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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 Motiejūnaite 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 (Motiejūnaite *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 (Motiejūnaite *et al.*, 2007), which binds the *dinJ-yafQ* palindrome (5′-CTGAATAAATATACAG-3′, –16 to –33 from the translation start site) (Prysak *et al.*, 2009) which overlaps the consensus LexA binding site (5′-TACTG$(TA)_5$CAGTA-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

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Table 1. *Escherichia coli* bacterial strains and plasmids used in this study.

Strains	Genotype	Source
MG1655	F^- λ^- *ilvG rfb*-50 *rph*-1	Blattner *et al.* (1997)
Δ6	MG1655 *ΔmazEF ΔrelBEF ΔchpB ΔyefM-yoeB ΔdinJ-yafQ ΔmqsRA* Ω Km^R	Wang *et al.* (2011)
Δ6 ΔKm^R	Δ6 ΔKm^R	Wang *et al.* (2011)
BW25113	*$lacI^q$ $rrnB_{T14}$ Δ$lacZ_{WJ16}$ hsdR514 Δ$araBAD_{AH33}$ Δ$rhaBAD_{LD78}$*	Datsenko and Wanner (2000)
BW25113 *rpoS*	BW25113 Δ*rpoS* Ω Km^R	Baba *et al.* (2006)
BW25113 *cspE*	BW25113 Δ*cspE* Ω Km^R	Baba *et al.* (2006)
DH5α	*luxS supE44 ΔlacU169*(*Δ80 lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Ren *et al.* (2004c)
Plasmids		
pCA24N	Cm^R; *$lacI^q$*, pCA24N	Kitagawa *et al.* (2005)
pCA24N-*dinJ*	Cm^R; *$lacI^q$*, pCA24N $P_{T5\text{-}lac}$::*$dinJ^+$*	Kitagawa *et al.* (2005)
pCA24N-*mqsA*	Cm^R; *$lacI^q$*, pCA24N $P_{T5\text{-}lac}$::*$mqsA^+$*	Kitagawa *et al.* (2005)
pCA24N-*lon*	Cm^R; *$lacI^q$*, pCA24N $P_{T5\text{-}lac}$::*lon^+*	Kitagawa *et al.* (2005)
pBS(Kan)	Km^R; pBS(Kan)	Canada *et al.* (2002)
pBS(Kan)-*dinJ*	Km^R; pBS(Kan)P_{lac}::*$dinJ^+$*	this study

Note that the Δ6 strains used in this study lack an insertion sequence in the promoter of *flhD* that gives the parent strain MG1655 artificially high motility. Cm^R and Km^R are chloramphenicol and kanamycin resistance respectively.

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 (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 on *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 H_2O_2 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.

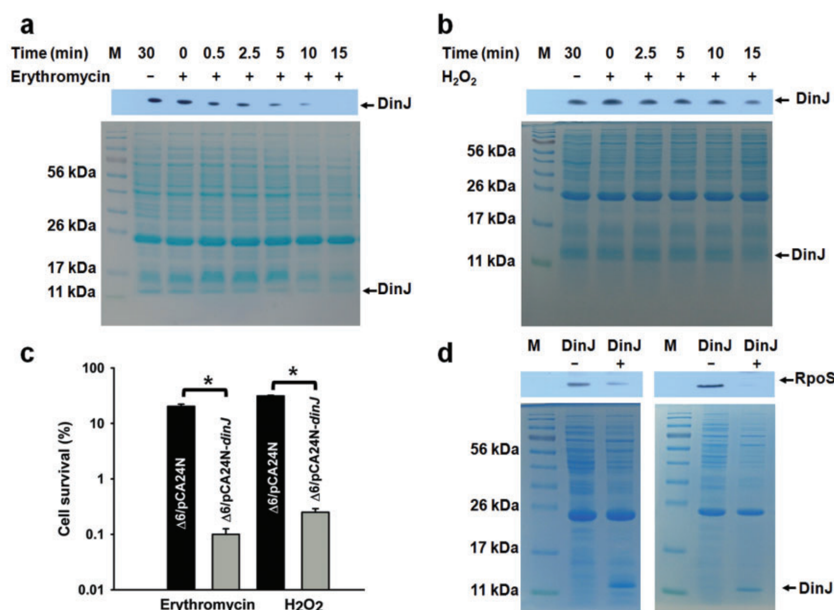

Fig. 1. DinJ is degraded during erythromycin stress and affects cell viability and RpoS levels. Lanes 3–8 in the upper panel (Western) show DinJ levels as detected by a His-tag antibody at different time points with 75 μg ml^{-1} erythromycin (A) and 20 mM H_2O_2 (B) stress for Δ6/pCA24N-*dinJ*. For both (A) and (B), the corresponding whole cell lysates were visualized by SDS-PAGE (lower panels). *dinJ* was induced from pCA24N-*dinJ* via 1 mM IPTG. Two independent cultures were used for each strain and one representative data set is shown here.
C. Percentage of cells which survive erythromycin (75 μg ml^{-1}) stress for 10 min or oxidative stress induced by 20 mM H_2O_2 for 10 min. Error bars indicate standard error of mean ($n = 2$). Significant changes are marked with an asterisk for $P < 0.05$.
D. Lanes 2 and 3 show RpoS levels as detected by an anti-RpoS antibody for Δ6/pCA24N-*dinJ* (DinJ+) and Δ6/pCA24N (DinJ-). Two independent cultures were used for each strain and both experiments are shown.

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 cellulose and curli (Ma and Wood, 2009), we found that producing DinJ decreased curli/cellulose production 5.4 ± 1.2-fold in the presence of erythromycin stress (7.5 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 H_2O_2 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 H_2O_2 to H_2O and O_2; hence, the reduced ability of the cells to decompose H_2O_2 when DinJ reduces RpoS was demonstrated by a dramatic reduction in oxygen bubbles upon addition of H_2O_2 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

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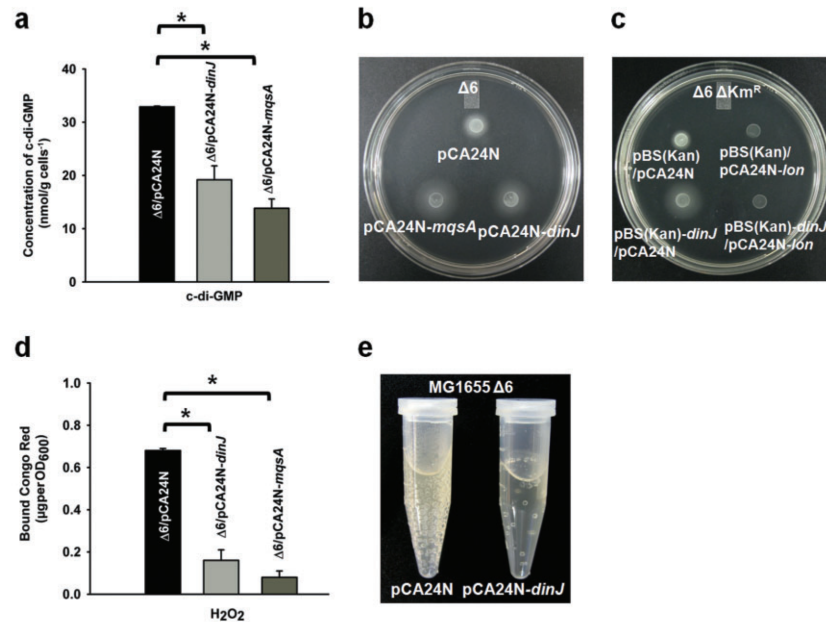

Fig. 2. DinJ reduces cyclic diguanylate thereby increasing swimming and decreasing cell adhesins and catalase activity.
A. Cyclic diguanylate concentrations in stationary-phase cultures (starving cells) of Δ6/pCA24N-*dinJ*, Δ6/pCA24N-*mqsA* and Δ6/pCA24N after 15 h at 37°C. Error bars indicate standard error of mean ($n = 2$). Significant changes are marked with an asterisk for $P < 0.05$.
B. Swimming motility after 12 h of growth at 37°C for Δ6 cells producing DinJ and MqsA via pCA24N-*dinJ* and pCA24N-*mqsA*. Two independent cultures were used, and a representative image is shown here.
C. Swimming motility after 12 h of growth at 37°C for Δ6 ΔKm^R cells producing DinJ from pBS(Kan)-*dinJ* and Lon from pCA24N-*lon* via 1 mM IPTG. Chloramphenicol (30 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) were used for maintaining the pCA24N-based and pBS(Kan)-based plasmids. Controls where *lon* was induced in the absence of DinJ were also included to show the direct effect of each gene on motility. Two independent cultures were used, and a representative image is shown here.
D. Cellulose/curli formation of Δ6/pCA24N-*dinJ*, Δ6/pCA24N-*mqsA* and Δ6/pCA24N after 180 min at 30°C with oxidative stress (2 mM H_2O_2). Error bars indicate standard error of mean ($n = 2$). Significant changes are marked with an asterisk for $P < 0.05$.
E. Images of Δ6/pCA24N-*dinJ* versus Δ6/pCA24N cultures (turbidity of 1) 10 min after adding 40 mM H_2O_2. Two independent cultures were used for each strain, and one representative data set is shown. Bubbles are oxygen produced by the decomposition of hydrogen peroxide by catalase: 2 H_2O_2 to 2 H_2O + O_2. DinJ was produced from pCA24N-*dinJ* via 0.5 mM IPTG for the c-di-GMP assay and via 1 mM IPTG for the stress assays.

differentially expressed during production of DinJ using a whole-transcriptome analysis. Exposure to erythromycin (75 μg 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 Δ6/pCA24N-*dinJ* versus Δ6/pCA24N with erythromycin stress and found that DinJ repressed *cspE* dramatically (114 ± 35-fold). Therefore, DinJ either directly or indirectly represses *cspE*, which results in a reduction in RpoS.

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 P*cspE* (from −123 to +53), both of which contain the consensus LexA binding sequence (5′-TACTG(TA)$_5$CAGTA-3′) (Fernández De Henestrosa *et al.*, 2000) as shown in Fig. S1. EMSA revealed that DinJ binds both P*cspE* 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 *rpoS* (Fig. 3D, promoter sequence shown in Fig. S2). Also, antitoxin MqsA failed to bind P*cspE* (Fig. 3E). To confirm that DinJ binds P*cspE*, we

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

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

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.

mutated the LexA binding sequence from CTGG to GACC and found that DinJ no longer binds P*cspE* (Fig. 3F). Therefore, DinJ represses *cspE* by directly binding it while it does not regulate *rpoS* directly.

DinJ depends on CspE to control RpoS levels and RpoS-controlled cell viability, swimming motility, cell adhesins, and catalase activity

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 BW25113*cspE*/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

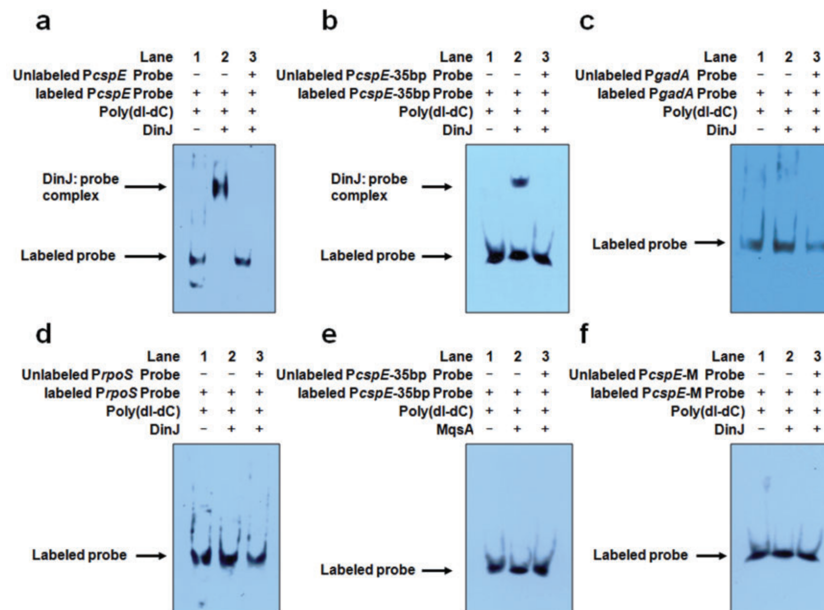

Fig. 3. DinJ binds P*cspE* at the LexA palindrome but does not bind P*rpoS*.
A. The 176 bp DNA fragment of the *cspE* promoter (P*cspE*) was incubated with DinJ. Lanes 1–3: 6 ng of biotin-labelled P*cspE*; Lane 2: addition of 1.2 μg DinJ; and Lane 3: addition of 1.2 μg DinJ and 600 ng of unlabelled P*cspE*.
B. The 35 bp DNA fragment of *cspE* promoter (P*cspE*-35bp) was incubated with DinJ. Lanes 1–3: 30 pg of biotin-labelled P*cspE*; Lane 2: addition of 1.2 μg DinJ; and Lane 3: addition of 1.2 μg DinJ and 12 ng of unlabelled P*cspE*.
C. The 185 bp DNA fragment of *gadA* promoter (P*gadA*) was incubated with DinJ as a negative control. Lanes 1–3: 6 ng of biotin-labelled P*gadA*; Lane 2: addition of 1.2 μg DinJ; and Lane 3: addition of 1.2 μg DinJ and 600 ng of unlabelled P*gadA*.
D. The 185 bp DNA fragment of *rpoS* promoter (P*rpoS*) was incubated with DinJ. Lanes 1 to 3: 6 ng of biotin-labelled P*rpoS*; Lane 2: addition of 1.2 μg DinJ; and Lane 3: addition of 1.2 μg DinJ and 600 ng unlabelled P*rpoS*.
E. The 35 bp DNA fragment of *cspE* promoter (P*cspE*-35bp) was incubated with MqsA as a negative control. Lanes 1–3: 30 pg of biotin-labelled P*cspE*; Lane 2: addition of 1.2 μg MqsA; and Lane 3: addition of 1.2 μg MqsA and 12 ng of unlabelled P*cspE*.
F. The mutated 35 bp *cspE* promoter (P*cspE*-M) lacking the LexA palindrome was incubated with DinJ. Lanes 1–3: 30 pg of biotin-labelled mutated P*cspE*; Lane 2: addition of 1.2 μg DinJ; and Lane 3: addition of 1.2 μg DinJ and 12 ng of unlabelled mutated P*cspE*. The unspecific competitor poly(dI-dC) was used for all samples.

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* demonstrate 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′-CTGGATGCGCTTTCAG-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

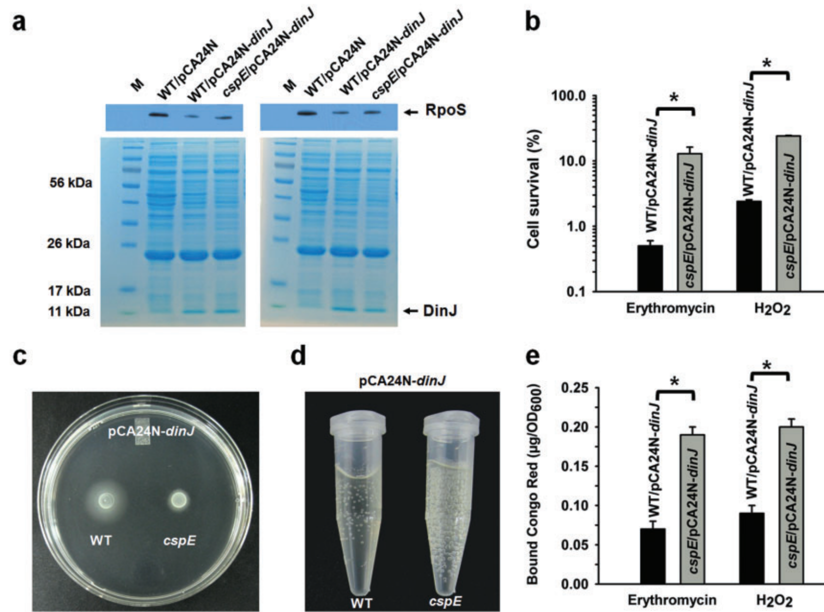

Fig. 4. DinJ requires CspE to influence RpoS levels and RpoS-controlled viability, motility, cell adhesins and catalase activity.
A. Lanes 2, 3 and 4 show RpoS levels as detected by an anti-RpoS antibody for BW25113/pCA24N, BW25113/pCA24N-*dinJ* and BW25113 *cspE*/pCA24N-*dinJ*. Two independent cultures were used for each strain and both experiments are shown.
B. Percentage of cells (BW25113 *cspE*/pCA24N-*dinJ* versus BW25113/pCA24N-*dinJ*) which survive erythromycin (75 μg ml^{-1}) stress for 10 min and oxidative stress induced by 20 mM H_2O_2 for 10 min. Error bars indicate standard error of mean ($n = 2$). Error bars indicate standard error of mean ($n = 2$). Significant changes are marked with an asterisk for $P < 0.05$.
C. Swimming motility after 12 h of growth at 37°C for BW25113 *cspE*/pCA24N-*dinJ* versus BW25113/pCA24N-*dinJ*. Two independent cultures were used, and a representative image is shown.
D. Images of BW25113 *cspE*/pCA24N-*dinJ* versus BW25113/pCA24N-*dinJ* cultures (turbidity of 1) 10 min after adding 40 mM H_2O_2. Bubbles are oxygen produced by the decomposition of hydrogen peroxide by catalase ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$). Two independent cultures were used, and a representative image is shown.
E. Curli/cellulose production for strains BW25113 *cspE*/pCA24N-*dinJ* versus BW25113/pCA24N-*dinJ* after 3 h of incubation with 7.5 μg ml^{-1} erythromycin or 2 mM H_2O_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 $P < 0.05$.

derepression of *cspE* transcription, which should result in an increase in RpoS. Hence, upon erythromycin stress, Lon is induced and degrades DinJ like other antitoxins, *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 *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.

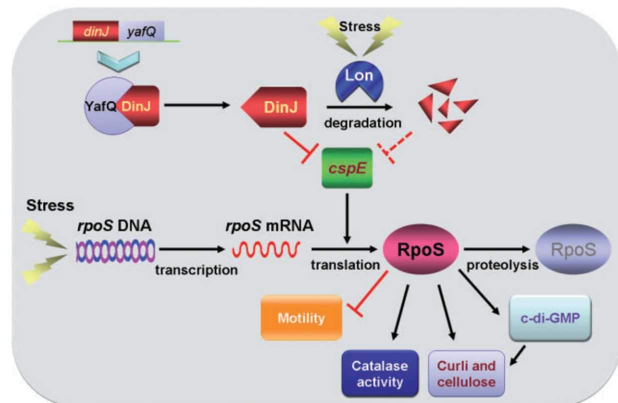

Fig. 5. Schematic of how DinJ impacts the general stress response. RpoS levels are reduced indirectly by DinJ through its control of *cspE*. Under erythromycin stress, DinJ is degraded by protease Lon, and *cspE* transcription is derepressed. The increased CspE levels lead to greater RpoS translation, which increases c-di-GMP concentrations, catalase activity and curli/cellulose production and decreases swimming. The lightning bolt indicates erythromycin stress, '→' indicates induction and '⊥' 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*-SacI-R. The PCR product was cloned into the multiple cloning site of pBS(Kan) (Canada *et al*., 2002) after double digestion with KpnI and SacI 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 H_2O_2 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 *cspE*/pCA24N-*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). Δ6/pCA24N-*dinJ*, Δ6/pCA24N and Δ6/pCA24N-*mqsA* (positive control) were grown in 1 l LB 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 H_2O_2 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 H_2O_2 (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 ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$).

RNA isolation and whole-transcriptome analysis

Whole-transcriptome analysis was performed using planktonic cells of Δ6/pCA24N-*dinJ* versus Δ6/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 (González 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-C_T *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 Δ6/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

manufacture's protocol. Purified DinJ was dialysed against buffer (25 mM Tris-HCl, pH 7.6) at 4°C overnight.

Electrophoretic mobility shift assays

To investigate binding of DinJ to promoter regions, EMSA was performed as described previously (Prysak *et al*., 2009) with some modification. Briefly, complementary oligonucleotides (35-mers) biotin labelled at the 3′ end (Table S1) were purchased from Integrated DNA technologies and were used to synthesize the wild-type LexA binding box and the corresponding mutated LexA binding box of the *cspE* promoter (from position −87 to −53) (Fig. S1). The combination of oligonucleotides was annealed at 65°C for 2 min and allowed to cool to room temperature. Promoter regions including the 176 bp fragment of P*cspE* (from position −123 to +53), the 185 bp fragment of P*rpoS* (from position −168 to +17), and the 185 bp fragment of P*gadA* (from position −205 to −21) were amplified using primers shown in Table S1, purified, and labelled with biotin using the biotin 3′ end DNA Labeling Kit (Pierce Biotechnology, Rockford, IL, USA). For the EMSA assay, biotin labelled target promoters were incubated with purified DinJ or MqsA (Brown and Page, 2010) either with or without unlabelled target DNA promoter for 120 min at room temperature in the reaction buffer [10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM $CaCl_2$, 100 mM NaCl, 1 mM DTT, 5% glycerol and 0.1 mg ml^{-1} BSA]. Samples were run on a 6% DNA retardation gel (Invitrogen) at 100 V in 0.5× TBE for 75 min at 4°C. The bound protein/DNA mixtures were then transferred to a nylon membrane at 380 mA for 60 min then UV cross-linked at 302 nm. Chemiluminescence was performed with the LightShift Chemiluminescent EMSA Kit (Thermo Scientific).

Western blot analysis

To investigate the degradation of DinJ under stress and the effect of DinJ on RpoS, Western blots were performed as described previously (Wang *et al*., 2011). To ascertain DinJ levels, Δ6/pCA24N-*dinJ* and Δ6/pCA24N were grown to a turbidity of 0.1, then 0.5 mM ITPG was added to induce *dinJ*. When the turbidity reached 1, 200 μg ml^{-1} rifampin was added to inhibit transcription, and the cell pellets were exposed to various stress conditions including 75 μg ml^{-1} erythromycin (samples taken at 0, 0.5, 2.5, 5, 10, 15 min), 20 mM H_2O_2 (0, 2.5, 5, 10, 15 min), 2 μg ml^{-1} mitomycin C (0, 5, 15, 30 min), 100 μg ml^{-1} ampicillin (0, 5, 15, 30 min), 50 μg ml^{-1} gentamicin (0, 5, 15, 30 min), 15 μg ml^{-1} tetracycline (0, 5, 15, 30 min), 200 μg ml^{-1} nalidixic acid (0, 5, 15, 30 min), heat (50°C, 0, 5, 15, 30 min) and acid (pH 2.5, 0, 5, 15, 30 min). Samples were processed with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich) and sonicated twice on ice for 15 s. Soluble protein samples in supernatants were obtained by centrifuging the cell pellets at 17 000 *g* for 4 min. The protein concentration was assayed by the BCA assay. The same amount of protein (2 μg) was loaded into each well of a 12% SDS-PAGE gel, then transferred to a PVDF membrane, which was then blocked with 4% BSA in TBST (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. The Western blots were probed with a 1:2000 dilution of primary antibodies raised against a His tag (Cell Signaling Technology), 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 a 1:2000 dilution of anti-RpoS monoclonal antibody (Neoclone) was used. To investigate how DinJ affects RpoS levels, Δ6/pCA24N-*dinJ* and Δ6/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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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 P*cspE* (EMSA) is underlined (176 bp, from position −123 to +53). The region 2 used for EMSA was amplified with primer P*cspE* (EMSA)-35mer and is indicated in lowercase (35 bp, from position −87 to −53). The consensus LexA binding site (5′-TACTG$(TA)_5$CAGTA-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 P*rpoS* (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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