-replete cells treated with methyl viologen, a chemical that produces reactive oxygen species, was measured (Figure 4D). The *yjeE*-complemented null (Figure 4D, triangles) and diploid strain (Figure 4D, circles) was grown in the absence (Figure 4D, open symbols) or presence (Figure 4D, closed symbols) of arabinose. YjeE-depleted cells exhibited a modest 2-fold sensitization to methyl viologen compared with YjeE-replete cells.

Using Deletions of Two-Component Response Regulators to Further Characterize the Mechanism of Norfloxacin Stimulation of P_{yjeE}

Two-component systems are regulatory mechanisms that consist of a sensor kinase and a transcriptional activator. These systems have widely characterized roles in diverse cellular processes; thus, deletions in two-component systems were used to shed light on the function of YjeE. The activity of the P_{yjeE} promoter was tested in a panel of 37 strains, each containing a single deletion of the transcriptional activator of a two-component system. If the transcriptional activator was not known, a deletion of the sensor kinase was tested instead (Oshima et al., 2002). The ability of 0.1 μM norfloxacin to induce P_{yjeE} was either increased or decreased in a number of the deletion strains compared with the isogenic parental strain. Gene deletions that had the greatest impact on norfloxacin stimulation of P_{yjeE} are shown in Figure 5. These chemical-genetic interactions occurred in deletions of regulators of respiration and central metabolism (*torR, narL, arcB, hydG*, and *uhpA*), ion or metal responses (*cusR* and *rcsD*), and oxidative stress responses (*cpxR* and *soxS*) (Figure 5).

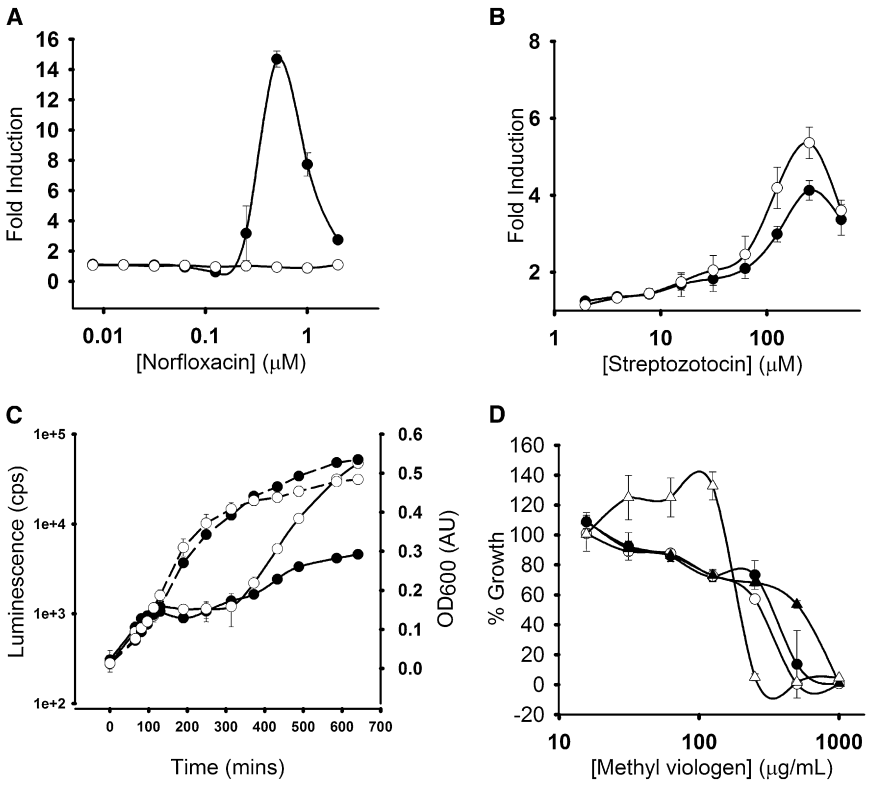

Figure 4. Characterization of the Mechanism of Stimulation of P_{yjeE}

(A and B) Norfloxacin-resistant (open circles) or wild-type (closed circles) *E .coli* MG1655 were treated with norfloxacin (A) or streptozotocin (B), and fold induction of OD_{600}-normalized luminescence of treated cells over untreated cells was plotted against compound concentration.

(C) Growth profiles (hashed lines) and promoter response (solid lines) of *E. coli* MG1655 treated with 0.5 μM norfloxacin (open circles) or untreated (closed circles). Drug was added at 120 min.

(D) Percentage growth of the *yjeE*-complemented null (triangles) and *yjeE* diploid strain (circles) treated with methyl viologen. Cells were grown in the presence (closed symbols) or absence (open symbols) of arabinose. Error bars represent the standard deviation of triplicates.

Growth of the *yjeE* Conditional Null under Anaerobic Stress

We reasoned that the abundance of interactions with two-component systems involved in aerobic and alternative respiration might reflect the physiological role of YjeE. Previously, we reported that the indispensability of *yjeE* could be partially suppressed by overexpression of *rstA*, an uncharacterized response regulator (Campbell et al., 2007). Oshima et al. (2002) found that deletion of *rstA* affected the expression of genes involved in alternate respiratory pathways in *E. coli* (*narL* and *dmsA*). In addition, Cabeza et al. (2007) found that overexpression of the homolog of *rstA* in *Salmonella* also affected the transcription of the alternative respiratory factor *narZ*. These observations, together with the findings of the two-component deletion experiment, suggested a role for *yjeE* in respiratory control.

To test this hypothesis, the dispensability of *yjeE* was examined under anaerobic conditions. The conditionally complemented *yjeE* null was grown in the presence or absence of arabinose (which induces expression of the complementing copy of *yjeE*) under anaerobic and aerobic conditions. As previously reported for aerobic conditions (Allali-Hassani et al., 2004), the conditional null was unable to grow in the absence of arabinose (Figure 6C and 6D). Remarkably, under anaerobic conditions, cells depleted of YjeE were able to survive and form single colonies (Figure 6I and 6J). *E. coli yjeE* has never been shown to be dispensable under any conditions. To ensure that the activity of the P_{BAD} promoter was not simply increased under anaerobic stress, a complemented null of a gene thought to be involved in ribosome biogenesis, *engA*, was tested. The *engA* conditional null was constructed in a manner identical to the *yjeE* conditional null (Bharat et al., 2006); however, this strain did not grow in the absence of arabinose under anaerobic conditions.

DISCUSSION

Herein, a chemical-genetic approach was used to probe the function a widely conserved but uncharacterized bacterial ATPase in *E. coli*, YjeE. A library of well-characterized bioactive small molecules was used to explore the transcriptional activity of the promoter of *yjeE*. The discovery of a profound norfloxacin-induced transcriptional activity led us

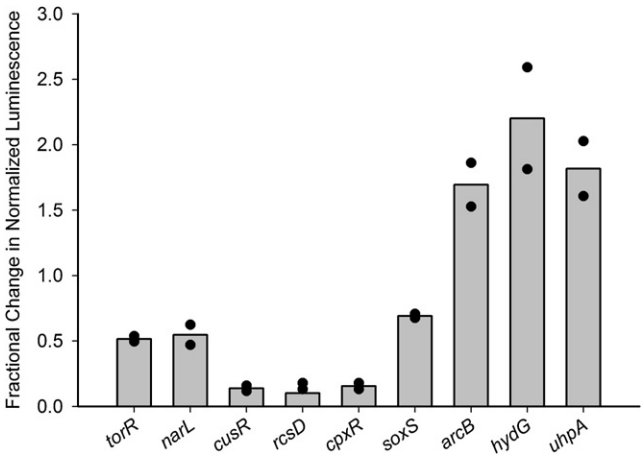

Figure 5. Norfloxacin Stimulation of P_{yjeE} in Various Deletions of Two-Component Response Regulators

Cells carrying a single deletion of either the response regulator or the histidine kinase of all known, nonessential two-component systems in *E. coli* were tested for the effect of the deletion on norfloxacin-stimulated activity of P_{yjeE}. Shown here are the genes whose deletions had the highest impact on norfloxacin induction of P_{yjeE}. Luminescence was measured 8 hr and 12 hr post addition of drug, and the maximal fractional change in luminescence of the deletion strain compared with the isogeneic wild-type strain was plotted. Values represent the average of duplicates.

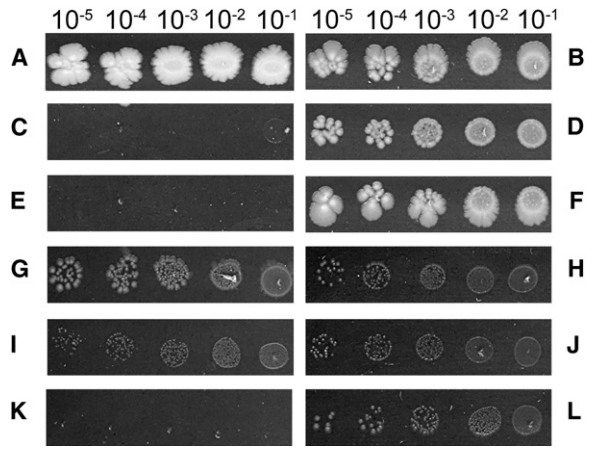

Figure 6. Phenotype of YjeE-Depleted Cells in the Absence or Presence of Oxygen

The *yjeE* diploid (A, B, G, and H), *yjeE*-complemented null (C, D, I, and J), and *engA*-complemented null (E, F, K, and L) were grown in the presence (A–F) or absence (G–L) of oxygen. In addition, all strains were grown in the presence (left panels) or absence (right panels) of arabinose. The dilutions, relative to an OD_{600} of 0.1, are indicated.

to investigate a role for YjeE in various *E. coli* stress responses mediated by two-component signal transduction systems. This approach led to the elucidation of a significant phenotype for this protein, namely, anaerobic gene dispensability.

The fluoroquinolone antibiotic norfloxacin, which is an inhibitor of DNA gyrase, was by far the most potent activator of P_{yjeE} and served as a useful chemical probe of the function of YjeE. Mutations in *gyrA*, which abrogate the binding of norfloxacin to DNA gyrase, abolished the stimulation of P_{yjeE} and suggested a possible role for YjeE in the recently elaborated oxidative stress response of bacteria to fluoroquinolones (Dwyer et al., 2007; Kohanski et al., 2007). A survey of deletions of two-component regulatory genes for those that influenced norfloxacin stimulation of P_{yjeE}, revealed that the majority have roles in respiration and central metabolism.

We found that the norfloxacin-stimulated activity of P_{yjeE} was augmented by a deletion in the aerobic respiratory regulator *arcB* and was negatively impacted by regulators of alternative respiratory pathways, *narL* and *torR*. Given that aerobic metabolism is known to negatively regulate alternative respiration in *E. coli* (Unden and Bongaerts, 1997), our findings suggested that YjeE has a role at the interface between these two pathways. SoxS is a response regulator whose regulon can lessen the effects of lethal oxygen radicals (Storz and Hengge-Aronis, 2000). Deletion of *soxS* negatively affected the ability of norfloxacin to stimulate P_{yjeE}, suggesting that YjeE may be involved in an oxidative stress response. Methyl viologen, a redox cycling agent that generates reactive oxygen species, was tested for its ability to activate P_{yjeE}; however, no increase in luminescence was observed (data not shown). Further, there was only a slight sensitization to methyl viologen when cells were depleted of YjeE. These findings suggested that if YjeE is involved in oxidative stress, its role may be in preventing the formation of toxic oxygen species, as opposed to shielding the cell from the effects of oxygen radicals. Apart from indispensability, the only other telling phenotypes for *yjeE* homologs have been reported in *Anabaena sp.*, where deletion of its homolog (*hetY*) affected the formation of heterocysts in response to nitrogen starvation (Yoon et al., 2003). During nitrogen starvation, the electron transport chain is hyperactive in order to consume intracellular oxygen, a potent inactivator of the nitrogen-fixing enzyme nitrogenase (Dalton and Postgate, 1969; Hochman and Burris, 1981). Incomplete differentiation to heterocysts caused by deletion of *yjeE*/*hetY* could well be explained by an inability of the cells to protect the nitrogenase from oxygen-dependent inactivation and is consistent with our observations that a lesion of *yjeE* has a negative interaction with oxygen-dependent cell physiology.

Deletion of the gene *cpxR* also had a negative effect on the norfloxacin-stimulated activity of P_{yjeE}. CpxR regulates the transcription of proteins in response to envelope stress (Ruiz and Silhavy, 2005). This finding was difficult to reconcile; however, CpxR is known to be responsive to copper (Yamamoto and Ishihama, 2006) and *cusR*, a regulator of copper homeostasis, also showed an inhibitory effect on norfloxacin-stimulated P_{yjeE} activity. At high concentrations in the periplasm, copper is known to cause promiscuous oxidation/reduction of thiol-containing proteins (Hiniker et al., 2005). CpxR drives the transcription of periplasmic chaperones and proteases that repair this damage (Raivio, 2005; Yamamoto and Ishihama, 2006). This is consistent with the interaction with *soxS* discussed previously, except that the link between the oxidative stress response and YjeE is unclear.

The norfloxacin-mediated stimulation of P_{yjeE} was particularly intriguing in light of recent mechanistic work describing the role of oxidative stress in the bactericidal effects of fluoroquinolones (Dwyer et al., 2007; Kohanski et al., 2007). The effect of norfloxacin on oxygen radical generation in cells is achieved by influencing the flux of NADH through the electron transport chain. Thus, we wondered if YjeE might have a proactive role in managing oxidative stress that was related to the respiratory chain. A physical interaction between YjeE and PflB in *E. coli* was previously reported (Butland et al., 2005). PflB is the β-subunit of pyruvate formate lyase that converts pyruvate to acetyl-CoA and formate under anaerobic conditions (Unden and Bongaerts, 1997). Formate is the preferred electron donor under anaerobic conditions in *E. coli*, and nitrate is the preferred electron acceptor (Unden and Bongaerts, 1997). The interaction discovered here between norfloxacin stimulation of P_{yjeE} and *narL*, a controller of nitrate reduction, is likewise supportive of the hypothesis that YjeE has a role in respiration. Further, *rstA*, the high-copy suppressor of the lethality of YjeE depletion, has a known interaction with nitrate respiration in Gram-negative organisms (Cabeza et al., 2007; Oshima et al., 2002). This represents another phenotypic link between *yjeE* and alternate respiratory pathways in *E. coli*.

We found that bacteriostatic compounds (chloramphenicols, tetracyclines, and spectinomycin) could induce P_{yjeE}, albeit 10-fold less than norfloxacin. The previous work by Kohanski et al. (2007) found that only bactericidal antibiotics lead to the production of toxic oxygen radicals, whereas bacteriostatic antibiotics did not. We tested kanamycin, a bactericidal antibiotic, specifically for its ability to influence the expression of P_{yjeE} and did not find any stimulation (data not shown). These observations suggested that the activity of P_{yjeE} may be more intimately linked

to the specific mechanism of the fluoroquinolone antibiotics (e.g., alteration of DNA topology or regulation of DNA replication by inhibition of DNA gyrase).

The level of transcription from certain promoters has been found to be linked to superhelicity of the promoter DNA. Microarray analysis found that 106 genes were upregulated in norfloxacin-treated cells (Peter et al., 2004). Although *yjeE* was not one of the upregulated genes, that experiment used 50,000 μM norfloxacin, whereas our experiments found maximal stimulation of P_{yjeE} at 0.5 μM norfloxacin, and the stimulation decreased past this concentration. The activity of P_{yjeE} was monitored as a function of growth and was found to be greatest as cells entered stationary phase, where DNA is in a more relaxed state (Dorman et al., 1988; Reyes-Dominguez et al., 2003). These findings suggest that if superhelicity does indeed affect the activity of P_{yjeE}, the stimulation happens at a low level of superhelicity over a narrow range of topological states.

The link between the expression of P_{yjeE} and superhelicity may be more indirect as suggested by the following findings. Dorman et al. (1988) found that negative supercoiling of DNA was increased during anaerobic growth in *E. coli*. *Salmonella typhimurium* can be made to be strictly anaerobic or aerobic by mutations in DNA topoisomerase I or *gyrA*, respectively (Yamamoto and Droffner, 1985). Finally, the expression of cytochrome bd oxidase and trimethylamine oxide reductase, enzymes involved in the electron transport chain, is influenced by DNA superhelicity (Ansaldi et al., 2007; Bebbington and Williams, 2001). We have observed that deletions of *arcB* and *torR*, two-component systems that govern the expression of the above enzymes, have an impact on the norfloxacin stimulation of the activity of P_{yjeE}. This is consistent with a role for YjeE in respiratory systems where DNA topology is used as a regulator of activity.

An alternative hypothesis is that YjeE is important for the production of critical components of aerobic metabolism. Recently, the function of YadR/ErpA, which is phenotypically similar to YjeE, has been elaborated. This protein is an Fe-S protein that was shown to be a chaperone for IspG by transferring Fe-S clusters to the protein in vitro (Loiseau et al., 2007). IspG is involved in isoprenoid biosynthesis, and an ErpA null is compromised for the formation of the ubiquinone and dimethyl/menaquinone; consequently, an ErpA null is essential under aerobic conditions but dispensable under anaerobic conditions. Such a mechanism—namely, the essential involvement in aerobic metabolism—could explain the anaerobic dispensability of *yjeE*.

The tractability of YjeE as an antimicrobial target is called into question by the finding that *yjeE* is nonessential in anaerobic conditions. The conditional null, however, requires 4 days to produce small colonies under anaerobic conditions in vitro. A *Staphylococcus aureus* deletion mutant of the putative ribosome biogenesis factor *yjeQ* has a slow growth phenotype and is unable to colonize mice in vivo (Campbell et al., 2006). The extreme slow growth phenotype of the *yjeE* null under anaerobic conditions suggests that an inhibitor of yjeE will likely result in attenuated infectiousness of bacteria in vivo, even under anaerobic conditions.

In summary, the promoter proximal to *yjeE* (P_{yjeE}) was shown to be responsive to the depletion of intracellular stores of YjeE. Profiling of the response of this promoter to bioactive molecules revealed that norfloxacin was a potent stimulator of transcription. This chemical-genetic interaction was used to uncover genetic relationships with two-component regulatory systems. Genes involved in respiration and oxidative stress responses were found to positively or negatively influence the stimulation of the *yjeE* promoter by norfloxacin. Taken together, these findings point to a role for YjeE in managing oxidative metabolism. Consistent with this hypothesis, YjeE was found to be dispensable in *E. coli* under anaerobic conditions. To the best of our knowledge, these findings represent the first definitive phenotypes for this mysterious and widely conserved bacterial ATPase.

SIGNIFICANCE

A significant fraction of sequenced genomes contain proteins of unknown function. Many of these proteins are conserved and essential, with high potential to be antimicrobial targets; however, the paucity of functional information hampers their development as such. Further, the essential phenotypes of these proteins provide manifold challenges in studying the effects of depletion. In this work, we have addressed these difficulties for one such protein, *E. coli* YjeE, using chemical probes of biological function. An annotated library of known bioactive molecules was successfully used to probe the promoter of gene *yjeE* and revealed functional relationships among *yjeE* and regulatory genes in *E. coli*. These relationships proved to have predictive power in finding a new phenotype for YjeE, which has provided a glimpse of its cellular function.

EXPERIMENTAL PROCEDURES

All restriction endonucleases and Vent® polymerase were purchased from New England Biolabs, Inc. (Beverly, MA). Oligonucleotides were from MOBIX (Hamilton, Ontario). Viewlux® 96-well plates for luminescence measurements were from Perkin Elmer (Boston, MA). All antibiotics used were purchased from Sigma-Aldrich (Oakville, Ontario).

Construction of pCS26-*bla*

The β-lactamase resistance cassette (*bla*) from pBluescript (Novagen; Madison, WI) was amplified with Vent polymerase using the primers 5′-GGGG GAGCTCATGAGTAAACTTGGTCTGACAGTTACC-3′ and 5′-AAAAGAAGAC GAAAGGGCGTGGCACTTTTCGGGGAAATGTGCGCGGAA CCCC-3′, where the underlined sequences indicate SacI and BbsI sites respectively. The kanamycin resistance cassette was excised from pCS26-pac by digestion with SacI and BbsI, and *bla* was ligated in its place (Bjarnason et al., 2003). The resistance cassette is transcribed in the opposite direction as the adjacent *luxCDABE* operon to ensure that it does not influence expression of the reporter.

Construction of pCS26-*bla*-P_{yjeE} and pCS26-*bla*-P_{yjeF}

The 750 bp regions upstream of the *yjeE* and *yjeF* translational start sites were amplified with Vent® polymerase using the primer pairs 5′-CCCC GGATCCGGTTTTTATCAGTCA CTTCCGG-3′,5′-GGGCTCGAGCGTATGACG GGGGAAGCGGCG-3′ and 5′-GGGGCTCGA GAGCTGGCAGTCATCGCAAC CG-3′,5′-CCCCGGATCCGTCAGAGCCCCTCGATCTCA ATCAGTTAGCG-3′, respectively, where the underlined sequences indicate BamHI and XhoI restriction sites. Each PCR product was digested with BamHI and XhoI and ligated into pCS26-*bla* using standard cloning techniques to yield pCS26-*bla*-P_{yjeE} and pCS26-*bla*-P_{yjeF}.

Promoter Profiling

A saturated culture grown overnight at 37°C was diluted 1:400 into fresh Luria Bertani media and incubated in 96-well Viewlux® plates at 37°C with rotation

at 250 rpm. Cells were treated with compounds from the Prestwick Chemical Library (Prestwick Chemical, Inc.; Washington, DC) dissolved in dimethylsulfoxide (DMSO) to a maximum final concentration of 1% (v/v). Low controls were treated with DMSO alone. The outer wells of the plates were not used to avoid edge effects due to evaporation. Compound addition was carried out by a Biomek FX Liquid Handler (Beckman Coulter; Fullerton, CA) fitted with a 96-channel head. Luminescence was measured in a Perkin Elmer Envision® luminometer with an ultrasensitive luminescence module. Data were plotted using SigmaPlot software (San Jose, CA).

Construction of a *gyrA* Chromosomal Mutant

A Ser83Leu/Asp87Tyr double mutation was introduced into the chromosome of wild-type *E. coli* MG1655 by transforming a 2 kb linear DNA fragment containing the mutations in the center of the sequence. To create this 2 kb product, a crossover PCR strategy was used where primers gyrA-A (5′-GATATGC CGCTCTTTTAAACTGG-3′) and gyrA-B (5′-TGGCTGCGCCATGCGGACGA TCGTGTAATAGACC GCCTGGTCACCATGGGGATGGT ATTTACC-3′) were used to amplify 1 kb upstream of the gyrA mutation site, and primers gyrA-C (5′-GGTAAATACCATCCCCATGGTGACCAGGCGGTCTATTACACGATCGTC CGCAT GGCGCAGCCA-3′) and gyrA-D (5′-CGTCCGGAATGGCTGGAGCCA GAG-3′) amplified 1 kb downstream. Primers gyrA-B and gyrA-C were complementary to each other, and both contained base substitutions to introduce the mutations (underlined in sequence). Primers gyrA-A and gyrA-D were used to join these two overlapping 1 kb products by crossover PCR. The 2 kb product was purified and transformed into electrocompetent *E. coli* MG1655 cells. Colonies containing the chromosomal mutations were selected on 20 μg/ml of nalidixic acid.

Deletions of Two-Component Systems

All single deletions were obtained from the Keio deletion collection (Baba et al., 2006). Only deletions of the transcriptional activator of each two-component system, as identified by Oshima et al. (2002), were tested. Where the transcriptional activator is not known, the deletion of the sensor histidine kinase was used instead.

Growth under Anaerobic Conditions

All strains were picked from a single colony and grown overnight in LB media supplemented with 50 μg/ml kanamycin. Cultures were diluted 1:400 into fresh LB and grown to an OD_{600} ~0.3 before diluting again to a final OD_{600} of 0.1. Cultures were then subjected to 10-fold serial dilutions, and 1 μl of each dilution was spotted onto LB agar (1.5% w/v) supplemented with 50 μg/ml kanamycin. Plates were grown under anaerobic conditions using the GasPak™ EZ Anaerobe Container System (Beckton Dickenson; Oakville, Ontario) according to the manufacturer's instructions for 4 days at 37°C.

SUPPLEMENTAL DATA

Supplemental Data include one figure and one table and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(08)00410-9.

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