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Role of a Zn-independent DksA in Zn homeostasis and stringent response

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Summary

DksA is a global transcriptional regulator that directly interacts with RNA polymerase (RNAP) and, in conjunction with an alarmone ppGpp, alters transcription initiation at target promoters. DksA proteins studied to date contain a canonical Cys-4 Zn-finger motif thought to be essential for their proper folding and thus activity. In addition to the canonical DksA protein, the *Pseudomonas aeruginosa* genome encodes a closely related paralogue DksA2 that lacks the Zn-finger motif. Here, we report that DksA2 can functionally substitute for the canonical DksA *in vivo* in *Escherichia coli* and *P. aeruginosa*. We also demonstrate that DksA2 affects transcription by the *E. coli* RNAP *in vitro* similarly to DksA. The *dksA2* gene is positioned downstream of a putative Zur binding site. Accordingly, we show that *dksA2* expression is repressed by the presence of exogenous Zn, deletion of Zur results in constitutive expression of *dksA2*, and Zur binds specifically to the promoter region of *dksA2*. We also found that deletion of *dksA2* confers a growth defect in the absence of Zn. Our data suggest that DksA2 plays a role in Zn homeostasis and serves as a back-up copy of the canonical Zn-dependent DksA in Zn-poor environments.

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Introduction

Tight control of zinc (Zn) homeostasis is critical for cell viability. Even though the activity of many essential proteins, such as DNA and RNA polymerases (RNAPs), ribosomal proteins and multiple metabolic enzymes, is strictly dependent upon Zn, excess Zn is toxic. In environments with varying Zn concentrations, specific genes are turned off or on to maintain the optimal intracellular Zn quota. In all three kingdoms, these genes encode a variety of Zn transport systems, chaperones and Zn-binding proteins (Blencowe and Morby, 2003).

Using both narrow and broad-specificity import systems, *Escherichia coli* can actively accumulate Zn(II) to a level of 200 000 atoms per cell (Outten and O'Halloran, 2001), which corresponds to 0.2 mM, a 1000-fold excess over the typical Zn concentration in the medium. However, biochemical measurements indicate that there is essentially no free Zn in an *E. coli* cell (Outten and O'Halloran, 2001), suggesting that, once imported, Zn becomes sequestered by cellular proteins. Zn-binding proteins account for 5% of the proteome (Andreini *et al.*, 2006), and ribosomes most likely constitute the largest Zn reservoir. Indeed, a rapidly growing *E. coli* cell contains as many as 50 000 ribosomes (Bremer and Dennis, 2008), each with approximately three bound Zn ions, thereby tying up 75% of all Zn. Other abundant proteins must sequester the remaining Zn pool; RNAP (present at ~2000 copies per cell and bound to two Zn ions) is one of many examples.

Zn frequently plays a key role as a catalytic and/or structural cofactor in proteins essential for viability. Under conditions of Zn limitation, for example upon entry into vertebrate hosts that sequester Zn to guard against infection (Kehl-Fie and Skaar, 2010), cells must be able to acquire sufficient Zn. Adaptation to Zn depletion depends primarily on Zur, a transcriptional repressor from the Fur family of proteins; Zur orthologues are present in many bacterial species (Lee and Helmann, 2007). In the presence of Zn, Zur binds to operator sequences upstream of target genes, preventing binding of RNAP and thus transcription initiation. Conversely, upon Zn depletion, repression by Zur is lifted and expression of target genes is increased.

Simulating Zn-depleted environments in the laboratory has proven difficult because common metal chelators

exhibit broad specificity that precludes targeted depletion of Zn from the culture medium. In most cases, the absence of the high-affinity Zn(II) transporter ZnuABC is required to observe growth defects linked to the deletion of genes involved in Zn homeostasis (Gabriel and Helmann, 2009; Petrarca *et al*., 2010). By growing *E. coli* under continuous culture conditions in a specially designed metal-free chemostat, sufficient Zn depletion was achieved to reveal growth defects in the wild-type *znuABC* background (Graham *et al*., 2009). However, this approach is labour-intensive and not amenable to a broad study of Zn homeostasis mechanisms across the bacterial kingdom. Fortunately, identification of putative Zur binding sites has proven to be a productive way to identify novel proteins involved in the adaptation to Zn-limited environments (Panina *et al*., 2003; Haas *et al*., 2009).

The ZnuABC transporter imports Zn(II) in an ATP-dependent manner and is thought to be the main target for Zur regulation (Patzer and Hantke, 2000). However, the Zur regulon in several bacteria is not limited to Zn transporters, and other mechanisms that allow adaptation to Zn-depleted environments have been described. For example, comparative genomic approaches led to the initial identification of four ribosomal protein paralogues (Makarova *et al*., 2001; Panina *et al*., 2003). In contrast to the main copies that contain Cys-4 Zn-ribbon motifs (and are thus called C^+), the C^- duplicates lack the key Cys residues, do not bind Zn and are repressed by Zur. When Zn is scarce, these C^- paralogues are expressed and substitute for the C^+ proteins in ribosomes (Natori *et al*., 2007; Gabriel and Helmann, 2009). This mechanism is proposed to increase cell survival in Zn-limiting conditions by supplying functional copies of Zn-free proteins for the newly made ribosomes, while at the same time liberating the pool of Zn through dissociation from the existing ribosomes, and subsequent degradation of the C^+ proteins.

Using a comparative genomic approach, we have recently identified six additional paralogues of Zn-dependent proteins in γ- and β-proteobacteria (Haas *et al*., 2009). It remains to be determined whether these proteins, whose genes are positioned downstream from putative Zur binding sites, simply replace their Zn-dependent counterparts when the activity of the latter is adversely affected by the absence of Zn or play distinct roles in the cell. In this work, we focus on the division of labour between two DksA paralogues (DksA and DksA2) in *Pseudomonas aeruginosa* and a possible role of DksA2 in Zn homeostasis.

DksA was initially identified in *E. coli* (EC) as a suppressor of the *dnaK* phenotype (Kang and Craig, 1990). Since then, DksA was shown to act synergistically with (p)ppGpp to control the bacterial response to stress and starvation (Paul *et al*., 2004; Potrykus and Cashel, 2008). The X-ray structure of EC DksA (Perederina *et al*., 2004) revealed that it belongs to a class of bacterial transcription factors (which includes GreA, GreB and GfhI) that bind within the RNAP secondary channel near the active site located at the base of this channel. These regulators share a common two-domain organization and the mode of binding to the RNAP (Stebbins *et al*., 1995; Perederina *et al*., 2004; Laptenko *et al*., 2006; Symersky *et al*., 2006): their coiled-coil domains extend towards, and modulate various activities of the RNAP active site whereas their globular domains likely bind to a β′ rim helices domain that lies at the entrance into the channel (Laptenko *et al*., 2003; Perederina *et al*., 2004; Vassylyeva *et al*., 2007). On the other hand, these proteins have very different effects on transcription: Gre factors act to rescue arrested [e.g. upon nucleotide misincorporation (Zenkin *et al*., 2006)] transcription elongation complexes, whereas DksA destabilizes initiation complexes (Paul *et al*., 2004; 2005).

The stringent response enables rapid and global change of gene expression following nutrient stress, which leads to a rapid increase in ppGpp levels (Magnusson *et al*., 2005; Srivatsan and Wang, 2008). DksA/ppGpp strongly inhibits transcription of rRNA genes while activating genes for amino acid biosynthesis and transport. Both effects utilize the main activity of DksA: destabilization of open promoter complexes. At the *rrn* promoters, open complexes are very unstable, and further destabilization essentially abolishes transcription of rRNA genes. Conversely, RNAP forms very stable complexes at amino acid promoters such as *hisG*. DksA and ppGpp destabilize these complexes and increase transcription *in vitro* (Paul *et al*., 2005). *In vivo*, a part of the control could be through liberating RNAP from *rrnB* promoters that account for 70% of the total RNA synthesis in rapidly growing cells (Zhou and Jin, 1998). The end result of this dual control is the restored balance between ribosome production and available amino acid pools. Interestingly, ppGpp and DksA may play independent, or even opposing, roles at some *E. coli* promoters (Magnusson *et al*., 2007; Aberg *et al*., 2008; Dalebroux *et al*., 2010) and during replication (Trautinger *et al*., 2005; Tehranchi *et al*., 2010).

DksA proteins characterized to date contain a canonical Cys-4 Zn-finger motif. Structural analysis of the EC DksA suggests that this motif plays a key role by maintaining the fold of the globular domain and its orientation relative to the 'catalytic' coiled coil (Perederina *et al*., 2004). The Zn(II) ion is chelated by two cysteines from each domain and cannot be mobilized after extensive dialysis in the presence of chelators; mutation of any of the four Cys residues renders DksA non-functional (Paul *et al*., 2004; Perron *et al*., 2005). However, several DksA paralogues that lack the canonical Zn-finger exist (Fig. 1). For example, *P. aeruginosa* encodes both the Zn-finger DksA and its C^- paralogue DksA2. The *dksA2* gene is located downstream of a putative Zur binding site (Haas *et al*.,

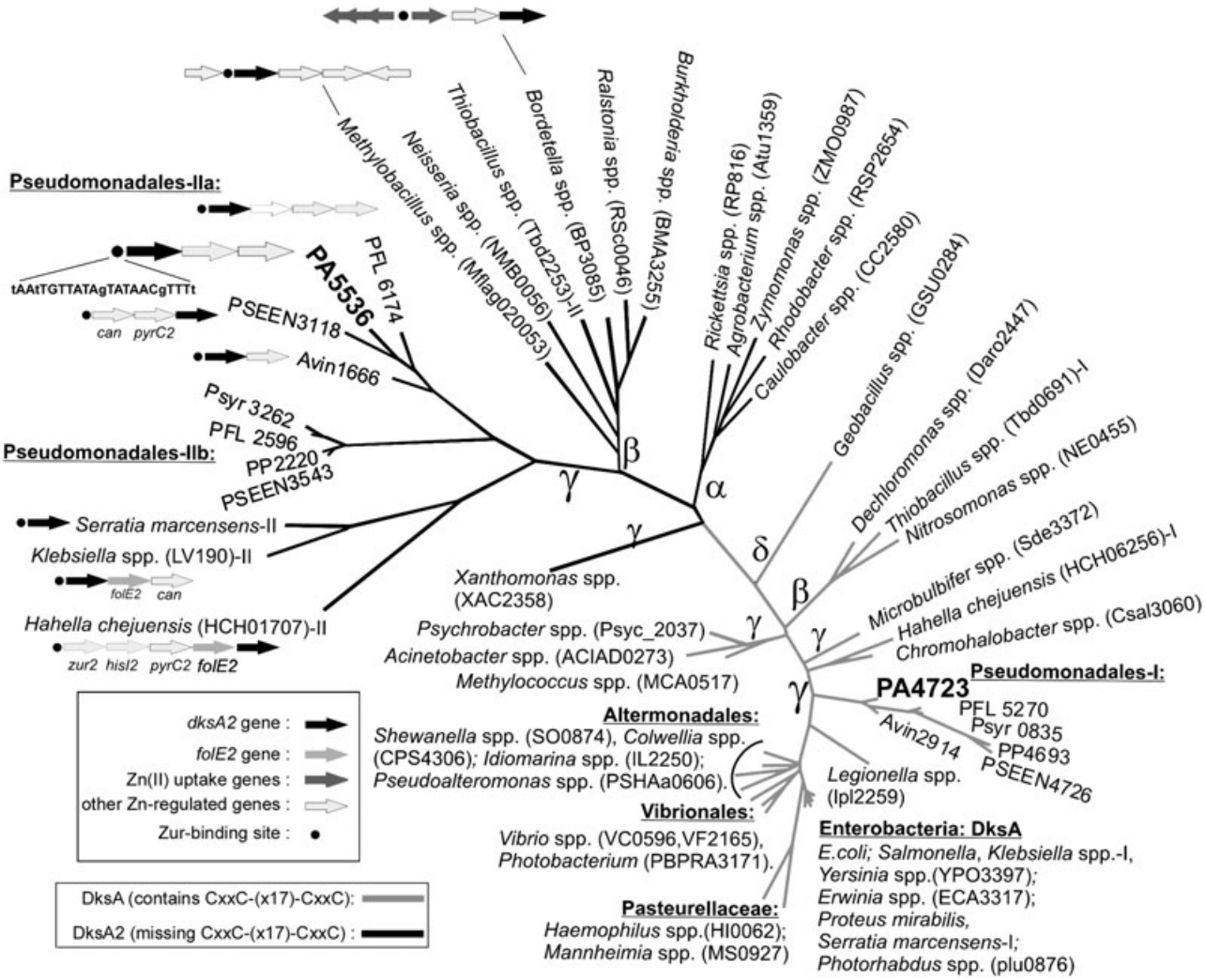

Fig. 1. Phylogenomic analysis of DksA and DksA2. Operon organization and location of putative Zur binding site are given for putatively Zur-regulated *dksA2* genes. Representative protein names for each branch are given in parentheses. To highlight that some organisms contain both DksA and DksA2, 'I' (for DksA) or 'II' (for DksA2) is given. For PA5536 (DksA2), in addition to the operon organization, the sequence of the predicted Zur binding site is indicated.

2009), suggesting that it may be induced upon Zn depletion. These observations suggest a model in which PA DksA and DksA2 have similar molecular mechanisms but act in different (Zn-rich and Zn-poor) conditions respectively. In this work, we set out to investigate whether DksA2 is a functional paralogue of DksA and is upregulated in Zn-depleted environments, as the model implies.

Results

Phylogeny of the DksA family of proteins

Proteins belonging to the DksA/TraR superfamily are present throughout the bacterial kingdom (Marchler-Bauer *et al*., 2009) and the majority of these proteins are of unknown function. In addition to the characterized DksA protein (PA4723), which has been shown to be involved in the stringent response, the *P. aeruginosa* genome (Stover *et al*., 2000) encodes four other proteins from this superfamily (Fig. S1). Three of these DksA-like proteins (PA4577, PA4870 and PA0612) contain the characteristic CxxC-(x17)-CxxC Zn-finger motif but otherwise have low sequence homology to DksA (24%, 16% and 14% identity respectively). The fifth DksA-like protein (PA5536), which we refer to here as DksA2, has significant sequence homology to DksA (34% identity) but contains a CxxT-(x17)-CxxA motif instead of the typical Cys-4 Zn-finger motif.

DksA2 is found in other bacteria and can be identified based on the presence of the Cxx[S/T]-(x17)-[C/S/T]xxA motif. We initially found *dksA2* as part of the computa-

tionally reconstructed Zur regulon in several γ- and β-proteobacteria (*Serratia, Klebsiella, Hahella, Pseudomonas* spp. and *Methylobacillus*) (Haas *et al*., 2009). As such, *dksA2* is often clustered physically on the chromosome with factors known to be involved in the response to Zn depletion, such as *znuABC* (Fig. 1). In genomes that contain both *dksA* and *dksA2*, such as *P. aeruginosa, dksA2* is usually found downstream of putative Zur binding sites (Fig. 1). This situation is similar to the distribution pattern of *folE* and *folE2* genes. The *folE2* gene is found downstream of putative Zur binding sites in genomes that contain both genes (Sankaran *et al*., 2009). Also like *folE2*, when only *dksA2* is present in a genome, Zur binding sites are not identified upstream. This appears to be the case for *dksA2* in several α- and β-proteobacteria (Fig. 1).

Exceptions to this pattern of distribution do occur in several *Pseudomonas* and *Bordetella* species. *Pseudomonas fluorescens* and *Pseudomonas entomophila* have one *dksA* and two *dksA2* genes (*dksA2-a* and *dksA2-b*); *dksA2-a* but not *dksA2-b* is predicted to be regulated by Zur. The presence of two *dksA2* genes could be explained by a late *Pseudomonas* clade-specific duplication of *dksA2* as some *Pseudomonas* spp. have subsequently lost one of the two *dksA2* paralogues. For instance, *P. aeruginosa* has retained *dksA2-a*, whereas *Pseudomonas putida* has kept *dksA2-b*. In several *Bordetella* species, the *dksA2* gene may be a part of a Zur-regulated operon even though the canonical *dksA* gene is not present.

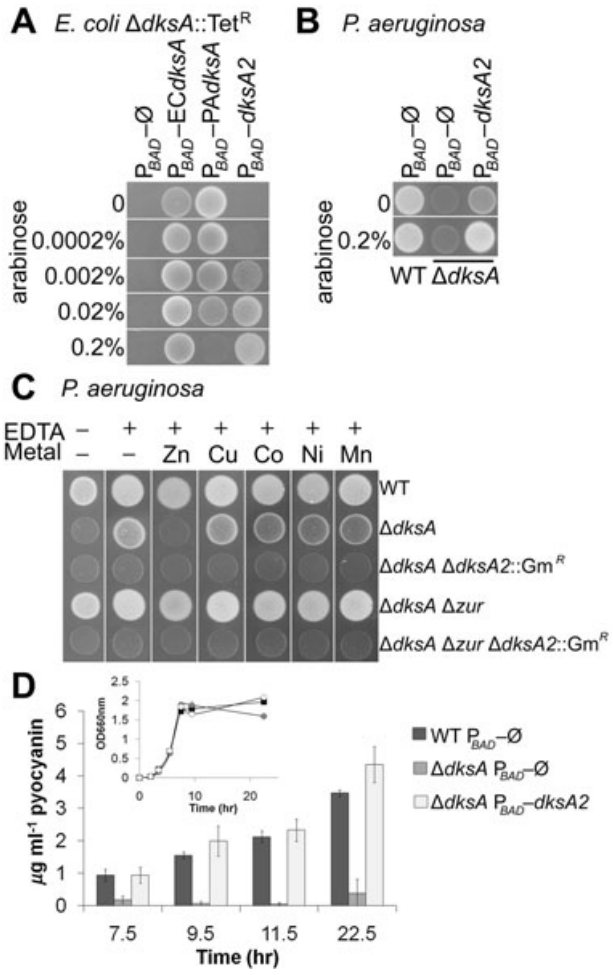

Fig. 2. Rescue of *E. coli* Δ*dksA*::Tet^R and *P. aeruginosa* Δ*dksA* phenotypes.
A. Growth of *E. coli* Δ*dksA*::Tet^R derivatives on M9 medium with the indicated constructs *in trans*.
B. Growth of *P. aeruginosa* derivatives on M9 medium with the indicated constructs *in trans*.
C. Growth of *P. aeruginosa* mutants on M9 medium ± 100 μM EDTA and ± 25 μM metal. Exactly 10^{-3} dilutions of normalized cultures are shown for (A)–(C).
D. Pyocyanin production of WT P_{BAD}–Ø, Δ*dksA* P_{BAD}–Ø and Δ*dksA* P_{BAD}–*dksA2* at various times during growth in LB at 37°C; corresponding growth curves are given in insert.

DksA2 in trans *rescues the amino acid auxotrophy of* E. coli ΔdksA

PA DksA (PA4723) has been the focus of several studies examining its roles in the quorum-sensing circuitry, rRNA transcription and survival during antibiotic stress (Branny *et al*., 2001; Jude *et al*., 2003; Perron *et al*., 2005; Viducic *et al*., 2006). DksA2 (PA5536), on the other hand, is annotated as a conserved hypothetical protein [*Pseudomonas* genome database (Winsor *et al*., 2009)]. In order to determine whether the DksA2 protein of *P. aeruginosa* can functionally replace DksA, we took advantage of the amino acid auxotrophy of a Δ*dksA E. coli* strain, which is unable to grow in minimal media lacking leucine, valine, glycine, phenylalanine or threonine (Brown *et al*., 2002). The *E. coli* genome does not contain a *dksA2* gene as discussed above.

As shown in Fig. 2A, *dksA2* expressed *in trans* from P_{BAD} was able to rescue the growth defect of the *E. coli* Δ*dksA* strain grown in minimal medium lacking casamino acids. Compared with EC *dksA* or PA *dksA*, where uninduced expression from P_{BAD} was sufficient for complementation, rescue by *dksA2* required a higher concentration of the inducer arabinose (0.002–0.2%). As previously shown for EC *dksA* (Potrykus *et al*., 2006), overexpression of PA *dksA* was toxic in the presence of 0.2% arabinose. Toxicity was not observed when expressing *dksA2* with the arabinose concentrations used in this study.

We next tested if changing the cysteines in the Cys-4 Zn-finger motif of the PA DksA to the corresponding residues in DksA2 would abolish the ability to rescue growth of the *E. coli* Δ*dksA* strain. Substitutions of Cys-132 and Cys-135 to Ser in PA DksA were previously found to abolish function (Perron *et al*., 2005). We substituted the

conserved Cys-114 and Cys-135 in PA DksA with Thr and Ala respectively. We found that both substitutions eliminated the ability of PA DksA to restore growth of *E. coli* Δ*dksA* (Fig. S2). This result is consistent with a key role of the Zn-finger motif in the DksA structure and thus, function.

DksA2 can functionally replace P. aeruginosa *DksA* in vivo

We then turned to phenotypic studies directly in *P. aeruginosa* PAO1 and assessed whether *dksA2* expression is regulated by Zn, as suggested by the presence of a putative Zur binding site upstream. Like in *E. coli*, deletion of the *dksA* gene in *P. aeruginosa* leads to a growth defect in M9 minimal media with glucose (0.2% w/v) as a sole carbon source (Jude *et al*., 2003), an effect which we reproduced in M9 minimal medium with glycerol as a sole carbon source (Figs 2B and S3A and B). We found that, similarly to the situation observed in a heterologous *E. coli* host, *dksA2* expressed from P_{BAD} complemented the growth phenotype of the *P. aeruginosa* Δ*dksA* strain (Figs 2B and S3A), strongly suggesting that DksA2 can functionally replace PA DksA.

We found that the growth defect of Δ*dksA* could be suppressed by the addition of 100 μM ethylenediaminetetraacetic acid (EDTA) or by combining the *dksA* deletion with the deletion of the gene encoding the Zur homologue (PA5499, *np20*) (Figs 2C and S3B and C). In both cases, suppression was dependent on the presence of *dksA2 in cis* (on the chromosome) (Figs 2C and S3B) or *in trans* (expressed from P_{BAD}) (Fig. S3E and F and S4). The addition of Zn, but not other transition metals tested, counteracted the suppression effect of EDTA (Figs 2C and S3D). In contrast, Zn did not affect growth of the Δ*dksA* Δ*zur* strain (Fig. 2C).

During these experiments, we noticed that pyocyanin was differentially produced in various strains and that these trends mimicked the growth defects observed above (Fig. S5). Pyocyanin is a secreted virulence factor that is thought to play a role in the tissue damage of infected hosts (Caldwell *et al*., 2009). The synthesis of this metabolite is regulated by quorum sensing, and PA DksA was initially characterized in a complementation screen of a quorum-sensing mutant (Branny *et al*., 2001). During growth in Luria broth at 37°C, the Δ*dksA* strain produced less than 10% of the pyocyanin produced by the parent strain (Fig. 2D). Pyocyanin production was restored by expressing *dksA2 in trans* (Fig. 2D).

DksA2 is repressed by Zn and Zur binds specifically to the dksA2 *promoter region*

These results, together with the promoter region organization of the *dksA2* gene (Fig. 1), suggest that *dksA2* is regulated by Zur in *P. aeruginosa*, and that its expression may be activated under Zn limitation. We therefore analysed the effect of EDTA and extracellular Zn on *dksA* and *dksA2* transcript levels by qRT-PCR and on DksA and DksA2 protein levels by Western blotting. As shown in Fig. 3A, the abundance of *dksA* transcript was not significantly affected by either EDTA or Zn and was found to gradually decrease throughout the growth cycle as previously described (Perron *et al*., 2005) (growth curves available in Fig. S6). In contrast, as shown in Fig. 3B, *dksA2* was not significantly expressed in the WT strain until late logarithmic/early stationary phase when grown in M9 medium, the same growth condition that gave rise to the Δ*dksA* growth defect described above. In the presence of EDTA, which was able to suppress the Δ*dksA* phenotype, the level of *dksA2* transcript was increased during early/mid logarithmic phase (Fig. 3B). The *dksA2* transcript was undetectable throughout the growth cycle when the cells were grown in the presence of 25 μM $ZnSO_4$ (Fig. 3B). In the absence of Zur, Zn failed to repress *dksA2* expression compared with the strain grown in M9 medium without supplementation (Fig. 3C). The abundance of DksA and DksA2 (as discerned from the Western blot) paralleled changes in the transcript levels (Fig. S7).

As expected from the expression results above, His_6-Zur was found to bind specifically to the promoter region of *dksA2* in the presence of Zn (Figs 3D and S8). In the absence of Zn, binding is observed but only in the presence of higher concentrations of purified His_6-Zur (Fig. S8A). In the presence of 100 μM EDTA, binding is abolished (Fig. S8A). Competition with the promoter DNA was achieved with specific competitor (annealed oligonucleotides containing the putative binding site) but not with non-specific competitor (control oligonucleotides which lack a consensus Zur binding site) (Fig. S8B).

Deletion of dksA2 *leads to an increased sensitivity to metal chelators*

We hypothesized that DksA2 may be a condition-specific functional variant of PA DksA, and our analysis suggests that *dksA2* is expressed during Zn depletion. Therefore, we wanted to test whether the deletion of *dksA2* gives rise to a growth defect under metal depletion conditions.

We compared the growth of WT and Δ*dksA2 P. aeruginosa* strains in the presence of metal chelators. The absence of *dksA2* resulted in an observable growth defect in the presence of EDTA or *N,N,N′,N*-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), the chelators used frequently to mimic Zn limitation (Figs 4 and S9). To confirm that this phenotype was due specifically to Zn depletion (as opposed to that of another metal), we

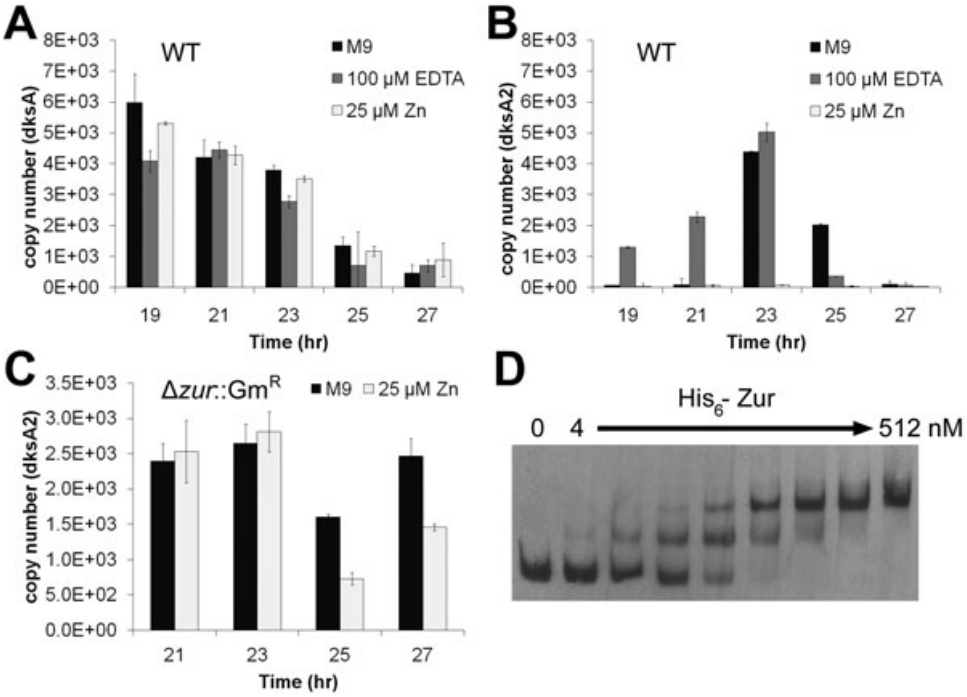

Fig. 3. Transcript abundance of PA DksA and DksA2 and binding of His_6-Zur to the promoter region of *dksA2*.
A and B. Average abundance of *dksA* (A) and *dksA2* (B) transcript in total mRNA extracted from *P. aeruginosa* at 19, 21, 23, 25 and 27 h of growth in M9 medium without supplementation (M9), plus 100 μM EDTA or plus 25 μM $ZnSO_4$.
C. Average abundance of *dksA2* transcript in total mRNA extracted from *P. aeruginosa* Δ*zur*::Gm^R at 21, 23, 25 and 27 h of growth in M9 medium without (M9) or with 25 μM $ZnSO_4$. Copy number refers to the number of *dksA* or *dksA2* transcripts per 0.1 ng of total mRNA. Error bars represent ± one standard deviation of three replicates.
D. Binding of purified His_6-Zur to the promoter region of *dksA2*. Increasing concentrations of Zur (4, 8, 16, 32, 64, 128, 256 and 512 nM; corresponding to the monomeric unit) were incubated in the presence of 100 μM $ZnSO_4$ with 1.5 ng of a biotin-labelled 223 bp fragment surrounding the putative Zur binding site.

deleted *znuA* in the Δ*dksA2* background. *znuA* encodes a homologue of the periplasmic chaperone component of the high-affinity Zn transporter ZnuABC, and its deletion impairs Zn uptake (Patzer and Hantke, 1998). Growth of WT, Δ*dksA2*, Δ*znuA*::Gm^R and Δ*dksA2* Δ*znuA*::Gm^R strains on minimal media were compared in the presence of various concentrations of EDTA and TPEN (Fig. S9). As shown in Fig. 4, deletion of *znuA* exacerbated the growth defect due to the deletion of *dksA2* in the presence of 1.5 mM EDTA or 5 μM TPEN. The growth defect was rescued by *dksA2* expressed *in trans* from P_{BAD} at 0.2% arabinose (Fig. 4). As further confirmation that the EDTA-sensitive phenotype of the *dksA2* mutant is due to depletion of Zn, 25 μM of various transition metals were added to the medium containing either 1.25 mM or 1.5 mM EDTA. Only the addition of Zn was able to suppress the growth defect caused by the presence of EDTA (Fig. S9C and D).

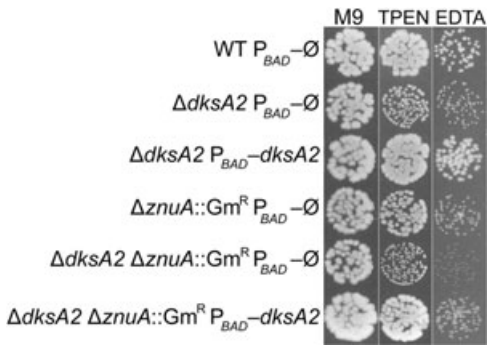

Fig. 4. Growth defect of *P. aeruginosa* Δ*dksA2* during metal depletion. Strains were grown on M9 medium with 0.2% arabinose without supplementation (M9), with 1.5 mM EDTA or 5 μM TPEN. The 10^{-5} dilution of normalized cultures is shown.

DksA2 inhibits transcription from the rrnB *P1 promoter* in vitro

The rescue experiments in *E. coli* and *P. aeruginosa* (see above) suggest that DksA2 can functionally replace DksA by binding to the RNAP and altering its properties. This idea is consistent with the common origin of the two proteins; other paralogous transcription regulators such as σ initiation factors (Burgess and Anthony, 2001) or NusG/RfaH elongation factors (Belogurov *et al.*, 2009) have been shown to bind to a shared target on RNAP,

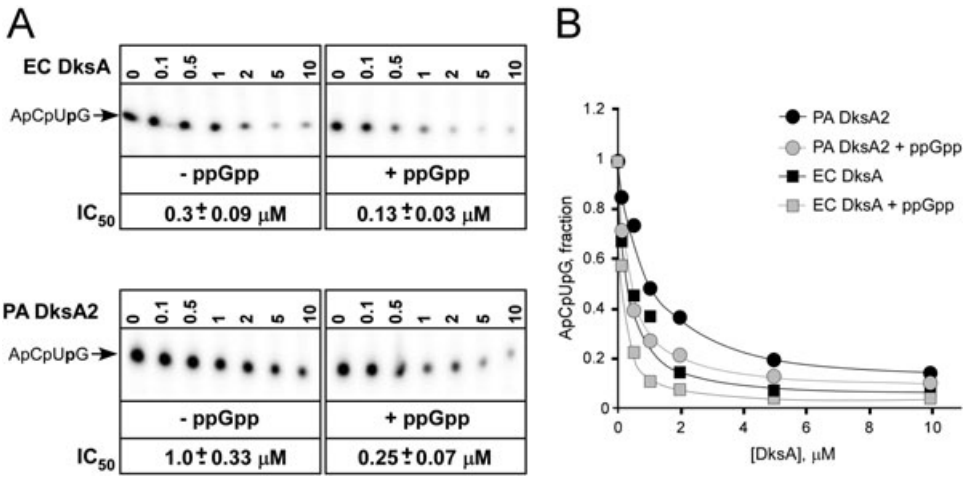

Fig. 5. Inhibition of transcription from the *E. coli rrnB* P1 promoter by DksA and DksA2.
A. *E. coli* DksA or *P. aeruginosa* DksA2 were incubated with RNAP for 15 min prior to the addition of the DNA template.
B. Different concentrations of DksA or DksA2 were added to RNAP in the presence or absence of 50 μM ppGpp. The concentration of DksA at which 50% of transcription is inhibited (IC_{50}) was calculated from three repeats using the Scientist software (MicroMath).

even though they have lower identity scores. It is also possible that DksA2 exerts its effect indirectly, through some alternative pathway. To distinguish between these possibilities, we tested the effect of DksA2 in a purified *in vitro* system.

The main targets of DksA are the ribosomal *rrn* promoters, which account for ~70% of the transcription in the cell. The EC DksA effect at the *rrnB* P1 promoter has been extensively studied both *in vivo* and *in vitro*. Since our genetic analysis indicates that DksA2 functions in *E. coli*, we used a well-established *in vitro* transcription assay to test the effect of DksA2 on initiation from the *E. coli rrnB* P1 promoter. We found that, similarly to DksA, DksA2 inhibited transcription from *rrnB* P1 (Fig. 5). The effects of both proteins were potentiated in the presence of 50 μM ppGpp. The concentration of DksA2 required for half-maximal inhibition was approximately threefold higher as compared with that of the EC DksA, suggesting that it binds more weakly to the EC RNAP (Fig. 5A). This is not surprising given that the DksA and RNAP regions that are proposed to mediate their interactions (Perederina *et al.*, 2004) display significant sequence divergence.

DksA2 destabilizes open promoter complexes

Transcription initiation is a multi-step process that proceeds through several intermediates, which differ in interactions between the RNAP and the promoter DNA. In the initial encounter complex, the double-stranded DNA is loosely bound on the RNAP surface via sequence-specific contacts to the σ factor. This closed promoter complex (called RP_c) is highly unstable and the RNAP is in equilibrium with the free enzyme in solution. Through a series of isomerization steps (which may differ at different promoters), the RNAP melts the promoter DNA segment between the −12 and +2 positions (relative to the transcription start site) to form a catalytically competent open promoter complex (RP_o).

Escherichia coli DksA was shown to reduce the RP_o stability at different promoters but the regulatory outcome of this destabilization differs depending on the specific features of the promoters. At promoters which form very stable open complexes (such as λ P_R with the half-life on the order of hours *in vitro*), EC DksA may not have any effect or act as an activator (Paul *et al.*, 2005; Łyzen *et al.*, 2009). In contrast, EC DksA (particularly in the presence of ppGpp) reduces transcription from promoters that form highly unstable open complexes (such as *rrnB* P1, $t_{1/2}$ on the order of seconds). To test the effect of DksA2 on the open complex stability we used a model *lac*UV5 promoter, which forms an RP_o with an intermediate, readily measurable half-life (Fig. 6A). To measure the rate of RP_o dissociation, we preformed the complexes in the presence or in the absence of EC DksA or DksA2 and then challenged them with heparin, a non-specific competitor that would trap the free RNAP that had dissociated from the DNA. The open promoter complex was resistant to heparin challenge whereas RP_c was highly sensitive. Following heparin addition, nucleotide substrates were added to measure the fraction of the remaining active RP_o (Fig. 6). We found that DksA2 destabilized *lac*UV5 open complexes ($t_{1/2}$ = 44 s in the absence of added factors) somewhat more efficiently than EC DksA; the half-life was decreased to 11 and 16 s respectively (Fig. 6B). As in the case of DksA (Paul *et al.*, 2005; Rutherford *et al.*, 2009), DksA2 destabilized complexes at another model promoter (Fig. S10).

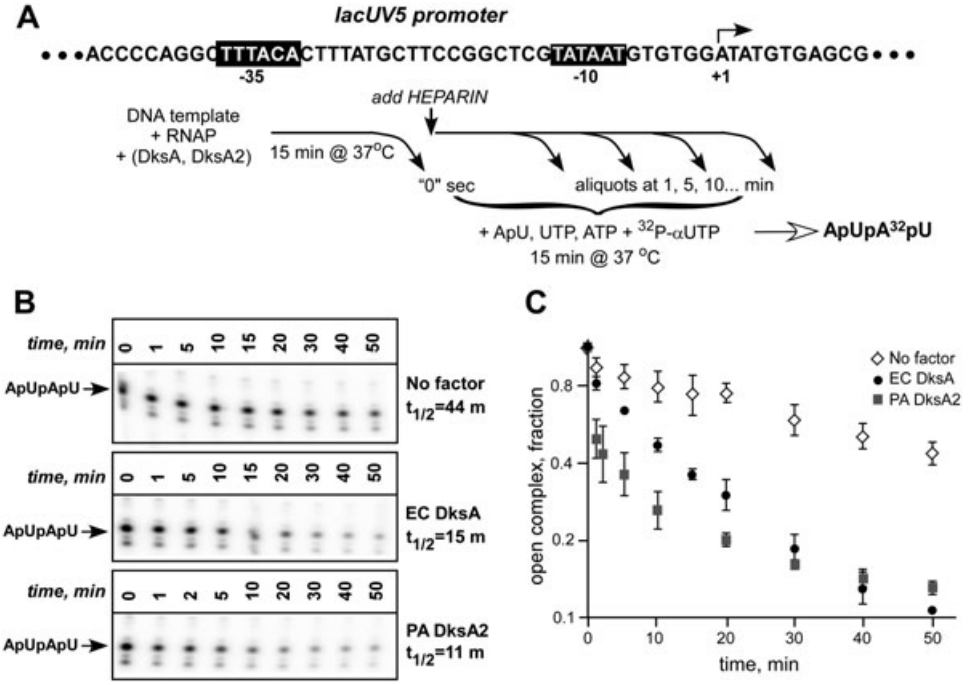

Fig. 6. Destabilization of the open promoter complex by DksA and DksA2 at the *lac*UV5 promoter.
A. Schematics of the assay.
B. Decay of the open complexes in presence or absence of 2 μM DksA or DksA2 was monitored by following the formation of short abortive transcripts radiolabelled with [α-^{32}P]-UMP at the selected times following the addition of heparin.
C. Quantification of the open complexes stability, with and without DksA proteins. The errors (±one standard deviation) were determined from three independent experiments.

DksA2 traps the closed promoter complex at rrnB *P1*

Heparin sensitivity of the T7A1 transcription complexes suggests that DksA2 affects a step in the open complex formation pathway. However, this pathway includes several intermediate complexes which can be variably sensitive to heparin challenge. We decided to evaluate the effect of DksA2 on the promoter complex formation pathway in more detail using *rrnB* P1 promoter, the main physiological target of DksA, as a model.

Studies in the Gourse lab revealed that RP_o formed at the *rrn* P1 promoters are highly unstable in the absence of the nucleotide substrates (Barker and Gourse, 2001); this property ties the rRNA synthesis to the cellular pool of ATP and GTP (Schneider *et al*., 2002), and thus the translational capacity of the cell. Additional level of regulation by ppGpp and DksA serves to co-ordinate the rRNA synthesis with the nutrient availability and other environmental factors. Gourse and co-authors argued that EC DksA inhibits transcription of the *rrn* genes by stabilizing a transcriptionally inactive promoter complex at *rrnB* P1 (Rutherford *et al*., 2009). Their DNase I footprinting analysis revealed that, in the absence of the initiating substrates which lock the complex in the open conformation, EC DksA shifted the downstream boundary of DNase I protection from ~+12 to ~+1 even at 37°C. This footprinting pattern is characteristic for the closed RP_c complex and readily explains the inhibitory effect of EC DksA at the *rrnB* P1 promoter.

Similar effects of the EC DksA and DksA2 on transcription from *rrnB* P1 (Fig. 5) suggest that the two proteins may share the molecular mechanism of inhibition. To test this idea, we performed DNase I footprinting analysis on a linear *rrnB* P1 promoter fragment (Fig. 7). As reported by Rutherford *et al*., the contacts between the RNAP and the downstream duplex DNA became destabilized upon the addition of DksA, as evidenced by the loss of protection downstream from the +1 position. Addition of DksA2 led to a similar loss of the downstream protection. As previously observed for EC DksA (Rutherford *et al*., 2009), DksA2 did not change the pattern of protection in the presence of ATP and CTP, the nucleotides incorporated at the +1 and +2 positions of the nascent RNA. These results support the hypothesis that DksA2 shares the molecular mechanism with the 'primary' DksA and can functionally substitute for it when Zn becomes scarce.

Purified DksA2 does not contain Zn(II)

Previously, EC DksA was found by ICP-MS (Inductively Coupled Plasma – Mass Spectrometry) to have one Zn per monomer and was crystallized with the Zn bound to

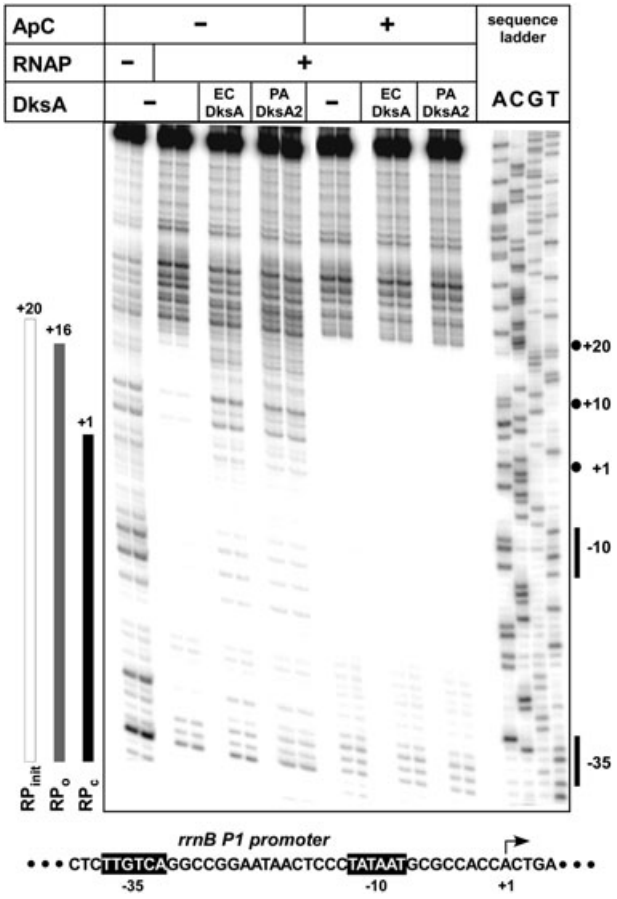

Fig. 7. Stabilization of the RPc complex at the *rrnB* P1 promoter by DksA and DksA2. A linear DNA fragment encompassing positions –60 to +10 of the *rrnB* P1 promoter was generated by PCR; the non-template DNA strand was end-labelled with [^{32}P]-ATP (see *Experimental procedures*). The footprint boundaries in the promoter region shown are indicated by black (RP_c), grey (RP_o) and white (RP_{init}, stabilized with ApC) bars on the left. DksA and DksA2 apparently destabilize the RP_o and shift the complex towards an RP_c-like state. The dideoxy-sequencing ladder is shown for reference.

the four cysteines of the Zn-finger motif (Paul *et al*., 2004; Perederina *et al*., 2004). As two of those four cysteines are absent from DksA2, Zn binding may have been lost. We tested EC DksA and PA DksA2 preparations for metal content using ICP-MS. As shown in Table 1, Zn was found in association with EC DksA (at a ratio of DksA to Zn of 1:0.67) but not with DksA2. Furthermore, X-ray crystallographic analysis of DksA2 (O. Tsodikov, pers. comm.) also failed to reveal a bound metal ion.

Discussion

Zn depletion can have detrimental effects on bacterial cell viability and can impede its ability to infect a vertebrate host. *P. aeruginosa* is a significant human opportunistic pathogen and the primary cause of mortality among cystic fibrosis patients. As part of the acute immune response, the host actively sequesters Zn, limiting its availability to the invading pathogen. One common solution is to induce the expression of a high-affinity Zn(II) transporter and transport available Zn from the environment. Indeed, high-affinity Zn(II) transporters have been shown to be required for virulence of several pathogens (Campoy *et al*., 2002; Kim *et al*., 2004; Yang *et al*., 2006; Ammendola *et al*., 2007; Davis *et al*., 2009; Dahiya and Stevenson, 2010). Other strategies may include expression of Zn-independent functional copies of key proteins, sometimes coupled to mobilization of protein-bound Zn (Panina *et al*., 2003; Akanuma *et al*., 2006; Gabriel and Helmann, 2009; Sankaran *et al*., 2009). Our analysis argues that *P. aeruginosa* utilizes the DksA2 protein as a part of the adaptation to Zn-limited environments.

DksA2 was initially discovered as an unknown protein putatively regulated by the Zn(II)-responsive transcription factor Zur (Haas *et al*., 2009). In support of this prediction, transcript analysis reveals that *dksA2* is expressed during metal depletion and repressed in the presence of Zn (Fig. 3B). This response is mediated by Zur, as expression of *dksA2* is not repressed by Zn in the absence of Zur and Zur was found to bind specifically to the promoter region of *dksA2* (Fig. 3C and D). These results suggest that DksA2 exerts its activity when cells encounter an environment poor in available Zn. In agreement with this hypothesis, DksA2 is induced during growth in cystic fibrosis sputum (Palmer *et al*., 2005). Sputum from cystic fibrosis patients was shown to have high levels of calprotectin (Gray *et al*., 2008), a neutrophil protein that chelates Zn making it unavailable to pathogens (Clohessy and Golden, 1995).

DksA2 lacks a conserved Zn-finger motif and, consequently, the bound Zn. Thus, DksA2 function should be independent of Zn, in contrast to DksA where it appears to be critical. Our growth assays and *in vitro* results reveal that DksA2 can (at least under some conditions) functionally replace DksA. These observations suggest a simple model wherein DksA2 substitutes for Zn-dependent DksA when Zn is scarce. This scenario is reminiscent of the

Table 1. ICP-MS analysis of EC DksA and DksA2.

Isotope	DksA	DksA2
K39	8.57[a]	30.0
Ca42	0	23.7
Mn55	0	0
Fe56	0.173	0.146
Fe57	0.0951	0.0877
Co59	0	0
Ni60	0	0
Cu63	0.118	0
Zn66	12.5	0

a. Units are in $\mu g\ l^{-1}$.

failsafe mechanism of action of a C⁻ S14 ribosomal protein paralogue. In this model, the S14 paralogue, which is missing the Zn-binding motif and does not require Zn, is expressed in the absence of Zn and enables active ribosome biosynthesis (Natori *et al*., 2007; Gabriel and Helmann, 2009).

By analogy, since Zn is likely required for the proper folding and function of DksA, the newly synthesized DksA would be inactive in a Zn-depleted environment, leading to defects in gene expression control. Under these conditions, it could be advantageous to express a back-up, Zn-independent DksA variant. Our data are consistent with a model in which DksA2 plays such a role in *P. aeruginosa*. *In vivo*, DksA2 compensates for the absence of DksA in both *E. coli* and *P. aeruginosa* during growth in minimal media and suppresses the pyocyanin deficiency phenotype of the *P. aeruginosa* Δ*dksA* strain (Fig. 2). These data suggest that DksA2 functions similarly to DksA in regulation of starvation and quorum sensing responses respectively. *In vitro*, purified DksA2 stabilizes the closed promoter complex at, and thus inhibits transcription from, the *rrnB* P1 promoter (Figs 5 and 7), the main target of DksA, as well as destabilizes open promoter complexes at the *lac*UV5 and T7A1 promoters (Figs 6 and S10), suggesting that DksA2 acts directly on RNAP.

These observations are consistent with a model where DksA (that requires Zn) can be functionally replaced with its paralogue DksA2 (that does not require Zn) when cellular Zn levels are low. However, it is quite possible that DksA2 plays additional roles in cellular physiology. For example, DksA2 may regulate promoters required for adaptation to various stresses, in essence acting as a specialized version of DksA. The growth defects observed in the presence of EDTA and TPEN could result from altered regulation at yet unknown promoters.

The discovery of the DksA paralogue, DksA2, adds an extra level onto the already complex global regulation of gene expression by RNAP-binding factors. Uniquely, DksA2 brings to light the potential for novel gene regulation during Zn limitation, a condition that triggers an increase in DksA2 levels (Fig. 3). It is also possible that DksA2 (or other DksA paralogues) may be upregulated in response to other environmental stresses, such as nutrient starvation or reactive oxygen species. The DksA family of regulators may be the key players of an elaborate gene expression programme designed to integrate diverse environmental cues and balance the cell's growth under a large variety of conditions.

Experimental procedures

Bioinformatics and phylogenetics

The complete genomes of proteobacteria were downloaded from GenBank (Benson *et al*., 2009). Identification of putative Zur binding sites and regulon reconstruction was performed using the RegPredict web server (http://regpredict.lbl.gov/RegPredict/) (Novichkov *et al*., 2010) as previously described (Schröder *et al*., 2010). The deduced regulatory interactions were stored in the RegPrecise database (http://regprecise.lbl.gov/RegPrecise) (Novichkov *et al*., 2010). Genomic neighbourhood of the *dksA* genes in bacterial genomes was analysed using the SEED database and its tools (Overbeek *et al*., 2005). The phylogenetic tree of the DksA family was constructed by the maximum likelihood method implemented in the PROML program of the PHYLIP package (Felsenstein, 1997) using multiple sequence alignments of protein sequences produced by the MUSCLE program (Edgar, 2004). Sequence identity between the DksA homologues of *P. aeruginosa* was determined with the Needleman–Wunsch Global Sequence Alignment Tool available at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Bacterial strains, plasmids and growth conditions

Escherichia coli K-12 MG1655 and *P. aeruginosa* PAO1 were used as WT strains and routinely grown at 37°C on LB-Lennox medium (LB) or minimal medium containing 1× M9 salts (Sambrook and Russell, 2001), 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 3 mg l^{-1} $FeSO_4 \times 7H_20$ and 0.2% (w/v) glycerol as the carbon source. Media were solidified with 15 g of agar per litre. Construction of *E. coli* Δ*dksA* strain was performed by P1 transduction of the *dksA*::Tet^R allele from strain RLG8124 (Rutherford *et al*., 2009) into MG1655 as described by Miller (1972). For plasmid maintenance in *E. coli*, the medium was supplemented with 100 μg ml^{-1} ampicillin (Amp), 10 μg ml^{-1} tetracycline (Tet) or 15 μg ml^{-1} gentamicin (Gm). For *P. aeruginosa*, 30 μg ml^{-1} Gm was used for marker selection and 200 μg ml^{-1} Amp was used for plasmid maintenance. Strains used in this study are listed in Table 2.

Plasmid construction

Genes of interest were amplified by polymerase chain reaction (PCR) using Phusion® High-Fidelity DNA polymerase (Finnzymes, Oy, Espoo, Finland) according to the manufacturer's guidelines from MG1655 or PAO1 genomic DNA using the primers listed in Table S1 in *Supporting information*. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA). PCR products were purified using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA, USA) and cloned into pBAD24 (Guzman *et al*., 1995) for expression in *E. coli* or pHERD20T (Qiu *et al*., 2008) for expression in *P. aeruginosa*. For site-specific mutagenesis of PA *dksA*, the megaprimer PCR method (Sambrook and Russell, 2001) was employed with primers listed in Table S1. Plasmids used in this study are listed in Table 2.

Construction of P. aeruginosa *mutants*

Primers used in gene deletion constructs are listed in Table S1 in *Supporting information*. For gene replacement, a *sacB*-based strategy (Schweizer and Hoang, 1995) was

Table 2. Strains and plasmids.

Strain	Genotype	Source
E. coli K12 MG1655	F, λ, *rph1*	*E. coli* Genetic Stock Center
P. aeruginosa PAO1	Wild-type strain	*P. aeruginosa* type strain (ATCC 33351)
VDC4098	MG1655 Δ*dksA::*Tet^R	This study
VDC4133	VDC4098 pBAD24	This study
VDC4241	VDC4098 pCH078	This study
VDC4263	VDC4098 pCH080	This study
VDC4614	VDC4098 pCH040	This study
VDC4239	VDC4098 pCH071	This study
VDC4240	VDC4098 pCH075	This study
VDC4489	PAO1 Δ*PA4723*	This study
VDC4525	VDC4489 Δ*PA5536::*Gm^R	This study
VDC4499	VDC4489 Δ*PA5499*	This study
VDC4510	VDC4499 Δ*PA5536::*Gm^R	This study
VDC4607	PAO1 pHERD20T	This study
VDC4666	VDC4489 pHERD20T	This study
VDC4667	VDC4489 pCH115	This study
VDC4610	VDC4525 pHERD20T	This study
VDC4611	VDC4525 pCH115	This study
VDC4612	VDC4510 pHERD20T	This study
VDC4613	VDC4510 pCH115	This study
VDC4640	PAO1 Δ*PA5499::*Gm^R	This study
VDC4615	PAO1 Δ*PA5536*	This study
VDC4635	VDC4615 Δ*PA5498::*Gm^R	This study
VDC4650	Δ*PA5498::*Gm^R pHERD20T	This study
VDC4652	VDC4635 pHERD20T	This study
VDC4653	VDC4635 pCH115	This study

Plasmid	Description	Reference
pBAD24	*E. coli* expression vector, Amp^R	Guzman *et al.* (1995)
pHERD20T	*P. aeruginosa* shuttle vector, Amp^R	Qiu *et al.* (2008)
pCH078	*PA4723* cloned into NcoI/XbaI sites of pBAD24	This study
pCH080	EC *dksA* cloned into NcoI/XbaI sites of pBAD24	This study
pCH040	*PA5536* cloned into NcoI/XbaI sites of pBAD24	This study
pCH071	Product of PA4723 C114T mutagenesis cloned into NcoI/XbaI sites of pBAD24	This study
pCH075	Product of PA4723 C135A mutagenesis cloned into NcoI/XbaI sites of pBAD24	This study
pCH115	*PA5536* cloned into NcoI/XbaI sites of pHERD20T	This study
pCH103	pEX18Tc derivative carrying *PA4723* deletion construct	This study
pCH107	pEX18Tc derivative carrying *PA5536* deletion construct	This study
pCH108	pEX18Tc derivative carrying *PA5499* deletion construct	This study
pCH131	pEX18Tc derivative carrying *PA5498* deletion construct	This study

employed. The gentamicin resistance gene was PCR-amplified from pEX18Gm with primers containing FRT sites at the 5′ end (Hoang *et al.*, 1998). Upstream and downstream regions flanking the gene of interest were PCR-amplified from PAO1 genomic DNA and gel extracted using the QIAquick gel extraction kit (Qiagen). Generation of the gene deletion construct was performed by PCR overlap extension as previously described (Choi and Schweizer, 2005) and cloned into pEX18Tc (Hoang *et al.*, 1998). The appropriate parent strain was transformed by electroporation as previously described (Choi *et al.*, 2006) with the following changes. Instead of using an overnight culture, 50 ml of LB was inoculated with 1 ml of overnight culture and grown for 2 h at 37°C then washed. Colonies were selected on gentamicin and screened for tetracycline sensitivity. If only single homologous recombination events occurred, Gm^R/Tet^R colonies were inoculated into 5 ml of LB, grown overnight, and plated onto LB (minus NaCl) and 10% (w/v) sucrose to select for excision of the plasmid from the chromosome. Deletions were confirmed by PCR using both primers external to the upstream and downstream flank regions and primers internal to the gene. When required, the gentamicin cassette was excised with FLP recombinase (Hoang *et al.*, 1998).

E. coli *and* P. aeruginosa *growth assays*

Overnight cultures grown in LB were washed with M9 medium and normalized to an optical density at 600 nm of 1.0 for *E. coli* and optical density at 660 nm (OD_{660}) of 1.0 for *P. aeruginosa*. Washed and normalized cultures were then serially diluted and 10 µl were plated onto appropriate media. Serial dilutions were also plated on minimal medium plus 0.2% (w/v) casamino acids (for the *E. coli* and *P. aeruginosa* Δ*dksA* rescue experiments) or on minimal medium without chelator (for the *P. aeruginosa* Δ*dksA2* Zn-depletion experiments) to ensure normalization of cell number between the different strains was achieved. Growth was imaged at 24 h

after plating for *E. coli* and 36 h for *P. aeruginosa*. For the Zn-depletion experiments, plates were imaged after 67 h. All experiments were repeated independently at least three times.

Pyocyanin assay

Overnight cultures of WT pHERD20T, Δ*dksA* pHERD20T and Δ*dksA* pCH115 grown in LB were washed, normalized to an OD_{660} of 1.0 and then diluted 1/100 into fresh LB. Pyocyanin was isolated from 5 ml of samples as described previously (Farrell *et al*., 2010) and detected at an absorbance of 520 nm in an acidic solution. The concentration of pyocyanin was calculated by multiplying the absorbance at 520 nm by 17.072 (Kurachi, 1958).

qRT-PCR and western blot analysis

One litre of cultures of PAO1 or Δ*PA5499::*Gm^R in M9 media with or without 100 μM EDTA or 25 μM $ZnSO_4$ were inoculated with 10 ml of an overnight culture grown in LB that was washed with M9 media and diluted to an OD_{660} of 1.0. Two samples were harvested at each time point from each culture. One sample was used for RNA isolation and subsequent qRT-PCR, while the other sample was analysed by Western blot as described below. For RNA isolation, 5 ml of culture was spun down and resuspended in TRIzol®-LS reagent (Invitrogen, Carlsbad, CA, USA) then froze at −80°C. Samples were thawed and RNA was extracted with chloroform and purified using the RNeasy mini kit (Qiagen). Trace DNA was removed with Turbo™ DNase (Ambion, Austin, TX, USA). qRT-PCR was performed using iScript cDNA synthesis kit and SYBR Green, according to the manufacturer's guidelines (Bio-Rad, Richmond, CA, USA). One nanogram of total mRNA was reverse transcribed then 1 μl of cDNA was added to a 20 μl SYBR Green reaction mix containing 2.5% DMSO. qPCR on the generated cDNA was conducted in the iCycler MyiQ™2 real-time system (Bio-Rad) in triplicate. Reactions containing 0.1 ng of total mRNA from each sample served as controls for DNA contamination. Primers used in the qPCR reactions are listed in Table S1. Standard curves were generated with serial dilutions of pCH078 for *dksA* and pCH0116 for *dksA2*. The cycling conditions were as follows: one cycle at 95°C for 3 min, 40 cycles of 95°C for 10 s and 58°C for 30 s. Product uniformity was determine using melt curves. Data were analysed using iQ™5 optical system software (Bio-Rad).

The other sample was spun down and pellets were frozen at −80°C. Pellets were thawed and cell concentrations were normalized. Gel electrophoresis was carried out using 15% sodium-dodecyl sulphate-polyacrylamide gels. Protein was transferred to an Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) and immunoblotted according to standard procedures using rabbit polyclonal antibodies (Harlan Laboratories, Indianapolis, IN, USA) specific for DksA or DksA2 as primary antibody. Specificity of primary antibodies was confirmed by blotting against whole-cell extracts from PAO1, Δ*dksA* and Δ*zur* grown in LB. For detection, anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate antibody developed in goat (Sigma-Aldrich, St Louis, MO, USA) with CPD-*Star* reagent was used. qRT-PCR and Western blot analysis were independently repeated twice.

Reagents for in vitro *assays*

All general reagents were obtained from Sigma-Aldrich and Fisher (Pittsburgh, PA, USA), NTPs from GE healthcare (Piscataway, NJ, USA), PCR reagents from Gene Choice (Frederick, MD, USA) restriction and modification enzymes from New England Biolabs (Ipswich, MA, USA), and [α-^{32}P]-NTPs from Perkin Elmer (Waltham, MA, USA). Oligonucleotides were obtained from Sigma-Aldrich. DNA purification kits were from Promega (Madison, WI, USA) and ppGpp from TriLink BioTechnologies (San Diego, CA, USA).

Protein purification

The *zur* gene (*P. aeruginosa, PA5499*) was cloned into the NdeI and HindIII sites of pET28a (Novagen, Darmstadt, Germany). The resulting vector was transformed into *E. coli* BL21(DE3). Overexpression of His_6-Zur was achieved as previously described (Gabriel *et al*., 2008) and cells were lysed in lysis buffer (20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, pH 7.3 and 2 mM DTT) using TEEN B Lysing matrix and a FastPrep-24 (MP Biomedicals, Aurora, OH, USA). Cleared lysate was loaded on a 25 ml Ni Sepharose High Performance column (GE healthcare, Waukesha, WI, USA). After washing with 10 vols of wash buffer (lysis buffer with 20 mM imidazole instead of 10 mM), His_6-Zur was eluted with a linear gradient of imidazole from 20 mM to 500 mM. His_6-Zur eluted around 250 mM imidazole. His_6-Zur-containing fractions were combined and stored at −20°C in 50% glycerol.

DksA and RNAP were purified as described previously (Artsimovitch *et al*., 2000; Vassylyeva *et al*., 2004). RNAP holoenzyme was prepared by mixing core RNAP with σ^{70} in a 1:4 molar ratio and incubating for 30 min at 32°C.

P. aeruginosa dksA2 gene was PCR-amplified from pCH040 and cloned between the NdeI and HindIII sites of pIA884, a derivate of pET28a (EMD Chemicals, Gibbstown, NJ, USA), which carries N-terminal 10-histidines tag followed by a TEV cleavage site, resulting in a DksA2-His fusion (pIA923). When induced in strain XJB, DksA2 was soluble and constituted ≈ 20% of total-cell protein. Cells were lysed by French press in a disruption buffer (50 mM Tris-HCl pH 6.9, 150 mM NaCl, 0.1 mM EDTA, 1 mM 2-mercaptoethanol). Cleared lysate was loaded on a Ni-NTA agarose (Qiagen) column. After four consecutive washes with 10 vols of the disruption buffer containing 0, 50 and 100 mM imidazole (pH 7.5), DksA2 was eluted with 250 mM imidazole and loaded on a heparin column (GE healthcare) equilibrated with HepA buffer (50 mM Tris-HCl pH 6.9, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 5% glycerol). Elution was carried out using 50–1500 mM NaCl gradient. DksA2 was eluted between 40 and 50 mSi (milli Siemens, units of conductivity). A final purification was performed on Resource Q (GE healthcare) equilibrated with HepA, elution was carried out with 75–1500 mM NaCl; DksA2 was eluted at 30 mSi. Purified DksA2 was incubated with the His-tagged TEV protease

overnight at room temperature and loaded again on resource Q column to remove the short His-tag, the TEV protease and the uncleaved protein.

Electrophoretic mobility shift assay (EMSA)

A fragment containing the promoter region of *dksA2* was amplified by PCR using *P. aeruginosa* genomic DNA as a template and Zur*dksA2*EMSAFor and Rev (Table S1). Zur*dksA2*EMSAFor was pre-labelled at the 5′ end with biotin. The PCR fragment was purified using a QIAquick spin column. EMSAs were performed as previously described (Gaballa and Helmann, 1998) except 1.5 ng of biotin-labelled DNA was used as the substrate. After electrophoresis, DNA was transferred to a positively charged nylon membrane (Roche, Indianapolis, IN, USA). Transferred DNA was cross-linked with a Fisher-Scientific FB-UVXL-1000 UV Crosslinker by using the optimal cross-link setting. Biotin-labelled DNA was detected using a Phototope-Star Detection Kit (NEB) following manufacturer's guidelines. Membranes were exposed to a Kodak X-ray film.

For EMSA competition assays, oligonucleotides (Table S1) corresponding to both strands of the putative Zur binding site were synthesized. As a control, oligonucleotides corresponding to 42 bp from the *PA4577* promoter region were synthesized. Annealing was carried out by mixing equimolar amounts of each complementary oligonucleotide in 1× ligase buffer. The mixture was heated at 98°C for 5 min then allowed to cool to room temperature.

In vitro *transcription*

Linear templates for all the *in vitro* experiments were generated by PCR amplification of the plasmids encoding the test promoters. Sequences of all the plasmids and oligonucleotides will be provided upon request. Plasmid pIA536 was used to amplify a 153 bp fragment containing the *rrnB* P1 promoter (encompassing positions –60 to +10 relative to the promoter start site). Plasmids pFW11 and pIA349 were used to generate linear templates encoding the *lac*UV5 and T7A1 promoters respectively. DksA, DksA2 (0.1–15 μM), ppGpp (50 μM) or Storage buffer (10 mM Tris-HCl pH 7.9, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 50% glycerol) were mixed with RNAP holoenzyme (30 nM), ApC (200 μM), UTP (200 μM), GTP (4 μM) and [α-^{32}P]-GTP (10 μCi of 3000 Ci mmol^{-1}) in 10 μl of Transcription buffer (20 mM Tris-HCl pH 7.9, 20 mM NaCl, 10 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 0.1 mM EDTA) at 37°C for 15 min. The DNA fragment was added and samples were incubated for an additional 15 min at 37°C. Reactions were stopped by the addition of an equal volume of Stop buffer (10 M urea, 20 mM EDTA and 45 mM Tris-borate, pH 8.3). Samples were separated by electrophoresis on 8% polyacrylamide, 7 M urea gels, and dried gels were visualized and quantified by phosphorimaging (ImageQuant software, Molecular Dynamics).

DNase I footprinting

The end-labelled 153 bp *rrn*B P1 template was generated by PCR with a non-template strand primer, which has been 5′-labelled with [γ-^{32}P]-ATP and polynucleotide kinase (Epicentre, Madison, WI, USA) prior to PCR. PCR products were gel-purified using a Promega kit. Sequencing reactions were performed using the same labelled primer with a SequiTherm kit (Epicentre). For DNase I footprinting experiments, wild-type *E. coli* RNAP holoenzyme (200 nM) was pre-incubated with DksA, DksA2 (2 μM final concentration) or an equal volume of Storage buffer for 15 min at 37°C in GB buffer (10 mM Tris-Acetate pH 7.9, 10 mM $MgCl_2$, 30 mM KCl, 1 mM DTT). The labelled *rrnB* P1 promoter fragment (10 nM) was added and the reaction was incubated for additional 15 min. Samples were shifted to room temperature (22°C) and treated with 0.002 U of DNase I (Roche) for 2 min. The reaction was stopped by the addition of an equal volume of Stop buffer. Samples were separated by electrophoresis on 6% polyacrylamide, 7 M urea gels, as described above.

Promoter complex stability assay

Linear 92 and 249 bp DNA fragments containing the *lac*UV5 (from –60 to +23) and T7 A1 (from –86 to +29) promoters (10 nM) were incubated with RNAP holoenzyme (30 nM) and DksA, DksA2 (1 μM each) or SB for 15 min at 37°C. For *lac*UV5, the assay was carried out in 100 μl of 40 mM Tris-Cl pH 7.9, 200 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT. The T7A1 assay was carried out in 100 μl of TGA buffer (40 mM Tris-Acetate, pH 7.9, 20 mM Na-Acetate, 2 mM Mg-Acetate, 5% glycerol, 1 mM DTT and 0.1 mM EDTA). Heparin (10 μg ml^{-1}) was added to sequester any free RNAP at time 0. Thereafter, reaction aliquots were withdrawn at various times and combined with nucleotide substrates. ApU (100 μM), ATP (500 μM), UTP (8 μM) and [α-^{32}P]-UTP (10 μCi of 3000 Ci mmol^{-1}) were used with *lac*UV5; ApU (100 μM), CTP (8 μM) and [α-^{32}P]-CTP (10 μCi of 3000 Ci mmol^{-1}) – with T7A1. Following a 15 min incubation at 37°C, reactions were quenched by the addition of an equal volume of Stop buffer. Samples were separated by electrophoresis on 8% polyacrylamide, 7 M urea gels, as described above.

ICP-MS analysis

Both DksA and DksA2 were dialysed against 20 mM Tris-Cl and 100 mM NaCl. Exactly 0.285 μM (5 mg l^{-1}) final concentration of each protein was analysed with ICP-MS at the Wisconsin State Laboratory of Hygiene Trace Element research group with an ELEMENT2 sector field ICP-MS (Thermo Scientific). Buffer was used as a blank.

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