Antitoxin DinJ influences the general stress response through transcript stabilizer CspE

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Summary
Antitoxins are becoming recognized as proteins that regulate more than their own synthesis; for example, we found previously that antitoxin MqsA of the Escherichia coli toxin/antitoxin (TA) pair MqsR/MqsA directly represses the gene encoding the stationary-phase sigma factor RpoS. Here, we investigated the physiological role of antitoxin DinJ of the YafQ/DinJ TA pair and found DinJ also affects the general stress response by decreasing RpoS levels. Corroborating the reduced RpoS levels upon producing DinJ, the RpoS-regulated phenotypes of catalase activity, cell adhesins and cyclic diguanylate decreased while swimming increased. Using a transcriptome search and DNA-binding assays, we determined that the mechanism by which DinJ reduces RpoS is by repressing cspE at the LexA palindrome; cold-shock protein CspE enhances translation of rpoS mRNA. Inactivation of CspE abolishes the ability of DinJ to influence RpoS. Hence, DinJ influences the general stress response indirectly by regulating cspE.

Introduction
The role of toxin–antitoxin (TA) pairs in bacterial physiology is becoming more clear as antitoxin MqsA actively participates in the general stress response of Escherichia coli by regulating more than its own promoter (Wang et al., 2011). MqsA helps mediate the general stress response via its repression of rpoS, which encodes the stationary-phase sigma factor RpoS; RpoS is the master regulator of the stress response (Pesavento et al., 2008) and controls 500 genes in E. coli (Hengge, 2008). By repressing rpoS, MqsA reduces the concentration of the internal messenger cyclic diguanylate (c-di-GMP), thereby increasing motility and decreasing biofilm formation and catalase activity (Wang et al., 2011). Upon oxidative stress, MqsA is rapidly degraded by Lon protease resulting in induction of rpoS, which in turn increases c-di-GMP, inhibits motility and increases cell adhesion and biofilm formation. Therefore, TA systems have an important impact on cell physiology by influencing such developmental cascades as the switch from planktonic cells to biofilm cells (Wang and Wood, 2011).

Thirty-seven chromosomal TA systems have been characterized in E. coli so far (Tan et al., 2011) and the YafQ/DinJ TA system was initially characterized by Motiejunaite and colleagues (2005). YafQ/DinJ are grouped in the RelE/RelB TA family (Gotfredsen and Gerdes, 1998), and the growth inhibition of toxin YafQ is counteracted by DinJ (Motiejunaite et al., 2005). YafQ is an endoribonuclease that associates with the ribosome through the 50S subunit and blocks translation elongation through mRNA cleavage at 5′-AAA-G/A-3′ sequences (Prysak et al., 2009). The gene that encodes the antitoxin, dinJ (damage inducible gene) was predicted to be regulated by the LexA repressor (Lewis et al., 1994); LexA acts as a transcriptional repressor of SOS-regulated genes but is inactivated in response to DNA damage resulting in their induction (Butala et al., 2009). DinJ is not well-characterized; for example, there is no structural and little physiological information about it, however DinJ is more stable in the absence of the Lon and ClpXP proteases (Prysak et al., 2009).

Palindromes are often present at TA promoters and are sites of antitoxin binding to confer auto-regulation (Gerdes et al., 2005). YafQ and DinJ form a stable complex (Motiejunaite et al., 2007), which binds the dinJ-yafQ palindromic sequence (5′-CTGAAATAATACAG-3′, −16 to −33 from the translation start site) (Prysak et al., 2009) which overlaps the consensus LexA binding site (5′-TACTG(TA)6CAGTA-3′) (Fernández De Henestrosa et al., 2000), suggesting that this module is regulated by DNA damage (Prysak et al., 2009). Although this is the only TA pair whose palindrome shares homology with the LexA binding site, the physiological role of this TA system in the general stress response has not been characterized.

The cold-shock proteins (Csp) of the CspA protein family consist of nine homologous proteins (CspA to CspI) that help the cell acclimate to low temperature conditions (Bae et al., 1999). However, CspE functions both at physiological temperatures and during cold shock (Phadtare et al., 2011).
et al., 2006). CspE is a single stranded nucleic acid-binding protein (Phadtare and Inouye, 1999) that plays a role in chromosome condensation by binding to distant DNA regions containing contiguous deoxythymine residues and dimerizing, thereby condensing the intervening DNA regions containing contiguous deoxythymine residues (Johnston et al., 2006). In addition, production of CspE increases RpoS through rpoS message stabilization (Phadtare et al., 2002; Phadtare et al., 2006). Therefore, CspE is involved in the regulation of RpoS, the global stress response regulator, as part of the complex stress response network of E. coli (Phadtare and Inouye, 2001; Phadtare et al., 2002).

In the present study, given that the palindrome recognized by DinJ is related to LexA and that antitoxin MqsA influences RpoS (Wang et al., 2011), we investigated whether antitoxin DinJ also influences the stress response. By using a genetic background devoid of the major E. coli TA pairs (the Δ6 strain of Wang et al., 2011), which lacks the MqsR/MqsA, MazF/MazE, RelE/RelB, ChpB, YoeB/YefM and YafQ/DinJ TA systems (Table 1), interpretation of the results was simplified. We find that the antitoxin DinJ reduces RpoS at the level of translation by repressing cspE, which encodes cold-shock protein CspE that facilitates the translation of rpoS mRNA. Hence, DinJ influences the general stress response indirectly by regulating cspE.

Results

DinJ reduces RpoS

Since antitoxin MqsA was shown to repress rpoS (Wang et al., 2011), we investigated the possible role of antitoxin DinJ in rpoS transcription and RpoS protein levels to see if this antitoxin also impacted the general stress response. We utilized strain Δ6 that is deleted for six sets of TA systems; hence, DinJ could be studied in a background without the best-studied TA systems. Since the effects of antitoxins on gene regulation should only be important during stress when proteases degrade the antitoxin and derepress genes (Wang et al., 2011; Wang and Wood, 2011), we investigated several stresses to identify a condition where DinJ was labile. Using heat, acid, oxidative and antibiotic stresses (erythromycin, gentamicin, mitomycin C, ampicillin, tetracycline and nalidixic acid), we determined that DinJ was degraded in the presence of erythromycin (75 μg ml⁻¹ for 10 min) with a half life of less than 2 min (Fig. 1A). In contrast, DinJ was stable in the presence of the other stresses with half lives greater than 15 min (hydrogen peroxide result shown in Fig. 1B). In addition, production of DinJ decreased cell viability by 220 ± 10-fold with erythromycin (Fig. 1C). Erythromycin, a macrolide antibiotic produced by Streptomyces erythreus (Weber et al., 1985), inhibits protein synthesis by binding to the 50S ribosomal subunit and inducing the dissociation of peptidyl-tRNAs from the ribosomes after the initiation of mRNA translation (Tenson et al., 2003). Hence, this condition was chosen to determine the impact of DinJ on cell physiology. In addition, DinJ also reduced cell viability under oxidative stress (20 mM H₂O₂ for 10 min) by 125 ± 20-fold (Fig. 1C). Therefore, DinJ reduces the ability of the cells to respond to erythromycin and oxidative stress, and DinJ is degraded during erythromycin stress.

Using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) to investigate whether DinJ affects rpoS, we applied erythromycin stress and found rpoS was repressed slightly (2.6 ± 0.2-fold). More significantly, production of DinJ dramatically decreased RpoS levels (Fig. 1D). Therefore, DinJ changes primarily RpoS levels in the cell, and the reduction in viability seen upon adding erythromycin is likely the result of reducing RpoS levels.
DinJ reduces cyclic diguanylate thereby increasing swimming and decreasing cell adhesins and catalase activity

To confirm that DinJ reduces RpoS, we investigated the phenotypes related to RpoS activity. Since RpoS is a positive regulator of diguanylate cyclases (Landini, 2009), it was expected that c-di-GMP concentrations should decrease in the presence of DinJ. As expected, upon overexpressing dinJ from a plasmid in stationary-phase cells, the intracellular c-di-GMP concentration decreased by 1.7 ± 0.2-fold (Fig. 2A). The positive control MqsA (Wang et al., 2011) also reduced c-di-GMP by 2.4 ± 0.3-fold (Fig. 2A). Therefore, DinJ reduces c-di-GMP concentrations.

Since c-di-GMP levels are reduced in the presence of antitoxin DinJ, motility should increase due to the lower RpoS levels since RpoS inhibits flhD, the master regulator of motility (Pesavento et al., 2008). As expected, production of DinJ in Δ6 increased motility by 1.7 ± 0.1-fold (Fig. 2B). The positive control MqsA also increased motility by 2.5 ± 0.1-fold (Fig. 2B). Moreover, producing Lon and DinJ simultaneously abolished the ability of DinJ to increase motility (Fig. 2C). Therefore, Lon degrades DinJ as it does for several antitoxins (Christensen et al., 2001; Wilbaux et al., 2007; Jorgensen et al., 2009; Kim et al., 2010; Wang et al., 2011), and DinJ increases swimming motility.

Given that RpoS levels are reduced, both curli and cellulose production should likewise be decreased since RpoS is a positive regulator of csgD (Pesavento et al., 2008). Using Congo red, a dye that binds to both cellulos and curli (Ma and Wood, 2009), we found that producing DinJ decreased curli/cellulose production by 1.7 ± 0.2-fold in the presence of erythromycin stress (75 μg ml⁻¹ for 3 h) when compared with an empty plasmid. Similar to the results under erythromycin stress, producing DinJ in the presence of oxidative stress decreased curli/cellulose production by 4 ± 1-fold (2 mM H₂O₂ for 180 min at 30°C) (Fig. 2D). We used oxidative stress along with erythromycin since RpoS is crucial for resistance to oxidative stress (Sammartano et al., 1986; Hengge-Aronis, 2002) and since it regulates the antioxidant activities of catalase and superoxide dismutase (Lacour and Landini, 2004). The positive control MqsA also decreased curli/cellulose by 8 ± 2-fold (Fig. 2D). Therefore, DinJ decreases adhesin formation.

Given that RpoS levels are reduced, catalase activity should be decreased when DinJ is overproduced since RpoS is a positive regulator of catalase activity. To confirm this, we checked catalase activity by both a colorimetric assay with lactoperoxidase and dicarboxidine (Macvanin and Hughes, 2010) and a bubble formation assay (Wang et al., 2011). As expected, overproduction of DinJ in MG1655 Δ6 decreased the catalase activity by a 13 ± 3-fold compared with the empty plasmid control (only trace catalase activity was seen in the DinJ-producing strain). Catalase converts H₂O₂ to H₂O and O₂; hence, the reduced ability of the cells to decompose H₂O₂ when DinJ reduces RpoS was demonstrated by a dramatic reduction in oxygen bubbles upon addition of H₂O₂ to Δ6 (Fig. 2E). Therefore, DinJ decreases catalase activity.

DinJ represses cspE

To determine the mechanism by which DinJ reduces RpoS levels, we investigated which genes were...
differentially expressed during production of DinJ using a whole-transcriptome analysis. Exposure to erythromycin (75 \( \mu \text{g ml}^{-1} \) for 10 min) significantly altered the expression of 925 genes as compared with the untreated control based on a cut-off ratio of 2.5 (Table 2). Of these, 822 genes were induced, while 103 genes were repressed. Consistent with the qRT-PCR results, rpoS was not changed upon production of DinJ (1.1-fold). Critically, some stress-related genes were significantly repressed by DinJ including the gene for the DNA-binding transcriptional repressor cspE (3.5-fold) and cspC (2.3-fold). Given that CspE is a positive regulator for RpoS and that the promoter of cspE contains a LexA/DinJ-like palindrome, we focused on it as a possible negative regulator of RpoS. To confirm that DinJ represses the transcription of cspE, we quantified the transcript levels of cspE using qRT-PCR for \( \Delta \delta / \text{pCA24N-dinJ} \) versus \( \Delta \delta / \text{pCA24N} \) after 180 min at 30°C with oxidative stress (2 mM H\(_2\)O\(_2\)). Error bars indicate standard error of mean (\( n = 2 \)). Significant changes are marked with an asterisk for \( P < 0.05 \).

DinJ binds the cspE promoter

To determine if antitoxin DinJ directly controls the transcription of cspE by binding, an electrophoretic mobility shift assay (EMSA) was used for the 35 bp (from –87 to –53) and 176 bp fragments of PcspE (from –123 to +53), both of which contain the consensus LexA binding sequence (5′-TACTG(TG)\(_5\)CAGTA-3′) (Fernández De Henestrosa et al., 2000) as shown in Fig. S1. EMSA revealed that DinJ binds both PcspE fragments (Fig. 3A and B). In contrast, for the negative control, at the same concentrations, there were no shifts for DinJ for the promoter of the negative control gadA (Fig. 3C) and also no shift for the promoter of rosE (Fig. 3D, promoter sequence shown in Fig. S2). Also, antitoxin MqsA failed to bind PcspE (Fig. 3E). To confirm that DinJ binds PcspE, we
DinJ regulates the general stress response

To confirm that DinJ controls indirectly the above phenotypes related to RpoS (resistance to erythromycin and hydrogen peroxide, cyclic diguanylate levels, swimming, cell adhesins and catalase activity) via its repression of cspE, we investigated whether DinJ was effective in the absence of CspE; i.e. we reasoned that deleting cspE would prevent DinJ from reducing RpoS levels and affecting these phenotypes. Using BW25113ΔcspE/pCA24N-dinJ versus BW25113/pCA24N-dinJ, as expected, producing DinJ reduced RpoS but only in the presence of CspE (Fig. 4A). Thus, CspE is required for DinJ to regulate RpoS protein levels.

Also as expected, we found that deleting cspE caused a 24 ± 2-fold increase in cell survival when DinJ was produced from pCA24N-dinJ under erythromycin stress compared with the wild-type strain (Fig. 4B). Similarly, deleting cspE also increased cell survival 10.2 ± 0.6-fold under oxidative stress (Fig. 4B). Hence, DinJ is not able to reduce RpoS levels without CspE so the cells survive better under stress without CspE. Also, deleting cspE abolished the increase in motility from DinJ (Fig. 4C). Furthermore, deleting cspE also prevented DinJ from reducing curli/cellulose under both erythromycin stress (2.6 ± 0.1-fold increase for cspE) and oxidative stress (2.1 ± 0.1-fold increase for cspE) (Fig. 4E). In addition, deleting cspE prevented DinJ from reducing catalase activity (3.73 ± 0.5-fold increase for cspE) and this is also illustrated by the larger bubble formation for the cspE strain (Fig. 4D). Together, these four sets of results convincingly show that CspE is crucial for DinJ to control phenotypes related to RpoS under the general stress response (cell survival with erythromycin and hydrogen peroxide, motility, production of adhesins and catalase activity).

Discussion

Previously, a single antitoxin, MqsA, was shown to regulate more than its own synthesis by regulating the general stress response through direct repression of rpoS via its mqsRA-like palindrome (Wang et al., 2011). As a result of this repression of rpoS, the concentration of the secondary messenger c-di-GMP is decreased, which results in

Table 2. Partial list of differentially expressed genes for Δ6/pCA24N-dinJ versus Δ6/pCA24N.

<table>
<thead>
<tr>
<th>Group and gene name</th>
<th>b number</th>
<th>Fold change</th>
<th>Description of encoded protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>osmB</td>
<td>b1283</td>
<td>−3.0</td>
<td>Osmotically and stress inducible lipoprotein</td>
</tr>
<tr>
<td>cspE</td>
<td>b0623</td>
<td>−3.5</td>
<td>DNA-binding transcriptional repressor, cold-shock protein</td>
</tr>
<tr>
<td>umuC</td>
<td>b1184</td>
<td>3.7</td>
<td>SOS mutagenesis and repair, DNA polymerase V, subunit D</td>
</tr>
<tr>
<td>umuD</td>
<td>b1183</td>
<td>4.0</td>
<td>DNA polymerase V subunit, error-prone repair</td>
</tr>
<tr>
<td>ybeS</td>
<td>b0646</td>
<td>3.0</td>
<td>Protein folding, heat shock protein binding</td>
</tr>
<tr>
<td>ygeS</td>
<td>b2851</td>
<td>3.0</td>
<td>Response to stress</td>
</tr>
<tr>
<td>Cell wall, membrane and motility/chemotaxis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>csgB</td>
<td>b1041</td>
<td>2.5</td>
<td>Currin nucleoprotein, homology with major currin, CsgA</td>
</tr>
<tr>
<td>eaeH</td>
<td>b0297</td>
<td>2.5</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>fliC</td>
<td>b1923</td>
<td>−6.1</td>
<td>Flagellar filament structural protein (flagellin)</td>
</tr>
<tr>
<td>fliK</td>
<td>b1943</td>
<td>3.2</td>
<td>Flagellar hook-length control protein</td>
</tr>
<tr>
<td>yaiC</td>
<td>b0385</td>
<td>2.8</td>
<td>Cellulose, biofilm, motility regulator, diguanylate cyclase; CsgD-regulated</td>
</tr>
<tr>
<td>yehD</td>
<td>b2111</td>
<td>2.6</td>
<td>Predicted fimbral-like adhesin protein</td>
</tr>
<tr>
<td>yfIP</td>
<td>b2632</td>
<td>3.0</td>
<td>Required for swarming phenotype</td>
</tr>
<tr>
<td>DNA replication, recombination and repair</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oraA</td>
<td>b2698</td>
<td>3.0</td>
<td>Blocks RecA filament extension: inhibitor of RecA ATPase, reduced resistance to DNA damage</td>
</tr>
<tr>
<td>recN</td>
<td>b2616</td>
<td>4.6</td>
<td>DNA Recombination and repair</td>
</tr>
<tr>
<td>rusA</td>
<td>b0550</td>
<td>2.6</td>
<td>DNA repair Endonuclease</td>
</tr>
<tr>
<td>yeeS</td>
<td>b2002</td>
<td>2.6</td>
<td>CP4-44 prophage; predicted DNA repair protein</td>
</tr>
<tr>
<td>Transport</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fruB</td>
<td>b2169</td>
<td>9.8</td>
<td>Fructosephosphotransferase enzyme III</td>
</tr>
<tr>
<td>yddl</td>
<td>b1472</td>
<td>4.0</td>
<td>Transport, OmpCFN porin family, N-terminal fragment</td>
</tr>
<tr>
<td>Metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fruK</td>
<td>b2168</td>
<td>4.0</td>
<td>Fructose-1-phosphate kinase; 1-phosphofructokinase</td>
</tr>
<tr>
<td>ybdK</td>
<td>b0581</td>
<td>4.3</td>
<td>Glutathione biosynthesis, cysteine ligase activity</td>
</tr>
</tbody>
</table>

Cells at 37°C had DinJ produced for 2 h via 1 mM IPTG then were adjusted to a turbidity of 1.0 and were exposed to erythromycin stress for 10 min in LB low-salt medium. The cut-off ratio was 2.5-fold.

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increased motility, decreased production of adhesins, reduced biofilm formation and reduced catalase activity (Wang et al., 2011). Here, our results show that DinJ regulates cspE, demonstrating that regulation of other genes may be a general feature of antitoxins. In addition, the results demonstrate that antitoxin DinJ influences RpoS activity via a novel, indirect mechanism: DinJ represses cspE which encodes the cold-shock protein CspE that induces translation of rpoS mRNA (Phadtare et al., 2002; Phadtare et al., 2006).

RpoS regulation is complex and includes regulation at the level of transcription, translation, protein stability and activity (Hengge, 2008). Positive regulators of rpoS translation include the cold-shock proteins CspC and CspE (Phadtare and Inouye, 2001), RNA binding protein Hfq (Soper et al., 2010), nucleoid protein HU (Balandina et al., 2001), and some small regulatory RNAs (DsrA, RprA and ArcZ) (Soper et al., 2010). Critically, our transcriptome study identified that DinJ represses cspE (Table 2) (these data were verified by qRT-PCR), and we showed that DinJ binds the promoter region of cspE at the LexA/CspE binding site (5'-CTGGATGGCGCTTTCCAG-3') to repress cspE. Mutagenesis of the LexA/CspE binding site and the lack of binding of DinJ to the mutated promoter confirmed that DinJ represses cspE.

Further evidence for DinJ repressing cspE was provided by the dependence on CspE for the effect of DinJ on RpoS-related phenotypes (resistance to erythromycin and hydrogen peroxide, swimming, cell adhesins and catalase activity). A schematic of our current understanding of how antitoxin DinJ mediates the general stress response is shown in Fig. 5.

Since our results show clearly that DinJ is degraded under erythromycin stress (Fig. 1A) but not under heat, acid, oxidative, gentamicin, mitomycin C, ampicillin, tetracycline and nalidixic acid stress, we have determined the first conditions that lead to the degradation of DinJ and have shown that Lon is required for this degradation (Fig. 2C). We note however that we do not fully understand the relevance of erythromycin stress for DinJ degradation. Consistent with our results indicating DinJ is degraded by Lon, Prysak and colleagues (2009) also showed that Lon protease should be involved in the degradation of DinJ. This degradation of DinJ should lead to
derepression of \textit{cspE} transcription, which should result in an increase in RpoS. Hence, upon erythromycin stress, Lon is induced and degrades DinJ like other antitoxins, \textit{cspE} is derepressed, RpoS levels increase, and the cell directs transcription towards stress-related genes, which includes increasing c-di-GMP concentrations and catalase activity.

In summary, our current results indicate that DinJ is involved in mediating the general stress response by indirect regulation of RpoS via its direct control of \textit{cspE}. The results provide additional proof that the ubiquitous TA systems are far more than genomic debris. Furthermore, they provide insights into how antitoxins allow the cell to respond to stress (it is not well understood how external stress is mediated to the inside of the cell). Also, the results suggest new methods for controlling cell behaviour such as persistence and antibiotic resistance since if antitoxins could be made to more readily bind their targets, then the cell would be less able to respond to stress (i.e. antibiotics) and less able to become dormant through the action of toxins.

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**Fig. 4.** DinJ requires CspE to influence RpoS levels and RpoS-controlled viability, motility, cell adhesins and catalase activity. A. Lanes 2, 3 and 4 show RpoS levels as detected by an anti-RpoS antibody for BW25113/pCA24N, BW25113/pCA24N-\textit{dinJ} and BW25113 \textit{cspEpCA24N-dinJ}. Two independent cultures were used for each strain and both experiments are shown. B. Percentage of cells (BW25113 \textit{cspEpCA24N-dinJ} versus BW25113/pCA24N-\textit{dinJ}) which survive erythromycin (75 \text{ mg}\text{ ml}^{-1}) stress for 10 min and oxidative stress induced by 20 mM H$_2$O$_2$ for 10 min. Error bars indicate standard error of mean (n = 2). Significant changes are marked with an asterisk for \textit{P} < 0.05. C. Swimming motility after 12 h of growth at 37°C for BW25113 \textit{cspEpCA24N-dinJ} versus BW25113/pCA24N-\textit{dinJ}. Two independent cultures were used, and a representative image is shown. D. Images of BW25113 \textit{cspEpCA24N-dinJ} versus BW25113/pCA24N-\textit{dinJ} cultures (turbidity of 1) 10 min after adding 40 mM H$_2$O$_2$. Bubbles are oxygen produced by the decomposition of hydrogen peroxide by catalase (2 H$_2$O$_2$ \rightarrow 2 H$_2$O + O$_2$). Two independent cultures were used, and a representative image is shown. E. Curli/cellulose production for strains BW25113 \textit{cspEpCA24N-dinJ} versus BW25113/pCA24N-\textit{dinJ} after 3 h of incubation with 7.5 \text{ mg}\text{ ml}^{-1} erythromycin or 2 mM H$_2$O$_2$. All assays were performed at 30°C. Error bars indicate standard error of mean (n = 2). Significant changes are marked with an asterisk for \textit{P} < 0.05.

**Fig. 5.** Schematic of how DinJ impacts the general stress response. RpoS levels are reduced indirectly by DinJ through its control of \textit{cspE}. Under erythromycin stress, DinJ is degraded by protease Lon, and \textit{cspE} transcription is derepressed. The increased CspE levels lead to greater RpoS translation, which increases c-di-GMP concentrations, catalase activity and curli/cellulose production and decreases swimming. The lightning bolt indicates erythromycin stress, ‘→’ indicates induction and ‘\textdownarrow’ indicates repression.
Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids are listed in Table 1. Luria–Bertani (LB) (Sambrook et al., 1989) at 37°C was used for all the experiments unless noted. For construction of pBS(Kan)-dinJ, dinJ was PCR-amplified from E. coli MG1655 chromosomal DNA as a template using front primer dinJ-KpnI-F (primers are shown in Table S1), and rear primer dinJ-Sacl-R. The PCR product was cloned into the multiple cloning site of pBS(Kan) (Canada et al., 2002) after double digestion with KpnI and SaccI restriction enzymes. The dinJ gene in pBS(Kan) is under the control of a lac promoter. The pBS(Kan)-dinJ plasmid was confirmed by DNA sequencing with pBS(Kan)-seq primer. Cell growth was assayed using the turbidity at 600 nm for shake flasks. Kanamycin with pBS(Kan)-lac dinJ KpnI-F (primers are shown in Table S1), and rear primer dinJ-Sacl-R. The PCR product was cloned into the multiple cloning site of pBS(Kan) (Canada et al., 2002) after double digestion with KpnI and SaccI restriction enzymes. The dinJ gene in pBS(Kan) is under the control of a lac promoter. The pBS(Kan)-dinJ plasmid was confirmed by DNA sequencing with pBS(Kan)-seq primer. Cell growth was assayed using the turbidity at 600 nm for shake flasks. Kanamycin (50 μg ml⁻¹) and chloramphenicol (30 μg ml⁻¹) were used to maintain the pBS(Kan)-based and pCA24N-based plasmids (Kitagawa et al., 2005).

Survival assays

 Overnight cultures were diluted to a turbidity of 0.05 and grown in LB low-salt medium (0.05% NaCl, 0.1% tryptone and 0.5% yeast extract) to a turbidity of 0.5, then 1 mM IPTG was used to induce dinJ for 2 h (low-salt medium was used to avoid osmotic stress). Cells were centrifuged and resuspended in LB to a turbidity of 1.0 and exposed to either erythromycin (75 μg ml⁻¹) for 10 min or 20 mM H₂O₂ for 10 min (Wang et al., 2011). To investigate how DinJ affects cell survival, Δ6/pCA24N-dinJ and Δ6/pCA24N were used. To investigate how DinJ affects cell survival in the absence of CspE, BW25113 cspEΔpCA24N-dinJ and BW25113/pCA24N-dinJ were used.

c-di-GMP assay

c-di-GMP was quantified using HPLC as described previously (Ueda and Wood, 2009). ΔΔ6/pCA24N-dinJ, Δ6/pCA24N and ΔΔ6/pCA24N-mqsA (positive control) were grown in 1 L medium for 2.5 h, then 0.5 mM IPTG was added to induce dinJ or mqsA for 15 h. A photodiode array detector (Waters, Milford, MA, USA) was used to detect nucleotides at 254 nm after the HPLC separation step. Synthetic c-di-GMP (BIOLOG Life Science Institute) was used as a standard and to verify the c-di-GMP peak via spiking. This experiment was performed with two independent cultures.

Swimming motility, curli/cellulose and catalase assays

Cell motility was examined on motility agar plates (1% tryptone, 0.25% NaCl and 0.3% agar) (Sperandio et al., 2002). Curli/cellulose production was quantified by the Congo red binding assay (Ma and Wood, 2009) performed at 30°C in the presence of erythromycin stress (7.5 μg ml⁻¹ erythromycin for 180 min) or oxidative stress (2 mM H₂O₂, for 180 min). IPTG (1 mM) was added in both assays to induce dinJ via the pCA24N-based plasmids. Catalase activity was quantified by a colorimetric assay using dicarboxidine/ lactoperoxidase to detect the remaining H₂O₂ (Macvanin and Hughes, 2010). Catalase activity was also tested by a bubble formation assay as described previously (Wang et al., 2011). Bubbles are oxygen produced by the decomposition of hydrogen peroxide by catalase (2 H₂O₂ → 2 H₂O + O₂).

RNA isolation and whole-transcriptome analysis

Whole-transcriptome analysis was performed using plasmidic cells of Δ6/pCA24N-dinJ versus ΔΔ6/pCA24N with 1 mM IPTG added at a turbidity of 0.5 for 2 h, then the cells were exposed to erythromycin (75 μg ml⁻¹) for 10 min and harvested as quickly as possible to avoid mRNA degradation. Total RNA was isolated from cells as described previously (Ren et al., 2004a) with a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) using a bead beater (Biospec, Bartlesville, OK, USA) and RNA later buffer (Applied Biosystems, Foster City, CA, USA) to stabilize the RNA. cDNA synthesis, fragmentation, hybridizations and data analysis were as described previously (Gonzalez Barrios et al., 2006). The E. coli GeneChip Genome 2.0 array (Affymetrix, Santa Clara, CA, USA; P/N 900551) was used, and if the gene with the larger transcription rate did not have a consistent transcription rate based on the 11 probe pairs (P < 0.05), these genes were not used. A gene was considered differentially expressed when the P-value for comparing two chips was < 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%). Since the standard deviation for expression ratio for all the genes was 2.0, genes were considered differentially expressed if they had greater than 2.5-fold changes for condition (Ren et al., 2004b). Gene functions were obtained from the Ecogene database (http://www.ecogene.org/). The microarray raw data are deposited at the Gene Expression Omnibus of the National Center for Biotechnology Information (GSE30692).

qRT-PCR

After isolating RNA using RNA later (Ambion), 50 ng of total RNA was used for qRT-PCR using the Power SYBR Green RNA-to-Ct 1-Step Kit and the StepOne Real-Time PCR System (Applied Biosystems). Primers were designed using Primer3 Input Software (v0.4.0) and are listed in Table S2. The housekeeping gene rrsG was used to normalize the gene expression data. The annealed temperature was 60°C for all the genes in this study. To investigate the rpoS and cspE mRNA changes by DinJ under erythromycin stress conditions, overnight cultures of Δ6/pCA24N-dinJ and ΔΔ6/pCA24N were inoculated into LB low-salt medium (0.05% NaCl) with an initial turbidity of 0.2 and grown to a turbidity of 0.5, then 1 mM IPTG was added for 2 h to induce dinJ until a turbidity ↓ 3.0. After diluting to a turbidity ↓ 1.0, cells were exposed to erythromycin (75 μg ml⁻¹) for 10 min.

Purification of DinJ

DinJ was produced in Δ6/pCA24N-dinJ via 1 mM IPTG at room temperature overnight. DinJ was purified using a Ni-NTA resin (Qiagen, Valencia, CA, USA) as described in the
When the turbidity reached 1, 200 μm exposed to various stress conditions including 75 μM IPTG added to inhibit transcription, and the cell pellets were washed with 1 mM EDTA, 5 mM CaCl₂, 100 mM NaCl, 1 mM DTT, 5% glycerol and 0.1 mg ml⁻¹ BSA. Samples were run on a 6% PAGE gel (Invitrogen) at 100 V in 0.5 M urea for 70 min at 4°C. The bound protein/DNA mixtures were then UV cross-linked at 302 nm. Chemiluminescence was performed as described previously (Prysak et al., 2009). To investigate DinJ on RpoS levels, strains were grown until a turbidity ~3.0 and then with a 1:20 000 dilution of horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Millipore). To ascertain RpoS levels, strains were grown until a turbidity ~3.0 and 1.200 dilution of horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Millipore). To ascertain how DinJ affects RpoS levels, ΔdinJ/pCA24N-dinJ and ΔdinJ/pCA24N were used. To investigate how DinJ affects RpoS levels in the absence of CspE, BW25113/cspE/pCA24N-dinJ, BW25113/pCA24N-dinJ and BW25113/pCA24N were used.

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References


Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The cspE promoter region. Sequences 500 bp upstream of the translation start of cspE are indicated in blue font. The Shine-Dalgarno sequence is in red, and the boxes indicate the −35 and −10 promoter regions. The region 1 used for EMSA amplified by primer PcspE (EMSA) is underlined (176 bp, from position −123 to +53). The region 2 used for EMSA was amplified with primer PcspE (EMSA)-35mer and is indicated in lowercase (35 bp, from position −87 to −53). The consensus LexA binding site (5′-TACTG(TA)5CAGTA-3′), which also is the DinJ binding site, is highlighted in green with the spacer indicated in yellow. The mutated four nucleotides in the palindrome are highlighted in red.

Fig. S2. The rpoS promoter region. Sequences 943 bp upstream of the translation start of rpoS are indicated in blue font. The Shine-Dalgarno sequence is in red, and boxes indicate the −35 and −10 promoter regions. The region used for EMSA amplified by primer PrpoS (EMSA) is underlined (185 bp, from position −168 to +17).

Table S1. Oligonucleotides (listed 5′ to 3′) used in this study for qRT-PCR, EMSA, cloning (restriction sites are underlined) and site directed mutagenesis (target mutated nucleotides are boxed). ‘f’ indicates forward primer and ‘r’ indicates reverse primer. ‘bio’ indicates that the primer was synthesized with biotin at the 3′ end.

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