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CspC regulates *rpoS* transcript levels and complements *hfq* deletions

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Abstract

The general stress response in *Escherichia coli* is activated by several stress agents, including entering the stationary growth phase. This response constitutes a complex regulatory network in which a large number of genes are induced and others are repressed. The stress response is regulated by the alternative sigma factor σ^S encoded by the *rpoS* gene. The *rpoS* transcripts are substrates of the RNA binding protein, Hfq, which is essential for its translation. The *rpoS* mRNA is also a substrate of the cold shock protein C (CspC) which stabilizes the transcripts. Here we demonstrate, using pull-down assays, that CspC interacts with Hfq via mRNA molecules. We also show that CspC acts on the 5′ UTR of the *rpoS* transcript, but its activity on *rpoS* is independent of Hfq. Moreover, we show that CspC suppresses the phenotypes of an *hfq* deletion. These results elucidate a new aspect in the post-transcriptional regulation of the stress response and will further our understanding of this complex network.

Keywords: Stress response; Transcript stability; CspC

1. Introduction

To cope with stress conditions, various molecular mechanisms have evolved in bacteria, leading to induction of a specific set of stress-related proteins. The general stress response is activated by several stress agents and leads to broad stress resistance. Activating agents include carbon starvation, extreme pH, high osmolarity, oxidative stress and entering the stationary growth phase (Weichart et al., 1993; Lange and Hengge-Aronis, 1994; Lee et al., 1995). The general stress response is regulated by the alternative sigma factor σ^S which is encoded by the *rpoS* gene. This sigma factor recruits the core RNA polymerase and initiates transcription from specific promoters of genes belonging to the general stress response. Genome-wide analyses of RpoS-dependent gene expression showed that up to 10% of the genes in *Escherichia coli* are under direct or indirect control of σ^S (Weichart et al., 1993; Patten et al., 2004).

The regulation of the general stress response is controlled by the cellular levels of σ^S, which are elevated when the stress response is induced. The synthesis of σ^S is under complex regulation which includes transcriptional and translational controls as well as proteolysis (Lange and Hengge-Aronis, 1994; Hengge-Aronis, 2002a). Transcription of *rpoS* is negatively controlled by cAMP-CRP (Lange and Hengge-Aronis, 1994) and positively controlled by a sensor kinase, BarA (Mukhopadhyay et al., 2000). Translation of *rpoS* in regulated by small non-coding RNAs and Hfq (Ruiz and Silhavy, 2003; Soper and Woodson, 2008) and degradation of RpoS is mediated by ClpXP and RssB (Pratt and Silhavy, 1996; Bouche et al., 1998). Hfq is an RNA binding protein, which is essential for the translation of *rpoS*. It binds many transcripts and affects their stability and translation, presumably by changing their secondary structure (Moll et al., 2003; Brennan and Link, 2007). Hfq was also shown to assists in bimolecular RNA–RNA interactions, thus mediating interactions of mRNA transcripts with small non-coding RNAs

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(Valentin-Hansen et al., 2004). Hfq is structurally and functionally similar to eukaryotic RNA-associated Sm proteins which are important post-transcriptional regulators (Valentin-Hansen et al., 2004). In *E. coli* deletion mutants of *hfq* have pleiotropic phenotypes including multiple stress sensitivity, mainly due to decreased translation of σ^S, resulting in reduced expression of σ^S-regulated genes (Tsui et al., 1994; Muffler et al., 1996, 1997). At the molecular level, Hfq stabilizes interactions between the small non-coding RNA DsrA and *rpoS* mRNA, frees the *rpoS* translation initiation region, thus reduces *rpoS* self-inhibition and increases translation (Lease and Belfort, 2000).

Another protein that affects the stability of *rpoS* transcripts is CspC, which belongs to the family of cold shock proteins (CSPs), known to be involved in mRNA stability and translation and serve as RNA chaperones (Jiang et al., 1997). CspC was shown to affect the *rpoS*-induced general stress response, through stabilization of the *rpoS* transcript, as its over-expression results in increased transcript stability of genes belonging to the σ^S regulon. (Phadtare and Inouye, 2001).

As both CspC and Hfq stabilize *rpoS* transcript, we investigated the possible interaction between the activities of these two proteins in regulating the RpoS-mediated stress response. Using co-immuno precipitation, we show that CspC and Hfq interact and that this interaction is, at least in part, mediated by RNA molecules. We also show that stabilization by CspC requires the *rpoS* leader sequence (UTR and first 90 bases), overlapping the binding sequence required for Hfq (Soper and Woodson, 2008). Yet, CspC is independent of Hfq in maintaining high level of *rpoS* transcripts and, moreover, CspC can compensate for the absence of Hfq.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All the experiments were carried out with *E. coli* K12 MG-1655 (wild type) and its derivatives (Table 1). Bacteria were grown in LB (Luria Bertani) broth at 37 °C. The medium was supplemented, when required, with 50 μg/ml kanamycin, 100 μg/ml Ampicillin, 0.2% arabinose or 0.2% glucose. Growth was followed by determination of OD_{600} using a WPA (Biowave, Ltd) spectrophotometer.

2.2. Genetic manipulations

Chromosomal epitope-tagging of Hfq was achieved as described by Uzzau et al. (Uzzau et al., 2001). Deletion strains were constructed using the one step inactivation system as described by Datsenko and Wanner (Datsenko and Wanner, 2000). A *lacZ* gene missing its promoter and first 8 amino acids was fused downstream to the 567 bases of the 5′ UTR and first 90 bases of the *rpoS* ORF. This construct was cloned into a pBAD24 plasmid under the arabinose-dependent promoter. Two gene fusions were constructed, one containing the *rpoS* UTR (567 bases) + the first 90 bases of the *rpoS* ORF, and the other construct had only the first 90 bases of the *rpoS* ORF.

2.3. Determination of acid tolerance

Cultures were grown exponentially for at least five generations, till stationary growth phase. Two hours after growth cessation the culture was subjected to low pH conditions (pH = 3.5 or pH = 1.5, as indicated) using hydrochloric acid and viable count was performed before that addition of acid (0 time) and at time intervals afterwards.

2.4. Assay for β-galactosidase activity

β-galactosidase activity was measured as described (Miller, 1972).

2.5. mRNA extraction

Cultures were grown exponentially for at least five generations, till stationary growth phase. Two hours after growth cessation RNA from 500 μl of a bacterial culture was stabilized using RNA protect reagent and extracted using the

Table 1
Bacterial strains and plasmids.

Plasmids	Genotype Description
pBAD24	Contains an arabinose-dependent promoter, Amp^R (Guzman et al., 1995)
pBAD:cspC	*cspC* ORF cloned into pBAD24
pBAD:lacZ	*lacZ* ORF cloned into pBAD24
pBAD::His-tagged cspC	His-tagged *cspC* cloned into pBAD24
pBAD:rpoS1	The *lacZ* ORF fused downstream to *rpoS* (UTR + first 90 bases of the ORF) and cloned into pBAD24
pBAD:rpoS2	The *lacZ* ORF fused downstream to *rpoS* (first 90 bases of the ORF) and cloned into pBAD24
pACYC:cspC	*cspC* ORF cloned downstream to the *ara* promoter in plasmid pACYC; Cm^R
Strains	
Wild Type	MG-1655 wild type strain of *E. coli* K12
hfq::FLAG	MG-1655 with chromosomal 3′ FLAG tag fused downstream of the *hfq* gene; Km^R
hfq::FLAG cspC:His	*hfq::FLAG* carrying *pBAD::His-tagged cspC*
Δ*hfq*	MG-1655*Δhfq*; Km^R
Δ*rpoS*	MG-1655*ΔrpoS*; Km^R
Δ*cspC*	MG-1655*ΔcspC*; Km^R
Δ*hfqΔcspC*	MG-1655*Δhfq;ΔcspC*; Km^R
MG-1655p*cspC*	MG-1655 carrying *pBAD:cspC*
Δ*hfq* p*cspC*	Δ*hfq* carrying *pBAD:cspC*
Δ*hfq* p*lacZ*	Δ*hfq* carrying *pBAD:lacZ*
Δ*rpoS* p*cspC*	Δ*rpoS* carrying *pBAD:cspC*
MG-1655 *pBAD:rpoS*1	MG-1655 carrying *pBAD:rpoS1* plasmid
MG-1655p*cspC; pBAD:rpoS1*	MG-1655 carrying both *pACYC:cspC* and *pBAD:rpoS*1 plasmids
Δ*cspC pBAD:rpoS1*	Δ*cspC* carrying *pBAD:rpoS1* plasmid
MG-1655 *pBAD:rpoS2*	MG-1655 carrying *pBAD:rpoS2* plasmid
Δ*cspC pBAD:rpoS*2	Δ*cspC* carrying *pBAD:rpoS2* plasmid

RNeasy minikit (QIAGEN). RNase free DNase set column (QIAGEN) was used to eliminate DNA contamination. RNA levels were quantified with Nano-drop spectrophotometer and were further evaluated by electrophoresis.

2.6. Reverse transcription and real time PCR

1 μg of total RNA was reverse-transcribed using random hexamers (Amersham) and ImPromII reverse transcriptase (Promega). Real time PCR reactions were performed using 500 nM of the following primers: rpoS F – CCTCTCGCCCGCCGGATGATC; rpoS R – CCAGCAACGCCAGACCACGATT; osmY F – CAAGACTGATTTCGA and osmY R – CGCTGTCATCCATGAAAT, in a 10 μl volume with SYBR green PCR master mix (Applied Biosystems). Reactions were run on a Rotorgene 6000 (Corbett) using the standard cycling parameters. The data represents three independent biological experiments.

2.7. Pull-down assay

Culture was grown in 37 °C with 0.2% arabinose for CspC induction. 30 ml of the culture ($O.D_{600} = 0.6$) were centrifuged for 10 min at 6500 G and then washed in 1 ml of TE-PMSF, suspended in 0.5 ml of PBS and sonicated. The lysate was centrifuged at 20,000 G for 30 min to precipitate membrane debris. The clear supernatant was supplemented with imidazole (to final concentration of 5 mM) and 10 μl Ni-NTA agarose resin (Qiagen). If required, RNase A (Qiagen) was added at this stage (to final concentration of 2 mg/ml). The tubes were kept at 4 °C while gently shaking for 1 h, washed with 1 ml of PBS containing imidazole (50 mM) and eluted with SDS-PAGE sample buffer. Fractions were analyzed by western blotting using anti-Flag and anti-His antibodies.

2.8. SDS-PAGE and western blotting

Gel electrophoresis was carried out using 12% SDS-PAGE. Immunoblots were performed according to standard procedures, using primary antibodies mouse anti-Flag (Sigma) and mouse anti-beta-gal (Promega), followed by HRP conjugated anti mouse IgG as secondary antibody, or using HRP conjugated anti-His antibodies (Roche). EZ-ECL chemiluminescence detection kit (Biological Industries) was used for detection.

3. Results

3.1. Effect of CspC on expression of rpoS

CspC is an RNA chaperone, previously shown to stabilize *rpoS* transcripts (Phadtare and Inouye, 2001; Phadtare et al., 2006). We examined the effect of CspC on expression of a *lacZ* gene fused downstream to the UTR and first 90 bases of the *rpoS* ORF. Indeed, expression of *rpoS* was higher in strains over-expressing CspC and is reduced in strains carrying a *cspC* deletion (Fig. 1). As expected, the CspC effect was more pronounced in stationary phase cells than in exponentially growing cells (Fig. 1).

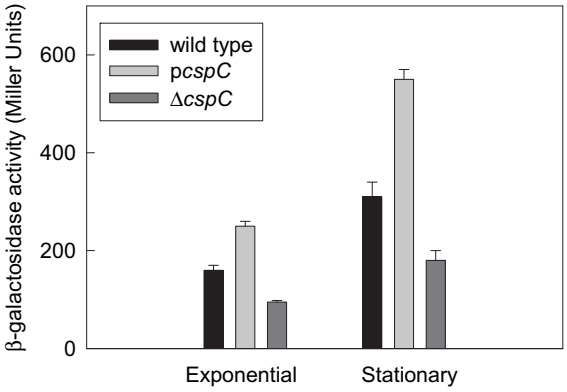

Fig. 1. Effect of CspC on *rpoS* expression. The expression of *rpoS* was determined using the *lacZ* gene as a reporter in the following strains: wild type MG-1655, a strain over-expressing CspC and a strain deleted for *cspC* (MG-1655 *pBAD:rpoS*1, MG-1655p*cspC2; pBAD:rpoS1* and *ΔcspC pBAD:rpoS1* respectively*)*. 0.2% Arabinose (Ara) was used to activate expression from the corresponding pBAD plasmid. The activity of β-galactosidase was determined as previously described (Miller, 1972).

3.2. CspC and hfq interact

Hfq is a positive regulator of *rpoS* mRNA and so is CspC (Muffler et al., 1996; Phadtare and Inouye, 2001). In order to learn about the interaction of CspC with *rpoS* transcripts and with Hfq, we performed co-immuno precipitation experiments, as described in Materials and Methods. A strain with an FLAG-tagged Hfq and His-tagged CspC (*hfq::FLAG cspC:His*, see Table 1) was grown in the presence of arabinose for *cspC* induction. The cell lysates were incubated with limiting amount of nickel beads for precipitation of the His-tagged CspC. After five washes Hfq could be detected only on the beads.

The results indicate that CspC was precipitated with the nickel beads and that Hfq was also pulled-down (Fig. 2, lane 1, two

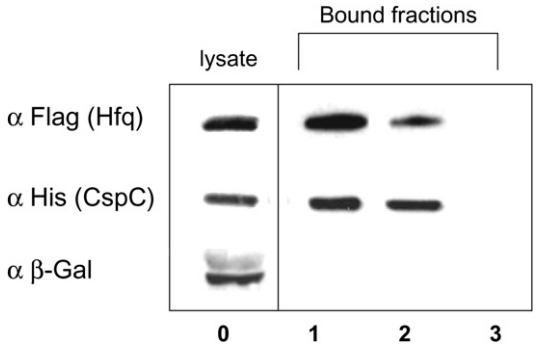

Fig. 2. Pull-down of Hfq with CspC. Lysates of strain *hfq::FLAG cspC:His* (see Table 1) were incubated with nickel beads for the precipitation of His-tagged CspC. The total lysate, as well as the fractions precipitated on the nickel beads were analyzed by western blots with three antibodies – anti-FLAG, anti-His and anti β-Gal antibodies to detect the presence of CspC, Hfq and β-Gal, respectively. The figure shows the western blot analysis of total lysate (left side, 0) and nickel-precipitated fractions: 1 – precipitates from nickel beads; 2 – as in lane 1 but with the addition of Rnase. Lane 3 represents the negative control, which was performed in the same was but with strain *hfq::FLAG* missing the *pBAD::His-tagged cspC* plasmid. The data presents a representative result of three independent experiments.

upper bands). Flag-tagged Hfq was found in the nickel beads only in the presence of bound his-tagged CspC. It is not precipitated with the nickel beads in a control strain – *hfq::FLAG*, missing the His-tag CspC (Fig. 2, lane 3). The specificity of the interaction between CspC and Hfq was shown by the finding that a control protein, such as β-galactosidase, was not co-precipitated with the his-tagged CspC on the nickel beads (Fig. 2, lower band).

3.3. CspC and hfq interact via RNA molecules

One possibility for the interaction between CspC and Hfq is that both bind mRNA which mediates this interaction. Indeed, the pull-down of Hfq by CspC was significantly reduced (61.8% ± 8.3% reduction) in the presence of RNase (Fig. 2, lane 2). As we assume that CspC and Hfq are both bound to *rpoS* transcripts we repeated the pull-down experiments with an *rpoS* deletion mutant. Indeed, we obtained a 30% decrease in the Hfq that was pulled-down with CspC. This result, however, may represent a minimal value, as the deletion *rpoS* mutant retained the UTR which is important for binding of both CspC and Hfq (see next paragraph).

3.4. CspC effect requires the rpoS UTR

The results presented in Fig. 2 indicate that CspC and Hfq interact through an RNA molecule. As it was previously shown (Soper and Woodson, 2008) that Hfq is bound to the 5′ UTR of the *rpoS* transcript, we examined the possibility that this UTR is also the binding site for CspC. The results presented in Fig. 3 support this hypothesis and indicate that the effect of CspC on *rpoS* expression depends on the presence of the *rpoS* 5′ UTR. Thus, in a *lacZ* fusion which contains only the 90 bases of the *rpoS* ORF the effect of CspC is minimal (Fig. 3B), in contrast to the fusion that also contains the 567 bases of the 5′ UTR (Fig. 3A).

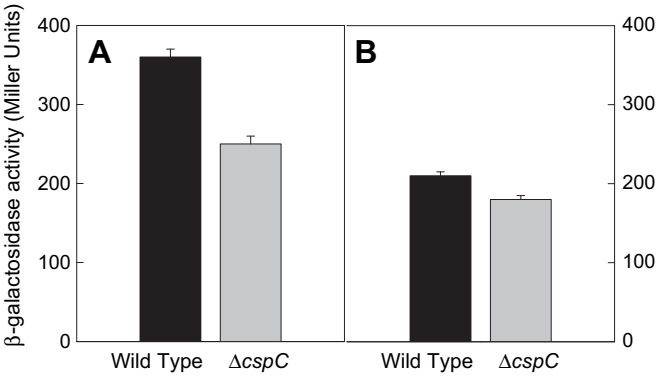

Fig. 3. Determination of the CspC responsive region in the *rpoS* transcript. The expression of *rpoS* was determined as described in Fig. 1. The results of the *lacZ* fusion downstream of the 567 bases of the 5′ UTR and first 90 bases of the *rpoS* ORF are presented in part A (MG-1655 *pBAD:rpoS*1 and *ΔcspC pBAD:rpoS1)*. The results in part B were obtained with a construct in which only the first 90 bases of the *rpoS* ORF were used for the *lacZ* fusion (MG-1655 *pBAD:rpoS*2 and *ΔcspC pBAD:rpoS2*).

3.5. CspC regulates RpoS levels independently of hfq

As both CspC and Hfq stabilize *rpoS* transcripts (Phadtare and Inouye, 2001; Phadtare et al., 2006), and as our results show that CspC and Hfq interact, we performed experiments to determine whether these two proteins are part of the same pathway or do they act independently of each other. We compared the levels of *rpoS* transcripts in a strain over-expressing CspC (MG-1655p*cspC*) with the rpoS transcript levels in the same strain but deleted for *hfq* (*Δhfq* p*cspC*). The levels of the *rpoS* transcripts were measured using real time PCR in conditions of activation and repression of CspC expression from the plasmid. The results, presented in Fig. 4A, indicate that over-expression of CspC resulted in an increase of *rpoS* transcript levels. This increase was independent of Hfq as it was also observed in the deletion strain (Fig. 4A). Over-expression of a control gene, *lacZ*, demonstrated that the effect of CspC on *rpoS* transcript levels is specific.

In order to examine whether the increase of *rpoS* transcript levels leads to increased σ^S we measured the transcript levels of *osmY*, a known *rpoS* substrate (Hengge-Aronis et al., 1993; Weichart et al., 1993). The results demonstrate that the increase in *rpoS* transcripts levels results in an increase of σ^S dependent transcription (Fig. 4B).

3.6. Over-expression of CspC protects against acid damage

A major function of σ^S is improving survival of bacteria under stress conditions. Stationary *E. coli* cultures, in which *rpoS* is highly expressed, show high resistance to lethal agents such as H_2O_2, heat and extreme pH (Hengge-Aronis, 2002b). Mutants deleted for *hfq*, in which the levels of RpoS are considerably reduced, are highly sensitive to these stresses (Tsui et al., 1994). As CspC increases the levels of *rpoS* transcripts and RpoS activity, we examined the possibility that its over-expression may complement an *hfq* deletion in respect to survival of environmental stresses. The results present in Fig. 5 demonstrate that, in fact, over-expression of *cspC* increases protection against acid conditions even in the absence of Hfq and can therefore complement an *hfq* deletion. This complementation is at the level of the *rpoS* transcripts, as the improving effect of CspC was abolished when *rpoS* was deleted (Fig. 5). These results further emphasize the versatile regulation of *rpoS* expression and the complex control of the important stress response.

3.7. Hfq and cspC mutants display an additive phenotype

In order to further examine the interactions between Hfq and CspC we constructed and studied a double mutant Δ*hfq* Δ*cspC*. We compared *rpoS* transcript levels as well as survival under acidic condition in wild type, Δ*cspC*, Δ*hfq* and the Δ*hfq* Δ*cspC* strains (see Table 1). In contrast to the experiment described in Fig. 5 with pH = 1.5, the experiment with the double mutant was performed in pH = 3.5 since the double mutant does not survive more acidic pH values. The double

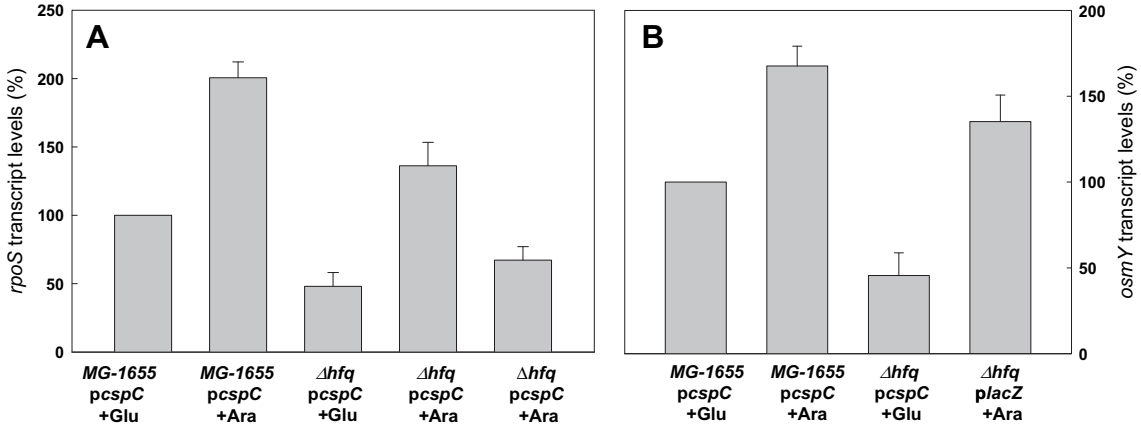

Fig. 4. Effect of CspC over-expression on rpoS and osmY mRNA levels. Comparison of mRNA levels of *rpoS* (A) and *osmY* (B) in stationary growth phase of indicated strains, measured using real time PCR. 0.2% Arabinose (Ara) or 0.2% Glucose (Glu) was used to activate or repress expression from the corresponding pBAD plasmid.

mutant exhibited a more severe phenotype than any of the single mutants, in respect to both *rpoS* transcript levels and the ability to cop with acid conditions (Fig. 6). These results are compatible with the data indicating that CspC and Hfq affect *rpoS* independently (Figs. 4 and 5).

4. Discussion

The stress response in *E. coli* constitutes a complex regulatory network which is mediated mainly by the stress response alternative sigma factor σ^S – RpoS. The levels of RpoS are tightly controlled at all levels – transcription, translation, activity, and protein degradation. We focused on two major regulators of σ^S – Hfq and CspC. The RNA binding protein, Hfq, is essential for the translation of *rpoS*, as it allows DsrA to bind *rpoS* and release its self-inhibition. Another factor that stabilizes *rpoS* mRNA is cold shock protein C (CspC), a member of RNA chaperones family

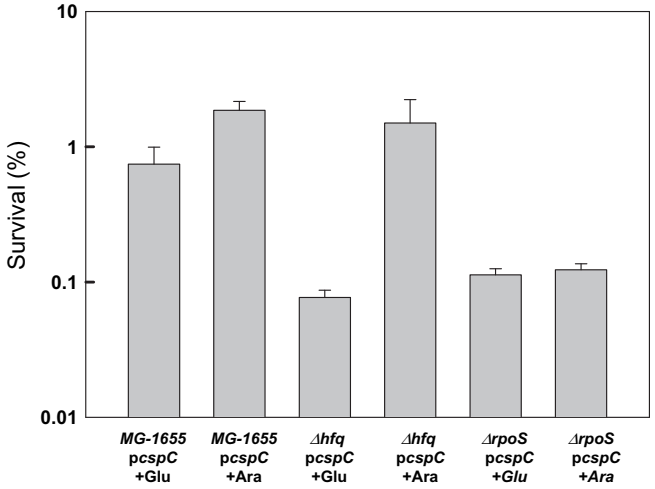

Fig. 5. Effect of CspC over-expression on survival following acidic stress. Survival percentage of indicated strains after 15 min exposure to low pH (pH = 1.5) as quantified by viable count. The data represents average results of three independent experiments.

(Phadtare and Inouye, 2001). Here we show that CspC interacts with the 5′ UTR of the *rpoS* transcript, which is also the site of Hfq activity, leading to an increase in the transcript levels. This increase in *rpoS* mRNA levels is independent of Hfq and is sufficient to complement an *hfq* deletion.

We could pull-down Hfq on nickel beads to which his-tagged CspC was attached. This pull-down was sensitive to the addition of RNase, indicating that the linkage of the two proteins was mediated by an RNA molecule, most likely mRNA.

Previous experiments (Phadtare and Inouye, 1999, 2001) using the SELEX approach of binding specific RNA/single stranded DNA sequences in vitro showed a binding of CspC to a 45-base-long ssDNA probes containing three tandem repeats of a consensus sequence (AGGGAGGGA). However, the *rpoS* transcript does not contain this sequence, suggesting that the in vivo binding of CspC may obey different rules. Moreover, it is calculated that CspC binds of the predicted consensus binding sequence with an affinity of about 2.5 mM. This value may be too low to allow binding which is strong enough to sustain the pull-down experiments, especially given the fact that the reported Kd values for Hfq-RNA binding are around 20 nM (Mikulecky et al., 2004). There are as yet no data on the affinity of CspC to natural, longer, mRNA and our results suggest that the affinity of CspC to natural mRNA substrates in vivo is higher than initially anticipated.

The effect of CspC on *rpoS* transcripts constitutes and additional level of control and protection of these transcripts. It was previously shown that Hfq, together with DsrA, is essential for translation of *rpoS* transcripts. Depletion of Hfq, by deletion of the gene, results in a slow growth rate and sensitivity to stress agents such as oxidative stress and low pH (Tsui et al., 1994; Soper and Woodson, 2008). We could show that the activity of CspC in increasing *rpoS* transcript levels is independent of Hfq. Moreover, CspC can complement the sensitivity of *hfq* deletion mutants to low pH, indicating the physiological significance of CspC as a chaperone of *rpoS* mRNA.

It was interesting to analyze the phenotype of a mutant lacking both Hfq and CspC. Indeed, these mutants are difficult

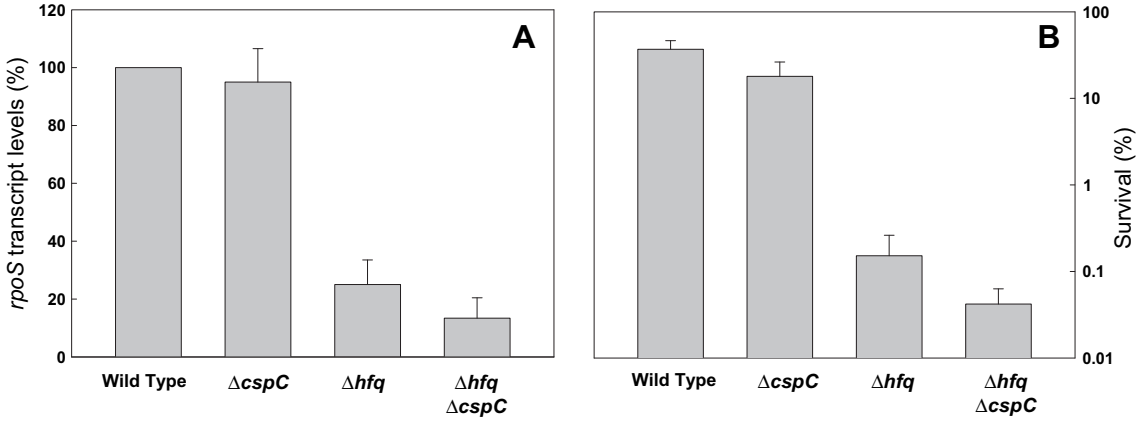

Fig. 6. *hfq* and *cspC* mutants display an additive phenotype. Comparison of mRNA levels of *rpoS* in stationary growth phase of indicated strains, measured using real time PCR. 0.2% Arabinose (Ara) or 0.2% Glucose (Glu) was used to activate or repress expression from the corresponding pBAD plasmid (A). Survival percentage of indicated strains after 15 min exposure to low pH (pH = 3.5) as quantified by viable count (B). The data represents average results of three independent experiments.

to obtain, and they grow very poorly. This additive effect of the two deleterious mutations also supports the notion that the two RNA stabilizing factors act independently.

Recently, CspC was shown to be a major regulator of the heat shock response (Shenhar et al., 2009). Here we show the importance of CspC in the general stress response and survival in acidic environments. These findings mark CspC as a major regulator of *E. coli* response to stress conditions.

In summary, we show that CspC and Hfq interact via RNA molecules, very likely via *rpoS* mRNA. We show that CspC is important for regulating the levels of *rpoS* transcripts, especially during stationary stress and low pH. We also demonstrate that the regulation on RpoS by CspC is physiologically important in *E. coli*, especially under stationary phase related stresses. Thus, the finding that CspC can compensate for the absence of Hfq, one of the main regulators of RpoS, elucidates a new aspect in the transcriptional regulation of the stress response and its complex network.

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