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A network of regulators promotes dehydration tolerance in *Escherichia coli*

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Summary

The ability to survive conditions of low water activity is critical for the survival of many bacteria in the environment and facilitates disease transmission through food and contaminated surfaces. However, the molecular mechanisms that enable bacteria to withstand this condition remain poorly understood. Here we describe a network of regulators in *Escherichia coli* that are important for this bacterium to survive dehydration. We found that the transcriptional regulator DksA and the general stress response regulator RpoS play a critical role. From a plasmid genomic library screen, we identified two additional regulators, Crl and ArcZ, that promote dehydration tolerance through modulation of RpoS. We also found that LexA, RecA, and ArcA contribute to survival. Our results identify key regulators that enable *E. coli* to tolerate dehydration and suggest a hierarchical network is involved in protection against cellular damage associated with this stress.

Introduction

Organisms from all kingdoms of life have the capacity to survive following periods of dehydration (Potts, 1994; Leprince and Buitink, 2015). Among prokaryotes, this stress resistance is critical for survival in niches with low or fluctuating water activity, such as soils and many foods (Potts, 2001; Billi and Potts, 2002; Garcia, 2011; Burgess, 2016). The response to dehydration has been best studied in highly dehydration-resistant organisms such as cyanobacteria and soil bacteria (Potts, 1999; Vriezen et al., 2007). In *Escherichia coli*, on the other hand, which has served as a model organism to establish numerous aspects of bacterial physiology and stress responses, the molecular genetics underlying dehydration tolerance has been relatively underexplored. While *E. coli* is naturally found in the mammalian gut, a well-hydrated environment, it is shed in animal feces and transported to other niches where it may encounter conditions of low water activity (Maule, 1997; Duffy et al., 2014). Compared to many other bacteria, *E. coli* is not highly resistant to dehydration, but it still has a significant ability to survive this condition. Indeed, several studies have identified *E. coli* as persistent members of soil microbial communities, and this bacterium

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has been shown to survive in manure and soil for up to several months (Temple, 1980; Byappanahalli et al., 2006; Habteselassie, 2008; Zhang and Yan, 2012). The capacity for at least a subpopulation to survive in low moisture environments is also clinically relevant, considering the low infectious dose of many pathogenic *E. coli*, which are frequently found in livestock and other agricultural settings (McClure, 2000; Duffy et al., 2014). In fact, several outbreaks of gastroenteritis have been associated with contamination of dried foods by *E. coli* (Hiramatsu et al., 2005; Burgess, 2016).

Bacterial cells are vulnerable to damage throughout the stages associated with dehydration and recovery: initial loss of water, storage under dry conditions, and rehydration. The removal of water initially increases osmotic stress and may eventually lead to protein misfolding and decreased cell membrane fluidity (Billi and Potts, 2002; Vriezen et al., 2007; Scherber et al., 2009). When water activity is low, bacteria are effectively in an inert state and thus unable to prevent the accumulation of damage to nucleic acids, proteins, and lipids (Potts, 2001). The rehydration process itself may also cause additional damage, such as lipid phase transitions that can compromise membrane permeability (Mille et al., 2003; Garcia, 2011). While many stresses have been proposed to accompany dehydration and recovery, the primary mechanisms involved in cell damage or death associated with this process are still largely unknown.

Previous studies in bacteria have identified a variety of pathways associated with dehydration tolerance, including the synthesis of protective compounds such as the disaccharide trehalose and extracellular matrix components, and repair mechanisms to combat DNA damage, oxidative stress, and envelope stress (reviewed in (Potts, 1994; Garcia, 2011; Burgess, 2016)). However, in most systems, the specific mechanisms that confer dehydration resistance and their regulation have not been established.

Here we identify several regulators that are important for *E. coli* to survive dehydration. We find that the general stress response sigma factor RpoS plays a critical role. In addition, we show that several other components that modulate or act in conjunction with RpoS—DksA, Crl, ArcZ, and ArcA—also contribute to dehydration tolerance. Identification of these regulators is a first step towards determining the specific types of cellular damage conferred by dehydration and the specific effectors that provide protection against this damage.

Results

Dehydration protocol

To study dehydration tolerance, we first developed a protocol for establishing a dehydrated environment and assaying cell viability in a reproducible fashion. We considered a number of factors, including the growth medium, growth phase, drying speed, surface material for drying, and drying time. Since bacteria in the external environment are generally thought to be in relatively low nutrient conditions, we opted to use stationary-phase cells from overnight cultures. We observed similar rates of survival when bacteria were dried on filter paper vs. polypropylene and therefore used 96-well polypropylene plates to facilitate performing multiple parallel experiments. Since growth medium osmolarity will increase during the drying phase, we used lysogeny broth (LB) without NaCl (denoted LBns), to

minimize the effects of osmotic stress. Overnight cultures grown in LBns were dried in polypropylene 96-well plates at 25 °C for either 24 hours or seven days, as indicated (Fig. 1A). The relative humidity across different experiments ranged from 1–10%, which is similar to the moisture content of dried manure and low-moisture foods (Himathongkham et al., 1999; Finn et al., 2013a). Cells were then rehydrated in LBns, and serial dilutions were plated on LB to determine cell viability. Percent survival was calculated based on the cell viability prior to drying, and fold change in survival was determined by normalizing percent survival rates to that of a reference strain. Using this protocol, a stationary-phase culture of the *E. coli* K-12 laboratory strain MG1655 had a mean survival of 2.1% after 24 hours of drying (Fig. 1B).

The transcriptional regulators DksA and RpoS are important for dehydration tolerance

A preliminary transposon sequencing screen suggested *dksA* might be important for dehydration tolerance. We therefore tested a *dksA* deletion and found a >500-fold decrease in survival compared to wild-type MG1655 after 24 hours of drying (Fig. 2). This survival defect could be complemented with a plasmid-borne copy of *dksA* (Fig. S1A). Since DksA often functions in coordination with the small molecule guanosine tetraphosphate (ppGpp), we tested whether ppGpp also played a role in dehydration tolerance. We found that deletion of *relA*, which results in low levels of ppGpp, as well as deletion of both *relA* and *spoT*, which eliminates ppGpp (ppGpp⁰), did not decrease survival (Fig. S2A). These results suggest that DksA contributes to dehydration tolerance independently of ppGpp.

To gain more insight into the role of DksA in dehydration tolerance, we screened for spontaneous suppressors of dehydration sensitivity in the *dksA* strain by conducting repeated cycles of dehydration and rehydration. After four cycles, we isolated several colonies with improved survival compared to the parent strain. Using whole-genome sequencing, we identified two independent mutations that partially improved survival: a point mutation in *rpoD* that causes the amino acid substitution A135D and a small deletion in the C-terminal region of *rpoC* (deletion of nucleotides 3451–3642) (Fig. S3). Although this screen failed to identify downstream genes regulated by DksA, these results are similar to the results of previous screens for suppressors of *dksA* amino acid auxotrophy, which only identified mutations in RNA polymerase (Brown et al., 2002; Rutherford et al., 2009).

DksA has been shown to be important for the full induction of the general stress response regulator RpoS (Webb et al., 1999; Brown et al., 2002). In addition, an *rpoS* deletion in *E. coli* O157:H7 was previously reported to affect survival following dehydration for four days using a drying method that differs from the method used in the present study (Stasic et al., 2012). We therefore asked whether RpoS plays a role in the dehydration tolerance described here, and whether the effect of DksA is through RpoS. We found that deletion of *rpoS* decreased survival by >100-fold (Fig. 2), and complementation with *rpoS* *in trans* confirmed that this result was due to the absence of *rpoS* (Fig. S1B). The fact that deleting *dksA* had a slightly stronger effect on survival than did deletion of *rpoS* (Fig. 2) suggests that DksA is not acting solely through RpoS in providing tolerance to dehydration. This is further supported by the observation that a double deletion of *dksA* and *rpoS* was ~9-fold lower than that of *rpoS* alone (Fig. 2). In light of the previously established effect of DksA on

RpoS levels (Webb et al., 1999; Brown et al., 2002), our results suggest that the decrease in survival of a *dksA* strain is primarily due to a decrease in RpoS expression, but DksA also contributes to dehydration tolerance independently of RpoS.

Pre-induction of *rpoS* improves dehydration tolerance

Previous studies have shown that stationary-phase cells are more resistant to dehydration than are exponential-phase cells (Welsh and Herbert, 1999; Gruzdev et al., 2012b). Since RpoS expression increases upon entry into stationary phase, we suspected that increased RpoS activity prior to drying may be critical for survival. To determine when RpoS activity is necessary to promote survival, we compared the effects of inducing *rpoS* transcription in an overnight culture before drying, at the time of rehydration, or during both periods. Transcription was controlled with a tetracycline-inducible *rpoS* (P_{tet} -*rpoS*) that was integrated into the chromosome in a *rpoS* *rssB* strain. The gene *rssB* was deleted to eliminate post-translational regulation of *rpoS* expression by the RpoS anti-sigma factor RssB (Muffler et al., 1996). In the absence of the inducer anhydrotetracycline (aTc), a derivative of tetracycline that does not inhibit *E. coli* growth at the low concentrations required for induction, mean survival following rehydration was 0.3% (Fig. S4). This survival rate for uninduced P_{tet} -*rpoS* was lower than that of wild-type cells (mean survival = 2.1%) but higher than that of a *rpoS* strain, which was >100-fold more sensitive than wild type (Fig. 2). This increased survival compared to a *rpoS* strain may be due to leaky expression of *rpoS* from the *tet* promoter. When we tested the effect of inducing *rpoS*, we found that pre-induction significantly improved survival by ~25-fold (Fig. 3). On the other hand, induction upon rehydration did not significantly improve survival, regardless of whether or not the cells had been pre-induced (Fig. 3).

Screens for additional genes important for dehydration tolerance

To identify additional factors that contribute to dehydration tolerance, we tested the effect of mutating genes that previously had been linked to dehydration survival in various bacteria, as well as selected genes from various stress responses or from the RpoS regulon (Cytryn et al., 2007; Battesti et al., 2011). Most of the mutants did not show a significant effect after 24 hours of drying (Fig. S2). For example, while increased intracellular trehalose concentrations have been correlated with improved dehydration tolerance (Welsh and Herbert, 1999), deletion of the trehalose biosynthesis genes *otsA* or *otsB* did not decrease survival and actually showed increased survival compared to wild type (Fig. S2A). We also tested whether induction of the extracytoplasmic sigma factor RpoE by envelope stress plays a role in dehydration tolerance. The *rpoE* gene is essential, and high levels of RpoE activity are also detrimental to cell growth. We therefore tested a strain that produces a mutant RpoE with decreased activity and that lacks the RpoE anti-sigma factor RseA, which mediates induction by envelope stress (Nicoloff et al., 2017). An overnight culture of this strain had ~4-fold lower RpoE activity compared to a wild-type strain, based on β -galactosidase measurements of an RpoE-dependent *rpoHp3-lacZ* reporter (Fig. S5). We found that this strain did not show a survival defect after 24 hours of drying (Fig. S2B), suggesting that induction of RpoE by envelope stress is not important for dehydration tolerance. In addition to RpoE, several other envelope stress response regulators that we tested, PspF, CpxR, BaeR, and RcsB, did not affect survival following dehydration (Fig. S2A).

Among the various stress response genes that we tested, we found that genes associated with the DNA damage response were important for survival. A strain producing a non-inducible variant of LexA, the repressor controlling the *E. coli* SOS system (Simmons et al., 2008) was significantly more sensitive to dehydration compared to a congenic strain that expresses wild-type LexA (Fig. S2C). In addition, we found that deleting the gene encoding RecA, which stimulates LexA auto-cleavage in response to DNA damage and also has additional roles in DNA repair, decreased survival after 24 hours of drying (Fig. S2A). These results suggest that DNA repair plays a role in dehydration tolerance, which is consistent with previous observations in *E. coli* and other bacteria (Asada, 1979; Mattimore and Battista, 1996; Humann et al., 2009; Aranda et al., 2011).

We also screened a genomic library from *Salmonella enterica* serovar Typhimurium, a species related to *E. coli* that is highly resistant to dehydration (Hiramatsu et al., 2005; Gruzdev et al., 2012a; Finn et al., 2013a). Compared to MG1655, *S. Typhimurium* 14028s was ten-fold more resistant to dehydration after 24 hours of drying and >1000-fold more resistant after one week (Fig. S6). The relative resistance of 14028s to MG1655 increased with the length of dehydration time up to at least a month, primarily due to a precipitous drop in survival of MG1655 (Fig. S6). We reasoned that we could use this large difference in survival to conduct a plasmid genomic library screen using *S. Typhimurium* genomic DNA to identify genes important for dehydration tolerance. We predicted that plasmids containing effector genes that prevent or repair dehydration-induced damage, or genes that increased RpoS levels and/or activity would improve the survival of MG1655. Although the genomic library comes from *Salmonella* rather than *E. coli*, we hypothesized that, given the similarity between the two species, the results could reveal similar pathways in *E. coli* or provide insights into the types of damage that *E. coli* encounters in the dehydration process and the repair pathways that mediate survival.

To perform the screen, *E. coli* MG1655 was transformed with a plasmid library constructed from *S. Typhimurium* 14028s genomic DNA and subjected to cycles of dehydration and rehydration to enrich for cells with improved dehydration survival. The screen was carried out in duplicate, with two independent transformations of the genomic library into MG1655. For each cycle, the cells were dried for one week. This period was chosen based on the rapid decrease in survival of MG1655 after prolonged drying (Fig. S6). Ideally, the level of killing for one cycle would provide a sufficiently stringent selection to recover plasmids that improve survival, but not so stringent as to prevent plasmids providing a relatively small level of protection from being recovered. After each cycle, beginning with the second cycle, individual colonies were randomly picked and tested for improved dehydration tolerance compared to MG1655 harboring the parent plasmid. Out of 223 colonies that were tested, two plasmids were isolated after the third and fourth cycles of drying that improved MG1655 survival by ~ten-fold after one week of drying.

Crl and ArcZ, both regulators of RpoS, contribute to dehydration tolerance

One of the plasmids isolated from the genomic library screen contained the gene *crl*, which encodes a small protein that enhances RpoS activity by promoting RpoS-RNA polymerase holoenzyme formation (Bougdour et al., 2004; Typas et al., 2007). Although most *E. coli*

strains have *crl*, our version of MG1655, which was obtained from the *E. coli* Genetic Stock Center (CGSC# 7740, MG1655(Seq)), contains an insertion sequence (IS1) element in *crl* (Freddolino et al., 2012).

To test the role of Crl in dehydration tolerance, we moved *crl* into MG1655 by P1 transduction from a *crl⁺* *E. coli* K-12 strain using a marker that is linked to the *crl* locus. Restoration of the *E. coli* *crl* gene increased survival after one week of drying by ~ten-fold (Fig. 4A). We also found that plasmid expression of *rpoS* completely rescued survival of a Crl-null strain (Fig. S7). Crl has also been shown to facilitate the association of other sigma factors with core RNA polymerase *in vitro* (Gaal et al., 2006). However, we found that disruption of *crl* had no effect in a *rpoS* background after 24 hours of drying (Fig. S8A), suggesting that Crl contributes to dehydration tolerance solely through RpoS.

Crl has been shown to regulate *csgD*, which encodes a regulator of biofilm formation that plays a role in dehydration resistance for *Salmonella* growing in colonies (Arnqvist et al., 1992; Barnhart and Chapman, 2006; White et al., 2006). We therefore tested the effects of deleting *csgD*. We found that *csgD* did not affect survival after 24 hours of drying, suggesting that Crl does not act through CsgD and that the CsgD regulon is not important for the dehydration tolerance of planktonic *E. coli* (Fig. S2D). These results are consistent with previous studies in *Salmonella* suggesting that extracellular matrix components are not important for dehydration tolerance of planktonic cells (Garmiri et al., 2008; Finn et al., 2013b).

The second plasmid that we isolated from our screen contained the gene for a small RNA, *arcZ*, which is also present in the *E. coli* genome. We therefore tested the effect of an *arcZ* deletion and observed a ~five-fold decrease in survival after one week of drying (Fig. 4B). ArcZ is among the core set of small RNAs in *Enterobacteriaceae* with several direct targets, most notably *rpoS* mRNA (Mandin and Gottesman, 2010). ArcZ stimulates translation of *rpoS* and is expressed in stationary phase during aerobic growth, although its expression is not dependent on RpoS. Transcriptomic studies indicate that ArcZ overexpression changes the expression of ~16% of *S. Typhimurium* genes, only 25% of which have been reported to be in the RpoS regulon, suggesting that ArcZ may also affect gene expression independently of RpoS (Papenfort et al., 2009). However, deletion of *arcZ* in a *rpoS* background showed a similar rate of survival after 24 hours of drying compared to *rpoS* alone, suggesting that ArcZ contributes to survival solely through RpoS (Fig. S8B).

Under low oxygen conditions, ArcZ is repressed by the ArcB/ArcA two-component system, enabling an additional layer of regulation of RpoS in low oxygen conditions (Mandin and Gottesman, 2010). Since cells entering stationary phase may deplete oxygen, which activates the ArcB/ArcA system (Sharma et al., 2013), we tested whether deletion of *arcA* affects dehydration tolerance. We hypothesized that the absence of ArcA would lead to increased ArcZ and thus higher survival. However, we found that deletion of *arcA* had the opposite effect, decreasing survival by ~three-fold after one week of drying (Fig. 5).

Natural variation in dehydration tolerance among *E. coli* isolates

To determine whether resistance to dehydration is conserved within *E. coli*, we compared the survival of MG1655 after one week of drying to that of representative *E. coli* strains from different phylogenetic groups (Fig 6). Most of the strains we tested had similar rates of survival compared to MG1655 *crI*⁺, although Nissle 1917 and CFT073 showed lower survival. Notably, several enterohemorrhagic *E. coli* (EHEC) strains were almost as resistant to dehydration as *S. Typhimurium*, which is consistent with a previous report of similar rates of survival between *E. coli* O157:H7 and *S. Typhimurium* in dried cow manure (Himathongkham et al., 1999).

Discussion

Our results reveal a group of interacting regulators that are important for *E. coli* to survive dehydration (Fig. 7). RpoS is a central part of this network, and its absence decreased survival by over two orders of magnitude. Furthermore, restricting *rpoS* transcription to the overnight culture prior to drying was sufficient to restore dehydration tolerance. This result suggests that the primary role of RpoS in dehydration tolerance is to protect cells from damage during the drying phase and/or to protect the cells from stresses associated with the early stages of rehydration before protein expression has restarted. We also identified several regulators of RpoS, namely DksA, Crl, ArcZ, and ArcA, that are important for survival, as well as LexA and RecA, which play a role in the SOS response and DNA repair.

Regulators of RpoS that contribute to survival after dehydration

DksA is highly conserved among bacteria and regulates transcription by binding directly to the secondary channel of RNA polymerase (Haugen et al., 2008; Osterberg et al., 2011; Parshin et al., 2015). Although DksA often functions in coordination with ppGpp, we found that a ppGpp⁰ strain was not more sensitive than the wild-type strain to dehydration, in contrast with the behavior of *dksA*. This difference between *dksA* and ppGpp⁰ strains may be due to differences in their regulation of RpoS. Indeed, overnight cultures of *dksA* and ppGpp⁰ strains have significantly different levels of RpoS (Webb et al., 1999; Brown et al., 2002). Thus, these results further support our hypothesis that induction of RpoS prior to drying is important for survival.

While the decreased levels of RpoS in a *dksA* strain may account for much of the sensitivity to dehydration of a *dksA* deletion, our results indicate that DksA also has an RpoS-independent role in dehydration tolerance. DksA may affect survival through its action on other sigma factors or through its role in transcription elongation, which may be critical for preventing the formation of arrested elongation complexes if the process of dehydration and rehydration results in the uncoupling of transcription and translation (Tehranchi et al., 2010; Zhang et al., 2014). In contrast with DksA, both Crl and ArcZ appear to act solely through RpoS, since deletion of either *crl* or *arcZ* had no effect on survival in a *rpoS* strain (Fig. S8). We note, however, that the genetic interactions described here between *rpoS* and the above genes may be due to indirect effects instead of direct interactions between these regulators and RpoS.

Although the ArcB/ArcA two-component system negatively regulates both RpoS and ArcZ (Mika and Hengge, 2005; Mandin and Gottesman, 2010), we found that an *arcA* deletion decreased survival following dehydration. ArcA is a global regulator that directly regulates at least roughly 100 operons and indirectly affects transcript levels of numerous additional genes (Salmon et al., 2005; Park et al., 2013), many of which are not clearly associated with the transition from aerobic to anaerobic metabolism. Given the complex and poorly understood effects of ArcA on *E. coli* physiology, it is difficult at this stage to speculate on the likely mechanism by which ArcA promotes dehydration tolerance.

Role of DNA damage response may depend on drying conditions

Previous studies have shown that drying bacteria below a critical water activity causes DNA damage (Asada et al., 1980; Dose et al., 1992; Potts, 1994). Consistent with these observations, we found that LexA and RecA are important for survival following dehydration. However, one study on dehydration of *E. coli* O157:H7 did not find a role for the SOS response (Stasic et al., 2012). In addition, a second study found that the SOS response was important for survival after freeze-drying, but drying alone did not induce SOS response genes (Rosen et al., 2016). The differences between our results and the results from these studies may reflect differences in drying protocols and the degree of dehydration that was achieved.

Many effectors of dehydration tolerance remain unknown

In our study, several genes that were previously associated with dehydration tolerance, including those involved in trehalose biosynthesis and envelope stress responses, did not affect survival after 24 hours of drying. The disaccharide trehalose is thought to improve survival by stabilizing proteins and cell membranes and inhibiting crystallization (Crowe et al., 1992). However, in the absence of osmotic stress prior to drying, deletion of trehalose biosynthesis genes did not affect survival in either *Rhizobium etli* or *Bradyrhizobium japonicum* (Sugawara et al., 2010; Reina-Bueno et al., 2012), which is consistent with our findings in *E. coli*. We also tested several envelope stress response systems to explore the possibility that altered lipid composition or repair of cell envelope damage might also be important for dehydration tolerance (Potts, 1994; Scherber et al., 2009; Li et al., 2012). None of the envelope stress response regulators that we tested, PspF, CpxR, BaeR, RcsB, and RpoE, affected survival. While this result does not rule out a role for envelope stress response pathways, our findings suggest that none of these pathways individually plays a significant role, at least for the dehydration conditions that we used.

Notably, our screen only identified regulators and did not identify any proteins that directly increase dehydration tolerance. This observation may simply reflect the fact that our screen was limited in scope and required multiple rounds of dehydration and rehydration. It is also possible, however, that multiple effectors may be required for survival, with each contributing a small effect on average. We also note that the challenge of identifying downstream effectors in the RpoS regulon is apparently not unique to dehydration, since the specific effectors for many other RpoS-mediated stress responses have not yet been identified (Battesti et al., 2011).

Potential role of RpoS in ex vivo environments

Inactivation of *crf* or *rpoS* has been shown to provide an advantage for growth and survival in stationary phase, a phenomenon known as GASP (Zambrano et al., 1993; Finkel and Kolter, 1999; Finkel, 2006). While *rpoS* and *crf* mutations are frequently found in laboratory strains (Atlung et al., 2002; Faure et al., 2004; Freddolino et al., 2012), the frequency of *rpoS* mutations is reportedly as low as 0.3–2% among natural isolates (Chiang et al., 2011; Snyder et al., 2012; Bleibtreu et al., 2014). In addition, recent studies find that RpoS is important for survival in farm environments and persistence in the soil. These observations suggest that in many of *E. coli*'s primary niches, stress resistance may be more critical for survival than is the ability to scavenge for nutrients (Parker et al., 2012; Somorin et al., 2016). Given the critical role played by RpoS and its modulators in dehydration tolerance, and the fluctuations in water activity that *E. coli* is likely to encounter in *ex vivo* settings, dehydration may well be a particularly important selective pressure for maintaining RpoS and for precise tuning of its activity.

Experimental procedures

Strains and Plasmids

Strains, plasmids, and primers are listed in Tables S1, S2, and S3, respectively. *E. coli* strains used in dehydration assays were derived from *E. coli* K-12 strain MG1655(Seq) (CGSC #7740), except where indicated in Fig. 6. Strain PM1460 was a gift from Susan Gottesman, strain SEA 13B88 was a gift from Pina Fratamico, strain CF15006 was a gift from Michael Cashel, and strains SEA001 and SEA6527 were gifts from Sarah Ades.

Media and growth conditions

Cultures were grown aerobically at 37 °C in LB medium without NaCl (LBns). LB (Miller) agar was used for growth on solid medium. The antibiotics ampicillin, kanamycin, chloramphenicol, and tetracycline were used at concentrations of 100 µg/ml, 25 µg/ml, 25 µg/ml and 15 µg/ml, respectively, unless otherwise indicated. Anhydrotetracycline (aTc) was used at a concentration of 100 ng/ml as indicated.

Strain Construction

Gene deletions were transferred to strains by transduction with P1_{vir} (Miller, 1992). The tetracycline-inducible *rpoS* strain was constructed by first integrating plasmid pAC6 into the att_λ site of strain SAM37, as described in (Haldimann and Wanner, 2001), to create strain AIC168. Strain AIC172 was then created by transduction from strain JW1223 into strain AIC168 to delete *rssB*. Antibiotic resistance cassettes flanked by FLP recombinase target (FRT) sites were removed using pCP20 (Cherepanov and Wackernagel, 1995). The *crf*⁺ strain was created by transduction from strain JW0255, which has a marker (*afuB*::(FRT-Kan-FRT)) that is linked to the *crf* locus. The *crf*⁺ and *crf*::IS1 alleles were determined by PCR using primers *crf*_MG1655_F and *crf*_MG1655_R.

Plasmid construction

Plasmid pAC3 was constructed by cloning *rpoS* with its native promoter into pBR322 using EcoRI and BamHI restriction sites. The *rpoS* gene was amplified by PCR using primers rpoS_EcoRI_u1 and rpoS_BamHI_l1. Plasmid pAC6 was constructed by cloning the *rpoS* coding sequence into pAS07 using KpnI and SpeI restriction sites. The *rpoS* was amplified by PCR using primers rpoS_KpnI_rbs_F2 and rpoS_SpeI_R2, and the vector backbone without *gfpmut3.1* was amplified by PCR using primers pAS07_U1 and pAS07_L1. Plasmid pAC15 was constructed by cloning *arcZ* with its native promoter into pBR322 using Gibson cloning with the NEB Gibson Assembly Master Mix (cat. E2611S) (Gibson et al., 2009). The vector was amplified by PCR using primers pBR322-arcZ-u1 and pBR322-arcZ-l1, and the insert was amplified by PCR using primers arcZ_EcoRI_u1 and arcZ_HindIII_l1.

Dehydration assay

10 μ L of overnight cultures grown in LBns were spotted in wells of a 96-well plate and air-dried for 24 hours, or longer as indicated, in a Rubbermaid Tupperware container containing commercial grade 6 mesh Drierite (W. A. Hammond DRIERITE CO. LTD, Xenia, OH) to achieve a relative humidity between 1 and 10% in a 25 °C incubator. This range refers to the variation in relative humidity between experiments; the variation over the course of an individual experiment was generally less than 1%. Relative humidity was recorded every 2–3 seconds using a DHT22 humidity sensor attached to an Arduino Uno microcontroller (Adafruit). An example of the relative humidity recorded for a one-week experiment is shown in Fig. S9. The initial number of CFUs/well was calculated by plating serial dilutions of the starting culture in triplicate on LB agar. 100 μ L of LBns was added to rehydrate the cells, and serial dilutions in PBS were plated in triplicate on LB agar to determine the number of colony forming units (CFUs) after dehydration. Percent survival was calculated by dividing the number of CFUs/well after dehydration by the initial number of CFUs/well. For each experiment, the fold change for each biological replicate was calculated by dividing the percent survival by the mean percent survival of a reference strain, as indicated, to normalize the values. The mean fold change for each strain (represented by horizontal lines in figures) was then calculated by combining all replicates from multiple independent experiments. Independent experiments refer to experiments performed on separate days. The Wilcoxon rank-sum test was performed using the R programming language (R Core Team, 2013). To test the effects of RpoS pre-induction, aTc was added to the overnight culture. Immediately before drying, the cells were washed twice with PBS to remove residual aTc and resuspended to the original volume in spent media that was obtained from a similar overnight culture that lacked aTc.

Spontaneous suppressor screen

A *dkcA* strain was grown overnight in LBns and subjected to repeated cycles of drying and rehydration. Cells were plated on LB agar after each cycle, and colonies were randomly selected to test for increased dehydration tolerance compared to the parent strain. After the fourth cycle of drying, several colonies showed improved survival after drying. The plasmid pRL27 was used to randomly insert a mini-Tn5 transposon and isolate kanamycin resistance insertions that were linked to the suppressor mutation. The mutation was then transduced to

a fresh background by P1 transduction, and dehydration tolerance was assessed. The mutations were identified by whole-genome sequencing. Genomic DNA was extracted using a Qiagen DNeasy Blood and Tissue Kit and eluted in 10mM Tris-HCl pH 8.5. Library preparation and sequencing were performed by the Center for AIDS Research SGA Service Center at the University of Pennsylvania. Single nucleotide polymorphisms (SNPs) were determined with FreeBayes on the Galaxy server (usegalaxy.org) (Afgan et al., 2016), by comparing the parent strain with each suppressor strain, and confirmed by Sanger sequencing.

Plasmid genomic library screen

Genomic DNA from *S. Typhimurium* 14028s was isolated using a Qiagen DNeasy Blood and Tissue Kit, partially digested with *Sau3aI*, and ligated into pBR322 digested with *BamHI*. The ligation was electroporated into Invitrogen ElectroMAX DH10b electrocompetent cells. Plasmid DNA was prepared and transformed into *E. coli* MG1655 by electroporation. To select clones with increased dehydration tolerance, the cells were subjected to repeated cycles of drying for seven days at a time. After each drying period, cells were rehydrated and plated on LB supplemented with 50 µg/ml ampicillin. Clones were randomly selected to test for improved survival after drying compared to MG1655 with the parent plasmid. Plasmids from clones with improved survival were isolated, re-transformed into a fresh background, and retested. Plasmids were sent to sequencing using primers pBR322-seq-For and pBR322-seq-Rev.

Construction of phylogenetic tree

Multiple sequence alignment of the seven genes used for MLST typing (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) (Wirth et al., 2006) was performed using MUSCLE (Edgar, 2004), and a phylogenetic tree was built using FastTree2 (Price et al., 2010). The tree was visualized using the interactive tree of life (iTOL) (Letunic and Bork, 2016). The tree scale denotes the number of nucleotide substitutions per site. The genome sequence for SEA 13B88 was not available and therefore was omitted from the phylogenetic tree.

β-galactosidase assay

Strains were first grown overnight from colonies inoculated in LBns. These cultures were then diluted 1:1000 in LBns the following day and grown overnight again. β-galactosidase activity was measured using the RpoE-dependent *rpoHp3::lacZ* reporter as described in (Miller, 1992).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Originality-significance statement

While many bacteria encounter conditions of low water activity, the cellular pathways that mediate their survival in these environments remain poorly characterized. Furthermore, relatively few genetic studies of dehydration tolerance have been performed on *Escherichia coli*, one of the best studied and genetically tractable organisms. Using *E. coli*, we developed a dehydration protocol and performed screens to identify genes that are important for surviving the process of dehydration and rehydration. We found that several interacting regulators play important roles, with RpoS a key component of this network. Unexpectedly, we also found that many envelope stress response regulators did not confer protection in our assay. These results are an important step towards identifying the specific types of cellular damage caused by dehydration and unraveling the mechanisms that enable bacteria to protect themselves from these stresses.

Figure 1. Dehydration protocol

A. Schematic of dehydration protocol. B. Distribution of percent survival of *E. coli* MG1655, referred to as “WT”, after 24 hours of drying. The horizontal line represents the mean percent survival pooled from eight independent experiments, and each dot represents a biological replicate.

Figure 2. DksA and RpoS are important for dehydration survival

Fold change in survival after 24 hours of drying, relative to WT. Horizontal lines represent the means pooled from three independent experiments, and each dot represents a biological replicate. Significance levels were determined based on a Wilcoxon rank-sum test (**p < 0.01). Strains used: MG1655, AIC56, SAM37, and AIC29.

Figure 3. Pre-induction of *rpoS* is protective against dehydration

Fold change in survival after 24 hours of drying, relative to no induction. Cells were washed twice with PBS and resuspended in spent media without anhydrotetracycline (aTc) prior to drying. *rpoS* expression was induced with 100 ng/ml aTc as indicated. Horizontal lines represent the means pooled from four independent experiments, and each dot represents a biological replicate. Significance levels were determined based on a Wilcoxon rank-sum test (** $p < 0.01$). Strain used: AIC172.

Figure 4. Genes identified from genomic library screen

A. Fold change in survival after one week of drying, relative to the *crl*⁺ strain. The *afuB*'::Kan mutation is linked to *crl* and was used to construct the *crl*⁺ strain. B. Fold change in survival after one week of drying, relative to WT. Horizontal lines represent the means pooled from three independent experiments, and each dot represents a biological replicate. Significance levels were determined based on a Wilcoxon rank-sum test (**p<0.01). Strains used: A) AIC171 and AIC170; B) MG1655 and AIC196.

Figure 5. ArcA plays a role in dehydration survival

Fold change in survival after one week of drying, relative to WT. Horizontal lines represent the means pooled from four independent experiments, and each dot represents a biological replicate. Significance levels were determined based on a Wilcoxon rank-sum test (** $p < 0.01$). Strains used: MG1655 and JES53.

Figure 6. Dehydration survival of various *E. coli* isolates and *S. Typhimurium* 14028s

A. Phylogenetic tree of *E. coli* isolates based on MLST genes. The tree scale indicates the number of nucleotide substitutions per site. B. Fold change in survival after one week of drying of representative *E. coli* isolates and *S. Typhimurium* 14028s relative to MG1655. EHEC refers to the O157:H7 enterohemorrhagic *E. coli* strains. Horizontal lines represent the means pooled from three independent experiments, and each dot represents a biological replicate. Strains used: MG1655, AIC171, HS, Nissle 1917, MP1, 042, E2348/69, CFT073, TW09308, TW10509, H10407, TUV93-0, EC4115, Sakai, JEONG-1266, SEA 13B88, and 14028s.

Figure 7. Summary of regulators identified in this study as contributing to *E. coli* dehydration tolerance

The thickness of each solid line denotes the proposed relative importance for dehydration tolerance, based on the effect of deleting the corresponding gene. The interactions between regulators are based on the comparison of pairwise deletions with single deletions, except for the negative regulation of RpoS and ArcZ by ArcA (denoted by dashed lines), which is from the literature (Mika and Hengge, 2005; Mandin and Gottesman, 2010). Note that the genetic interactions described here may be indirect. DksA has a small effect that is independent of RpoS, whereas Crl and ArcZ act solely through RpoS. While ArcA has been shown to repress RpoS and ArcZ, deletion of *arcA* decreased survival, suggesting that the net effect of ArcA's activities promotes dehydration tolerance. LexA also contributes to dehydration tolerance, indicating a role for DNA repair.