

# Copper Stress Causes an *in Vivo* Requirement for the *Escherichia coli* Disulfide Isomerase DsbC\*

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In *Escherichia coli*, the periplasmic disulfide oxidoreductase DsbA is thought to be a powerful but nonspecific oxidant, joining cysteines together the moment they enter the periplasm. DsbC, the primary disulfide isomerase, likely resolves incorrect disulfides. Given the reliance of protein function on correct disulfide bonds, it is surprising that no phenotype has been established for null mutations in *dsbC*. Here we demonstrate that mutations in the entire DsbC disulfide isomerization pathway cause an increased sensitivity to the redox-active metal copper. We find that copper catalyzes periplasmic disulfide bond formation under aerobic conditions and that copper catalyzes the formation of disulfide-bonded oligomers *in vitro*, which DsbC can resolve. Our data suggest that the copper sensitivity of *dsbC*<sup>−</sup> strains arises from the inability of the cell to rearrange copper-catalyzed non-native disulfides in the absence of functional DsbC. Absence of functional DsbA augments the deleterious effects of copper on a *dsbC*<sup>−</sup> strain, even though the *dsbA*<sup>−</sup> single mutant is unaffected by copper. This may indicate that DsbA successfully competes with copper and forms disulfide bonds more accurately than copper does. These findings lead us to a model in which DsbA may be significantly more accurate in disulfide oxidation than previously thought, and in which the primary role of DsbC may be to rearrange incorrect disulfide bonds that are formed during certain oxidative stresses.

Most periplasmic *Escherichia coli* proteins contain at least two cysteine residues and many are stable and active only when these cysteines form their native disulfide bond pairings (1). In *E. coli*, a family of thiol-disulfide oxidoreductases ensures that periplasmic and secreted proteins form correct disulfide bonds. DsbA is the primary disulfide oxidant in the periplasm. It rapidly donates its disulfide directly to substrate proteins and oxidizes them (2). DsbA is believed to act as a relatively nonspecific oxidant, joining any two cysteines that approach each other (3). A *dsbA*<sup>−</sup> strain shows several *in vivo* phenotypes, including attenuated virulence and loss of motility, because of the absence of disulfide bonds in proteins involved in these pathways (4, 5).

DsbC, a second periplasmic thiol-disulfide oxidoreductase, appears to function as a disulfide isomerase both *in vitro* and *in vivo*. *In vitro*, DsbC has been shown to rearrange non-native disulfides in well studied isomerization substrates such as BPTI and RNase A (6, 7). *In vivo*, DsbC

is required for full activity of a handful of proteins containing at least one non-consecutive disulfide bond (1). We have found that the periplasmic proteins RNase I (four disulfides, one non-consecutive) and MepA (three disulfides, two non-consecutive) require DsbC for their stability and, in the case of RNase I, *in vivo* activity (1). Berkmen *et al.* (3) recently showed that the folding of Agp (three consecutive disulfides) becomes DsbC-dependent with the introduction of a non-consecutive disulfide bond. These results suggest that the principal role of DsbC under non-stress conditions is to rearrange disulfide bonds that DsbA forms incorrectly.

A key question remaining in disulfide biology is the relative importance of disulfide oxidation and disulfide isomerization during *in vivo* protein folding. The disulfide oxidation pathway in the *E. coli* periplasm is well characterized, with about 25 proteins identified that require DsbA for correct folding and functioning (2, 8). In contrast, only three *in vivo* substrates have been found for DsbC, and none have been found for the second periplasmic disulfide isomerase DsbG (1, 3). Whereas a *dsbA*<sup>−</sup> strain shows pleiotropic phenotypes, no consistent phenotype has yet been found for a *dsbC*<sup>−</sup> or *dsbG*<sup>−</sup> strain. This suggests that disulfide isomerization may be less important under non-stress conditions than previously believed.

Here we show that mutants lacking any part of the DsbC disulfide isomerization pathway are less viable than isogenic wild-type strains under oxidative copper stress conditions. We show that copper, a redox-active metal, catalyzes the formation of disulfide bonds *in vivo*, and appears to introduce incorrect disulfides more frequently than DsbA does. Our data suggest that the copper sensitivity of *dsbC*<sup>−</sup> strains arises from the inability of the cell to rearrange copper-catalyzed non-native disulfide bonds in the absence of functional DsbC. Intriguingly, this may indicate a role for DsbC in combating periplasmic oxidative stress.

## MATERIALS AND METHODS

**Bacterial Strains and Growth Conditions**—The bacterial strains used in this study are listed in TABLE ONE. For copper sensitivity assays, bacteria were grown in Brain Heart Infusion (BHI)<sup>3</sup> media (Difco). For motility assays, *malF-lacZ* assays, and the alkaline phosphatase assay, an M63 minimal media was used (13.6 g of KH<sub>2</sub>PO<sub>4</sub>/2 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>/0.5 mg FeSO<sub>4</sub>·7H<sub>2</sub>O, pH 7.0) with 0.4% glucose, 0.1% casamino acids, 1 mM MgSO<sub>4</sub>, 2 μg/ml thiamine, 2 μg/ml biotin, 2 μg/ml nicotinamide, and 0.2 μg/ml riboflavin. Media was supplemented with 100 μg/ml X-gal and/or CuCl<sub>2</sub>, AgNO<sub>3</sub>, MnCl<sub>2</sub>, ZnCl<sub>2</sub>, NiSO<sub>4</sub>, CoCl<sub>2</sub>, or iron(III) citrate to a final concentration between 0.05 mM and 30 mM as required for the assays. Strains were grown at 34 °C rather than 37 °C as we found that

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<sup>3</sup> The abbreviations used are: BHI, brain heart infusion; DTT, dithiothreitol; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; drRNase A, denatured and reduced RNase A.

TABLE ONE

## Strains used in this study

Strain	Relevant genotype	Source
AH50	MC1000 <i>phoR</i> $\Delta$ <i>ara714</i> <i>leu</i> <sup>+</sup> <i>phoA68</i>	Ref. 9
AH55	AH50 <i>dsbA</i> ::kan1	Ref. 9
AH65	AH50 $\Delta$ <i>dsbC</i> ::cm	Ref. 9
AH66	AH50 <i>dsbG</i> ::kan	Ref. 18
AH358	AH50 $\Delta$ <i>dsbC</i> ::cm <i>dsbA</i> ::kan1	This study
AH392	AH50 <i>dsbD</i> ::cm	Ref. 9
AH396	AH50 <i>dsbD</i> ::cm, <i>dsbA</i> ::kan1	Ref. 15
JCB169	MC4100	Lab strain
AH51	MC4100 <i>dsbC</i> ::kan	This study
BL21	F- <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm lon</i>	Novagen
AH131	BL21 <i>dsbC</i> ::kan	Ref. 1
JCB816	MC1000 <i>phoR</i> $\lambda$ 102	Ref. 2
JCB817	JCB 816 <i>dsbA</i> ::kan1	Ref. 2
JCB818	JCB 816 <i>dsbA</i> ::kan1 <i>dsbB</i> ::kan	Ref. 2

the copper phenotype was more pronounced at this temperature. The BL21 *dsbC*<sup>-</sup> strain AH131 was created by P1 transduction of *dsbC*::kan moved out of SR3324 (MC4100 *dsbC*::kan obtained from George Georgiou) into wild-type BL21. AH358 (AH50  $\Delta$ *dsbC*::cm *dsbA*::kan1) was created by P1 transduction of *dsbA*::kan1 out of JCB571 (Bardwell laboratory strain) into AH65 (AH50  $\Delta$ *dsbC*::cm). Anaerobic growth was performed in an anaerobic chamber using BD BBL GasPak Anaerobic System Envelope and BBL GasPak Anaerobic System Indicator.

**Alkaline Phosphatase Assay**—Alkaline phosphatase assays were performed as previously described (9). The values shown represent the average of at least three separate experiments.

**In Vitro Copper Oxidation**—DsbC was purified as described previously using nickel chromatography via a His<sub>6</sub> tag (10). It was reduced for 10 min on ice with 10 mM dithiothreitol (DTT), and the DTT was removed by buffer exchange into 25 mM Hepes pH 7.5 using a Nap 5 column (Amersham Biosciences). Bovine pancreatic RNase A was purchased from Sigma, reduced and denatured in 7 M guanidinium chloride, 40 mM Hepes (pH 7.5), 120 mM dithiothreitol, and 0.2 mM EDTA at 25 °C for 2 h, and buffer-exchanged into 0.1% acetic acid. Denatured and reduced RNase A (drRNase A) was quantified using  $\epsilon_{275.5} = 9,300 \text{ M}^{-1} \text{ cm}^{-1}$ , whereas native RNase A was quantified using  $\epsilon_{275.5} = 9,800 \text{ M}^{-1} \text{ cm}^{-1}$  (Swiss Protein Database). The thiol content of reduced DsbC and drRNase A was measured with 5,5'-dithiobis(2-nitrobenzoic acid) as described, and both were found to be >90% reduced (11). Proteins were flash-frozen and stored at  $\leq -20$  °C until use.

For oxidation by copper/H<sub>2</sub>O<sub>2</sub>, native RNase A or drRNase A was diluted into 300 mM NaCl, 50 mM sodium phosphate, pH 6 to a final concentration of 50  $\mu\text{M}$  in the presence of 50  $\mu\text{M}$  CuCl<sub>2</sub>, 2 mM H<sub>2</sub>O<sub>2</sub>, 50  $\mu\text{M}$  CuCl<sub>2</sub>/2 mM H<sub>2</sub>O<sub>2</sub>, or buffer alone and incubated at 25 °C for 30 min. CuCl<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> was removed and buffer was exchanged by gel filtration to 300 mM NaCl, 50 mM sodium phosphate, pH 7.5. The protein concentration after gel filtration was measured by a Bradford assay (12); the thiol oxidation status was measured with 5,5'-dithiobis(2-nitrobenzoic acid). Samples were diluted to 20  $\mu\text{M}$ , incubated with or without 20  $\mu\text{M}$  reduced DsbC for 2 h at 25 °C, and run under reducing and non-reducing conditions on a 14% Tris-glycine gel (Invitrogen). To test the ability of DsbC to restore activity to copper-oxidized RNase A, 200- $\mu\text{l}$  aliquots of the above samples were added to 4 mM cCMP (final concentration) in 400  $\mu\text{l}$  (final volume) of 300 mM NaCl, 50 mM sodium phosphate, pH 7.5. The hydrolysis of cCMP was followed at 296 nm for 300 s and the initial hydrolysis rate for the first 30 s of the measurement was recorded. The initial hydrolysis rate of an equimolar amount of native

RNase A was set to 100% activity and all other samples expressed as percent native RNase A activity.

## RESULTS

**The DsbC Disulfide Isomerization Pathway Is Involved in Copper Resistance**—Given the importance of proper protein folding to the well being of the cell, it is surprising that no clear phenotype has yet been found for null mutations in the *E. coli* principal disulfide isomerase, DsbC. Missiakas *et al.* (13, 14) reported that *dsbC*<sup>-</sup> strains are benzylpenicillin and DTT-sensitive. However, we and others have been unable to repeat the DTT sensitivity (data not shown).<sup>4</sup> We can reproduce *dsbC*<sup>-</sup> benzylpenicillin sensitivity but only in certain strain backgrounds (data not shown). Previous work by Rietsch *et al.* (9) demonstrated that *dsbD*<sup>-</sup> strains are sensitive to the redox-active metal copper and that thioredoxin mutants are also sensitive to copper, but less so than a *dsbD*<sup>-</sup> strain. It is known that thioredoxin passes electrons to DsbD, maintaining DsbD in reduced and active form (15). DsbD then passes electrons to a number of periplasmic proteins, including DsbC (16). Thus, it seemed possible that the entire disulfide isomerization pathway might be involved in copper resistance and that *dsbC*<sup>-</sup> strains would also be copper-sensitive, despite previous reports that did not observe *dsbC*<sup>-</sup> copper sensitivity (13).

To test this, we compared the ability of wild-type strains and strains lacking individual *dsb* genes to grow on various concentrations of CuCl<sub>2</sub> (strains listed in TABLE ONE). In agreement with Rietsch *et al.* (9), we found that a mutation in *dsbD* causes increased sensitivity to copper (TABLE TWO). Whereas the MC1000-derived wild-type strain AH50 grew on BHI-copper plates at 11 mM CuCl<sub>2</sub>, the isogenic *dsbD*<sup>-</sup> strain, AH392, was unable to form single colonies at 8 mM CuCl<sub>2</sub>. Importantly, we found that *dsbC*<sup>-</sup> strains were also copper-sensitive, exhibiting the same copper-sensitive phenotype as the *dsbD*<sup>-</sup> strain. To ensure that our result was not dependent on strain background, we compared the copper sensitivity of wild-type and *dsbC*<sup>-</sup> MC4100 and BL21 strains and found that the *dsbC*<sup>-</sup> mutant was copper-sensitive in all three strain backgrounds tested (data not shown). We also reintroduced wild-type DsbC expressed from the pBAD33 plasmid into the *dsbC*<sup>-</sup> strain, and found that DsbC expressed from this plasmid restored copper resistance to wild-type levels. We note that our strongest phenotype is found when the strains are grown on brain heart infusion (BHI) agar, a very

<sup>4</sup>G. Georgiou, personal communication.

**TABLE TWO**  
**Copper sensitivities of null mutations in the *E. coli* periplasmic disulfide oxidoreductases**

Strain	Genotype	8 mM CuCl <sub>2</sub>	4 mM CuCl <sub>2</sub>
AH50	Wild-type	++++ <sup>a</sup>	++++ <sup>a</sup>
AH55	<i>dsbA</i> <sup>-</sup>	+++	+++
AH392	<i>dsbD</i> <sup>-</sup>	-	+++
AH65	<i>dsbC</i> <sup>-</sup>	-	+++
AH66	<i>dsbG</i> <sup>-</sup>	+++	+++
AH396	<i>dsbA</i> <sup>-</sup> <i>dsbD</i> <sup>-</sup>	-	-
AH358	<i>dsbA</i> <sup>-</sup> <i>dsbC</i> <sup>-</sup>	-	-

<sup>a</sup> Viability relative to wild type, ranging from wild-type-sized single colonies (++++) to not viable (-).

rich media. The experiments of Metheringham *et al.* (17) that failed to show *dsbC*<sup>-</sup> copper sensitivity were done on LB agar, where we see a weaker, but still clear, phenotype.

DsbG is a second putative periplasmic disulfide isomerase that is 30% identical to DsbC at the amino acid level (18). We compared the copper sensitivity of *dsbG*<sup>-</sup> and wild-type strains and found no appreciable difference between the wild-type and *dsbG*<sup>-</sup> cells, both in MC1000 and in BL21. At 11 mM CuCl<sub>2</sub>, *dsbG*<sup>-</sup> colonies were slightly smaller than wild-type colonies; however, the effect was much less dramatic than for *dsbC*<sup>-</sup> strains, which failed to grow at all under these conditions. When wild-type DsbG was overexpressed from the pBAD33 plasmid in the *dsbC*<sup>-</sup> strain, DsbG could only partially rescue *dsbC*<sup>-</sup> copper sensitivity. We conclude that of the two disulfide isomerases, DsbC is chiefly responsible for maintaining wild-type levels of copper resistance *in vivo*, but that DsbG may play a small role as well.

To determine whether a *dsbC*<sup>-</sup> strain shows increased sensitivity to other metals, the relative growth of isogenic *dsbC*<sup>-</sup> and wild-type strains were tested on BHI plates supplemented with silver(I) nitrate, manganese(II) chloride, iron(III) citrate, zinc(II) chloride, nickel(II) sulfate, or cobalt(II) chloride. All metals were tested in increments of 1 mM or less until the lethal concentration of the metal in the wild-type strain was reached. AH65 (*dsbC*<sup>-</sup>) showed no increased sensitivity to any of these metals relative to AH50, the isogenic wild type. Additionally, under anaerobic conditions, wild-type and *dsbC*<sup>-</sup> strains were equally resistant to copper treatment.

**Copper Catalyzes Disulfide Bond Formation in Vivo**—Two mechanisms can explain the requirement for the DsbC disulfide isomerization pathway under conditions of copper stress. One possibility is that there exists at least one DsbC substrate protein that becomes essential under copper stress conditions but that is not essential during normal growth. In this case, the substrate would always require disulfide isomerization by DsbC for its correct folding but would only become essential in the presence of copper. Copper is a toxic metal, so periplasmic or membrane proteins involved in copper homeostasis are appropriate candidates. To be consistent with our observations that *dsbC*<sup>-</sup> strains are not copper-sensitive when grown anaerobically and are not sensitive to other metals tested, the substrate should be important to copper resistance under aerobic but not anaerobic conditions and should not be involved in resistance to any of the other metals tested. To require DsbC for folding, the substrate should have three or more cysteine residues and need disulfide isomerization to achieve its native structure. A number of proteins are involved in copper resistance (see Rensing and Grass for a review, Ref. 19), but we have not found any that meet these criteria. Many copper resistance proteins are important both aerobically and anaerobically (such as the P-type ATPase CopA), and others are involved in resistance to multiple metals (the Cus proteins are important for silver as well as copper resistance). The multi-copper oxidase

CueO functions in copper resistance only under aerobic conditions and contains three cysteine residues. However, the crystal structure of this protein has been solved, and reveals no disulfide bonds (20). While it is possible that DsbC does act on a copper homeostasis protein, we have been unable to find a likely candidate among the known copper resistance proteins.

A second possibility is that copper stress may lead to incorrect disulfide bonds in proteins that do not normally require disulfide isomerization. In this case, copper stress might cause DsbC to become essential because copper is catalyzing the formation of non-native disulfide bonds. It has recently been shown that copper can form *in vitro* disulfides in protein substrates (21, 22). To examine whether copper can catalyze disulfide bonds *in vivo*, we looked at the ability of copper to complement a *dsbA*<sup>-</sup> phenotype.

A *dsbA*<sup>-</sup> strain exhibits a number of phenotypes caused by a generalized loss of disulfide bond formation. On minimal media, a *dsbA*<sup>-</sup> strain is unable to form the single disulfide in the flagellar motor protein FlgI that is required for FlgI folding. Therefore, *dsbA*<sup>-</sup> strains are not motile while wild-type strains are motile (23). We tested the ability of copper to restore motility to a *dsbA*<sup>-</sup> strain. In the absence of copper, only the *dsbA*<sup>+</sup> strain JCB816 exhibited motility, while JCB817, the *dsbA*<sup>-</sup> strain, was completely non-motile (TABLE THREE). In the presence of 0.2 mM copper, however, the *dsbA*<sup>-</sup> strain became nearly as motile as wild type, showing that copper was able to form the single disulfide in FlgI and restore motility.

The ability of copper to introduce disulfides in FlgI is possibly specific to this protein and may not apply to other periplasmic or membrane proteins. To determine whether copper introduces disulfides more widely, we looked at disulfide formation in a disulfide detector protein, a *malF-lacZ* fusion construct (23). This disulfide detector consists of  $\beta$ -galactosidase, normally a cytoplasmic protein with four thiol groups, fused to the inner membrane protein MalF (24). In the presence of functional DsbA, it is believed that non-native disulfide bonds are formed in  $\beta$ -galactosidase, causing it to be inactive (25). In the absence of a periplasmic disulfide oxidant,  $\beta$ -galactosidase retains its reduced thiol groups and can fold to its active form (25). Consistent with previous work, JCB816, a wild-type strain expressing the MalF-LacZ fusion protein, was white on X-gal, indicating that  $\beta$ -galactosidase was inactive in this strain and thus contained non-native disulfides (TABLE THREE). In contrast, JCB817, a *dsbA*<sup>-</sup> strain isogenic with JCB816, was blue on X-gal, illustrating that disulfide bonds could not form. In the presence of 0.1 mM CuCl<sub>2</sub>, the *dsbA*<sup>-</sup> strain JCB817 became white on X-gal, suggesting that copper was catalyzing disulfide bond formation in the MalF-LacZ protein.

To examine whether copper rescue of disulfide bond formation depends on DsbB, the native reoxidant of DsbA, we repeated the motility and MalF-LacZ assays using a *dsbA*<sup>-</sup>*dsbB*<sup>-</sup> double mutant, JCB818. JCB818 was non-motile on minimal motility medium but also nearly as motile as wild type in the presence of 0.2 mM copper (TABLE THREE). Similarly, JCB818 was blue on X-gal alone and white on X-gal-0.1 mM CuCl<sub>2</sub> plates. These findings indicate that copper can bypass the entire DsbA-DsbB pathway and form *de novo* disulfides in periplasmic proteins.

**Disulfide Bond Formation and DsbC<sup>-</sup> Sensitivity Are Unique to Aerobic Copper Stress**—The ability of copper to complement a *dsbA*<sup>-</sup>*dsbB*<sup>-</sup> strain demonstrates that copper forms *de novo* disulfide bonds *in vivo*. If copper-catalyzed disulfide formation causes *dsbC*<sup>-</sup> copper sensitivity, then metals that do not cause *dsbC*<sup>-</sup> sensitivity should not catalyze disulfide bond formation. We therefore tested the metals that did not cause *dsbC*<sup>-</sup> sensitivity for their ability to form



TABLE THREE

## Copper complements mutations in the DsbA-DsbB pathway

Strain	Genotype	Phenotype on minimal media		Phenotype on minimal media + CuCl <sub>2</sub>	
		Motility	MalF-βgal	Motility	MalF-βgal
JCB816	Wild-type	++++ <sup>a</sup>	White	++++ <sup>a</sup>	White
JCB817	<i>dsbA</i> <sup>-</sup>	-	Blue	+++	White
JCB818	<i>dsbA</i> <sup>-</sup> <i>dsbB</i> <sup>-</sup>	-	Blue	+++	White

<sup>a</sup> Motility relative to wild type, ranging from wild-type levels of motility (++++) to non-motile (-).

disulfide bonds by measuring their ability to both inhibit MalF-LacZ activity and restore motility in a *dsbA*<sup>-</sup> strain. Silver(I) nitrate, manganese(II) chloride, iron(III) citrate, zinc(II) chloride, nickel(II) sulfate, and cobalt(II) chloride were each examined at a range of concentrations from 0 mM to the lethal concentration for that metal. Addition of silver(I) nitrate, manganese(II) chloride, or iron(III) citrate did not inhibit β-galactosidase activity at any concentration tested. Although zinc(II) chloride, nickel(II) sulfate, and cobalt(II) chloride were able to inhibit β-galactosidase activity in the MalF-LacZ assay, none of the metals could restore motility in the *dsbA*<sup>-</sup> strain at any concentration tested. Furthermore, the wild-type strain remained motile in the presence of these metals. Of the metals tested, only copper could both abolish MalF-LacZ activity and rescue motility in a *dsbA*<sup>-</sup> strain. This indicates that *in vivo* disulfide bond formation is not a universal property of metal cations.

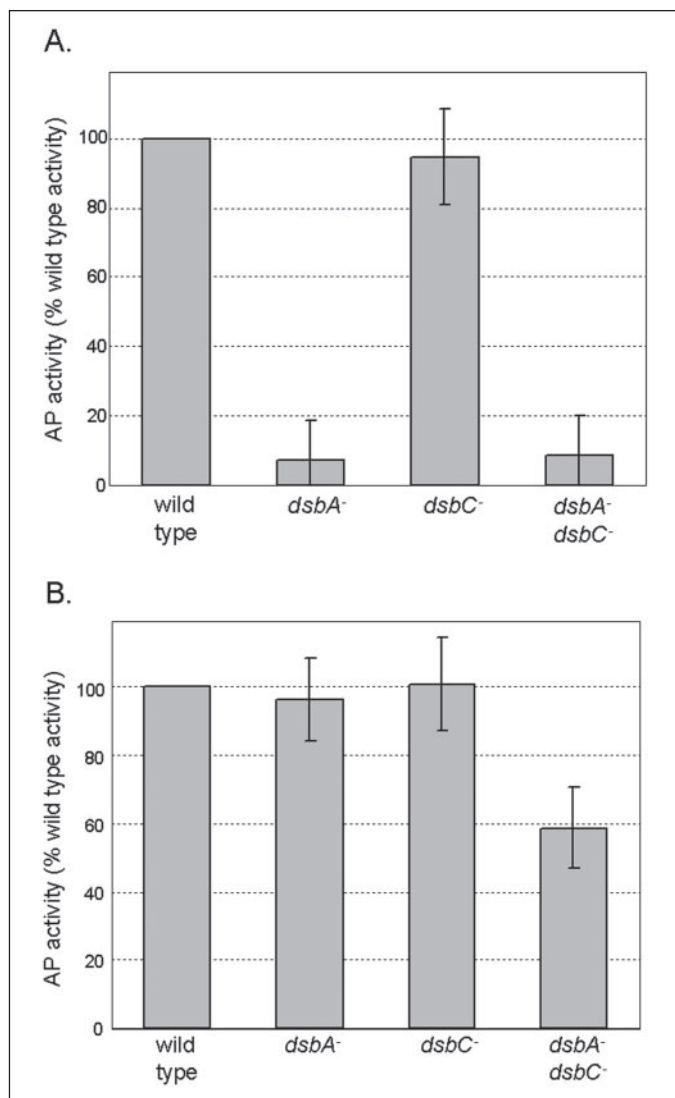
We postulated that copper complementation of a *dsbA*<sup>-</sup> strain could be due to the specific redox properties of copper. The conversion of Cu<sup>2+</sup> to Cu<sup>1+</sup> has a redox potential of 0.153 V, likely allowing Cu<sup>2+</sup> to oxidize free thiol groups and then become re-oxidized by oxygen species. Most other metals have properties that do not allow them to redox cycle: they either have redox potentials that do not allow re-oxidation by oxygen species, do not have multiple redox states, or are largely insoluble under physiologic conditions. Redox cycling of copper likely requires the presence of molecular oxygen species in order to re-oxidize copper after it has oxidized thiol groups. We tested the ability of copper to restore disulfide bond formation in the absence of oxygen species by testing motility under anaerobic conditions. Under anaerobic conditions, copper could not restore motility to a *dsbA*<sup>-</sup> strain at any copper concentration tested, supporting the idea that *in vivo* disulfide bond formation by copper relies on the presence of molecular oxygen. This was in good agreement with our finding that, under anaerobic conditions, wild-type and *dsbC*<sup>-</sup> strains were equally resistant to copper treatment.

**Copper Forms Non-native Disulfide Bonds *in Vivo***—Our motility and MalF-LacZ assays indicate that copper catalyzes periplasmic disulfide bond formation. To address whether copper forms a high proportion of non-native disulfide bonds in the cell, we examined the effect of copper on a strain with an increased number of periplasmic proteins harboring exposed thiol groups. If copper forms incorrect disulfide bonds between any free thiol groups, this strain might be especially sensitive to the effects of copper in the absence of a disulfide isomerase such as DsbC. A *dsbA*<sup>-</sup> strain exactly fits these criteria because it lacks DsbA and therefore acquires disulfides more slowly than a wild-type strain (2). If copper forms wrong disulfides, we expect that copper sensitivity of any mutation in the DsbC disulfide isomerization system will be augmented when combined with a *dsbA*<sup>-</sup> mutation. We therefore compared the copper sensitivity of isogenic *dsbA*<sup>-</sup>, *dsbC*<sup>-</sup>, *dsbA*<sup>-</sup> *dsbC*<sup>-</sup> and wild-type strains by streaking them out on copper-BHI plates (TABLE TWO). The *dsbA*<sup>-</sup> strain was not sensitive to copper relative to wild type, both were viable on 11 mM CuCl<sub>2</sub> plates, whereas the *dsbC*<sup>-</sup> strain was viable on 7 mM CuCl<sub>2</sub>. The *dsbA*<sup>-</sup> *dsbC*<sup>-</sup> strain was viable only to 3 mM CuCl<sub>2</sub>,

a much lower concentration than would be predicted by the copper sensitivities of the *dsbA*<sup>-</sup> and *dsbC*<sup>-</sup> single mutants. Similarly, the *dsbA*<sup>-</sup> *dsbD*<sup>-</sup> strain was also viable only to 3 mM CuCl<sub>2</sub>, though the *dsbD*<sup>-</sup> strain was viable to 7 mM. These results show that the absence of DsbA does indeed increase copper sensitivity of a *dsbC*<sup>-</sup> strain, perhaps because of the increased number of exposed thiol groups in the absence of DsbA.

If copper catalyzes incorrect disulfides more frequently than DsbA does, we expect that it will only poorly substitute for DsbA *in vivo*. This should be especially apparent when the DsbA substrate protein has more than two cysteines (which could therefore form non-native disulfides) and when DsbC is absent and cannot rearrange non-native disulfides. We tested the ability of copper to rescue disulfide bond formation in isogenic *dsbA*<sup>-</sup>, *dsbC*<sup>-</sup>, *dsbA*<sup>-</sup> *dsbC*<sup>-</sup>, and wild-type strains by measuring their alkaline phosphatase activity in the presence and absence of copper. Alkaline phosphatase is a periplasmic protein that contains four cysteine residues that form two consecutive disulfide bonds in the native structure of the protein. In minimal media in the absence of DsbA, these disulfides are not formed and the protein is degraded (2). In minimal media without copper, wild-type and *dsbC*<sup>-</sup> strains showed the same level of alkaline phosphatase activity (Fig. 1A), consistent with previous results (9). In the absence of copper, the *dsbA*<sup>-</sup> and *dsbA*<sup>-</sup> *dsbC*<sup>-</sup> strains both showed <10% of the activity of the wild type, also similar to earlier work (9). In the presence of 0.05 mM copper (Fig. 1B), the highest copper concentration at which the growth rate of the *dsbA*<sup>-</sup> *dsbC*<sup>-</sup> strain in minimal media was unaffected, alkaline phosphatase activity in the *dsbA*<sup>-</sup> strain was equal to that of the wild-type strain, showing that copper can fully complement a *dsbA*<sup>-</sup> strain when functional DsbC is present. In the *dsbC*<sup>-</sup> strain in the presence of copper, alkaline phosphatase activity was also similar to the wild-type strain in the presence of copper, suggesting that DsbA correctly oxidizes alkaline phosphatase thus preventing incorrect oxidation by copper. The *dsbA*<sup>-</sup> *dsbC*<sup>-</sup> strain, in contrast, showed only partial complementation by copper, with copper restoring alkaline phosphatase activity to ~60% of wild-type levels. This suggests that in the absence of both DsbA and DsbC, copper may form a large number of non-native disulfide bonds in alkaline phosphatase that cannot be rearranged.

**DsbC Rearranges Copper-catalyzed Non-native Disulfide Bonds**—To test directly whether copper forms non-native disulfide bonds that are substrates for the DsbC isomerase activity, we examined the effect of copper on *in vitro* refolding of drRNase A. RNase A contains four disulfides and is a standard substrate for *in vitro* disulfide isomerization assays in part because its folding intermediates are soluble (26). We used 50 μM CuCl<sub>2</sub> to oxidize 50 μM drRNase A, which contains 200 μM disulfides (4 disulfide bonds per RNase A molecule). Thus, copper was required to redox-cycle in order to fully oxidize the RNase. Because *in vivo* disulfide formation by copper occurred only aerobically, we used H<sub>2</sub>O<sub>2</sub> *in vitro* to recapitulate the *in vivo* re-oxidation of Cu<sup>1+</sup> to Cu<sup>2+</sup> by oxygen species. After incubation with copper and H<sub>2</sub>O<sub>2</sub>, drRNase A was >98% oxidized. This fully oxidized form of RNase A had no activity, identical to the fully denatured and reduced RNase A (Fig. 2A). Thus,

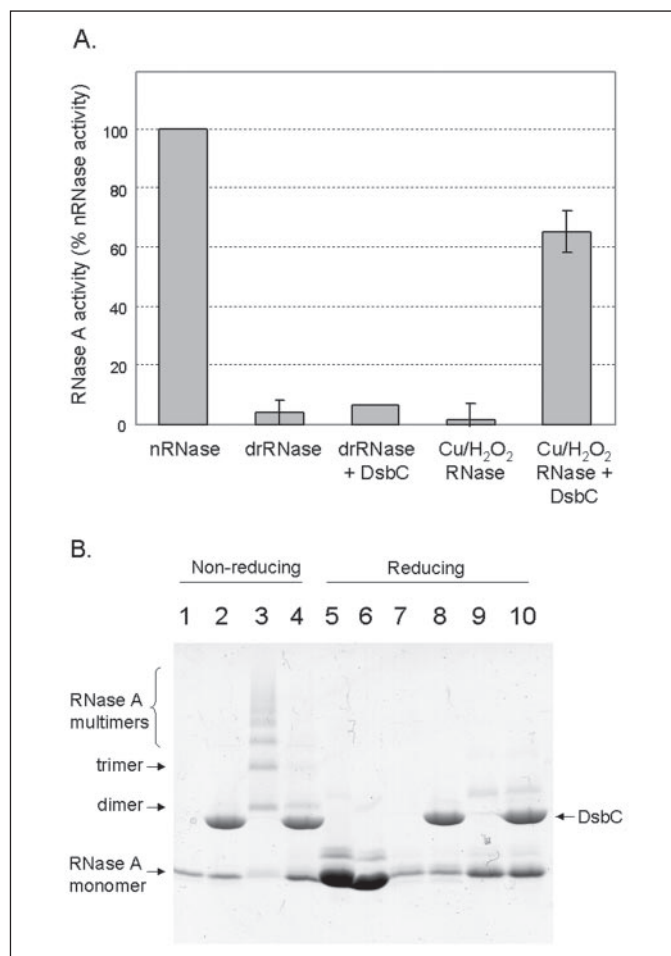


**FIGURE 1. Relative alkaline phosphatase activity of periplasmic disulfide oxidoreductase mutants.** Bars represent average activity of at least three experiments with wild type set to 100% activity; error bars show standard deviations. *A*, alkaline phosphatase activity of wild type and *dsb* mutant strains in M63 minimal media was performed as previously described (9). *B*, alkaline phosphatase activity of wild-type and *dsb* mutant strains in M63 minimal media + 0.05 mM CuCl<sub>2</sub>.

the CuCl<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>-treated RNase A likely represents a scrambled version of RNase A with essentially none of the RNase A achieving native disulfide connectivity. To determine whether DsbC could isomerize CuCl<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>-treated RNase A and cause it to regain activity, we removed copper and H<sub>2</sub>O<sub>2</sub> and incubated 20 μM CuCl<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>-treated RNase A with 20 μM reduced DsbC. Incubation with DsbC allowed RNase A to regain ~60% of activity. Thus, treatment of drRNase A with copper and an oxidant causes misoxidation of RNase A. This misoxidized form of RNase A can be rearranged by DsbC to form active RNase A.

We next used SDS-PAGE to visualize copper-induced changes in RNase A structure. After incubation with copper and H<sub>2</sub>O<sub>2</sub>, drRNase A formed higher molecular weight species whose molecular weights correspond to dimers, trimers, and higher-level multimers (Fig. 2*B*, lane 3). These species were visible on a gel under non-reducing conditions (Fig. 2*B*, lane 3) but disappeared under reducing conditions (Fig. 2*B*, lane 9), indicating that intermolecular disulfides connect these oligomers. Because RNase A normally contains no intermolecular disulfide bonds, these disulfides must be non-native. Treatment of drRNase A with cop-

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**FIGURE 2. DsbC isomerizes copper-catalyzed non-native disulfide bonds in RNase A.** *A*, RNase A activity of copper-scrambled RNase A is significantly restored by incubation with DsbC. Assay was performed as described under "Materials and Methods." Activity is shown as percent activity of equimolar native RNase A. *B*, DsbC resolves non-native intermolecular disulfides introduced by CuCl<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> treatment. Assay was performed as described under "Materials and Methods." Lanes 1–4 are run under non-reducing conditions, lanes 5–10 are run under reducing conditions. Lane 5, native RNase A standard. Lane 6, drRNase A standard. Native RNase A treated with CuCl<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> and incubated without DsbC (lanes 1 and 7) and with DsbC (lanes 2 and 8). drRNase A treated with CuCl<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> and incubated without DsbC (lanes 3 and 9) and with DsbC (lanes 4 and 10).

per alone, H<sub>2</sub>O<sub>2</sub> alone, or with the *E. coli* periplasmic disulfide oxidase, DsbA, did not cause intermolecular disulfide formation (data not shown). Similarly, treatment of native RNase A with copper/H<sub>2</sub>O<sub>2</sub> (Fig. 2*B*, lane 1) did not form intermolecular disulfides. To test the effect of DsbC on these disulfide-linked RNase A oligomers, we removed copper and H<sub>2</sub>O<sub>2</sub> by gel filtration and incubated the RNase A oligomers with reduced DsbC. DsbC was able to resolve the non-native intermolecular disulfide bonds in RNase A, as shown by the disappearance of oligomers and appearance of monomeric RNase A (Fig. 2*B*, lane 4).

## DISCUSSION

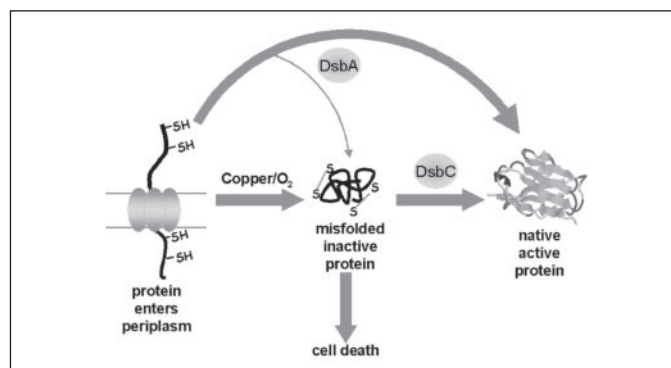
In this work, we show that *E. coli* lacking the periplasmic disulfide isomerase *dsbC* are sensitive to the redox-active metal copper. This is the first clear phenotype reported for a disulfide isomerase-deficient bacterial strain and suggests that disulfide isomerization plays a key role in protecting the cell against oxidative copper stress. Copper has been shown to act as a cysteine oxidant *in vitro* (21, 22) and can catalyze the *in vitro* formation of non-native disulfide bonds in lens membrane proteins and aldolase reductase, leading to loss of activity (27, 28). Thus, a possible mechanism of *dsbC*<sup>-</sup> copper sensitivity involves copper-cata-

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lyzed formation of non-native disulfide bonds in periplasmic and membrane proteins. If copper forms incorrect disulfide bonds frequently or in proteins essential for cell viability, DsbC may become essential because it is required to rearrange non-native disulfides. For this mechanism to be true, copper must catalyze disulfide bond formation in periplasmic and/or membrane proteins *in vivo*. We have found that copper forms *in vivo* disulfides in the periplasmic proteins FlgI and alkaline phosphatase as well as in the membrane-fusion protein MalF-LacZ. *In vitro*, copper forms non-native intermolecular disulfide bonds in RNase A that inhibit its activity and DsbC rearranges these non-native disulfides, allowing RNase A to regain activity. These results support the idea that *dsbC*<sup>-</sup> copper sensitivity may indeed be due to copper-catalyzed non-native disulfide bond formation. A second possibility is that DsbC may directly interact with copper to counteract copper toxicity. We have found, using thiol-trapping techniques, that DsbC becomes partially oxidized *in vivo* in the presence of copper (data not shown). The proportion of oxidized DsbC appears to increase slowly over time. This result is compatible with the scenario in which DsbC is required to rearrange copper-catalyzed non-native disulfide bonds: Because copper appears to slowly and incompletely oxidize DsbC, enough functional DsbC may be present to isomerize substrates. However, another possibility is that DsbC directly reduces copper, becoming oxidized, and DsbD recycles oxidized DsbC to allow it to re-reduce copper. This mechanism is more difficult to reconcile with the increased copper sensitivity of a *dsbA*<sup>-</sup>*dsbC*<sup>-</sup> strain as compared with a *dsbC*<sup>-</sup> strain, but should also be considered.

Bacterial strains do not require the disulfide isomerase DsbC under normal conditions but do require DsbC when treated with copper. This seems to imply that disulfide isomerization becomes especially important for bacterial survival under oxidative copper stress conditions. This is a surprising observation given how important disulfide bonds are to protein activity. In the current model of periplasmic protein folding, DsbA is thought to be a powerful but nonspecific oxidant, joining any two cysteines in a protein as the protein is secreted into the periplasmic space. There is some evidence supporting this model: when CcrA, a metallo- $\beta$ -lactamase from *Bacteroides fragilis* that normally contains no disulfides, is expressed in the *E. coli* periplasm, DsbA introduces a non-native disulfide bond (29). Additionally, DsbA introduces a non-native disulfide bond into a mutant version of alkaline phosphatase that is missing its first cysteine (30). Here we show that although both copper and DsbA catalyze disulfide bond formation *in vivo*, DsbA appears to form correct disulfide bonds more frequently than copper does. This suggests that DsbA may be more accurate in performing *in vivo* disulfide bond oxidation than previously thought.

The question of how DsbA could “know” how to form correct disulfide bonds and avoid forming incorrect disulfide bonds is intriguing. We postulate that there are at least two factors that make DsbA more accurate than copper in forming disulfides in the cell. First, the particular disulfide connectivity of periplasmic proteins might allow DsbA to form correct disulfides more frequently than expected. We previously performed a bioinformatics search of the periplasmic and membrane *E. coli* proteins catalogued in the Swiss Protein Data base (1). This search revealed that of the ~700 periplasmic and membrane proteins listed, more than half have two or more cysteine residues, suggesting that DsbA may form disulfides in >300 substrates. Of these possible DsbA substrates, only a handful (<10) are known to have non-consecutive disulfides. There is increasing evidence that DsbA interacts with proteins to form consecutive disulfide bonds, regardless of their native disulfide connectivity (3). If this is true, DsbA will form native disulfides in nearly all known *E. coli* substrates and DsbC will be important for the



**FIGURE 3. Model of the possible roles of the disulfide oxidase DsbA and the disulfide isomerase DsbC in periplasmic protein folding.** In this model, DsbA forms correct disulfide bonds in substrate proteins most of the time; thus, the role of DsbC under non-stress conditions is limited. However, under stress conditions that form non-native disulfides, such as aerobic copper stress, disulfide isomerization becomes essential, and DsbC is required. In the absence of DsbC, proteins with non-native disulfides cannot be transformed to natively folded, active proteins; this eventually causes cell death through an unknown mechanism.

folding of only a few proteins under non-stress conditions. This possibility also appears consistent with previous work showing that DsbA introduces a non-native disulfide into mutant alkaline phosphatase missing its first cysteine: in this case DsbA would form a consecutive, but non-native, disulfide.

Second, the reaction mechanisms by which DsbA and copper likely catalyze disulfide bond formation are very different. DsbA, a protein with peptide binding ability, attacks a free thiol on a substrate protein to rapidly form a mixed disulfide, which is then attacked by a second free thiol within that substrate protein, causing transfer of the DsbA disulfide to the substrate (8). By oxidizing substrates via a mixed-disulfide intermediate, DsbA is very likely to form an intramolecular disulfide bond. In contrast, copper can react with cysteines to form much more reactive thiol radicals (21). These radicals are so reactive that they could rapidly interact with any free thiol, on any protein, causing the formation of a high proportion of incorrect disulfides, including intermolecular disulfides. Consistent with these mechanisms, our RNase A gel assay shows that copper/H<sub>2</sub>O<sub>2</sub> forms intermolecular disulfide bonds in RNase A, whereas the DsbA/DsbB system catalyzes only intramolecular disulfide bonds. However, we must note that we have not identified the copper-generated species that interact with free thiol groups *in vivo* to catalyze disulfide bond formation. It is known that copper and H<sub>2</sub>O<sub>2</sub> can react to form highly reactive hydroxyl radicals (21). Thus, we cannot be certain that our *in vitro* assay using copper and H<sub>2</sub>O<sub>2</sub> exactly recapitulates the *in vivo* effects of copper. Further investigation into the precise mechanism of *in vivo* copper-catalyzed disulfide bond formation would be useful.

What then, are the roles of disulfide oxidation and isomerization during protein folding? Prior to this work, it was thought that DsbC primarily functions to resolve the disulfide bonds that DsbA makes incorrectly. However, our work suggests that disulfide isomerization becomes especially important for *E. coli* when they are subjected to oxidative copper stress leading to incorrect disulfide formation. This implies that the principal role of DsbC may be to rearrange wrong disulfides formed by extremely nonspecific oxidants like copper.

This leads us to one possible model of the roles of DsbA and DsbC during *in vivo* protein folding (Fig. 3). In this model, DsbA forms correct disulfide bonds in substrate proteins very frequently, and the role of disulfide isomerization under non-stress conditions is limited. However, under stress conditions that form non-native disulfides, disulfide isomerization becomes essential and DsbC is required. Without DsbC,



cell death occurs as a result of this non-native disulfide bond formation, either via a build-up of toxic aggregates or due to the misfolding of essential cellular proteins.

Consistent with this model, recent work shows that copper stress causes an increase in expression of the Cpx regulon, which responds to an accumulation of misfolded proteins in the periplasm (31). Interestingly, DsbA synthesis is known to be up-regulated 5–10 fold by the Cpx pathway (32). Up-regulation of DsbA under copper stress is consistent with our model, in which DsbA forms largely correct disulfides and can compete with copper to correctly oxidize thiol groups. Furthermore, both the *dsbC* and *dsbG* genes have been reported to be stress-inducible: DsbC is controlled by  $\sigma^E$ , which, like Cpx, responds to the accumulation of misfolded proteins in the cell envelope (33). The related isomerase DsbG is downstream of a putative OxyR binding site, which suggests that DsbG disulfide isomerization could also be involved in responding to oxidative stress that causes non-native disulfide bonds (34).

We find it intriguing that our RNase A gel assay reveals that DsbC can rearrange intermolecular disulfides. This is the first evidence of a role for DsbC in resolving disulfide-bonded complexes of proteins and suggests a set of possible *in vivo* substrates for disulfide isomerases that has not yet been widely considered. It also indicates one possible set of substrates, those that contain incorrect intermolecular disulfides, which might lead to a requirement for DsbC under oxidative copper stress conditions.

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## REFERENCES

- Hiniker, A., and Bardwell, J. C. (2004) *J. Biol. Chem.* **279**, 12967–12973
- Bardwell, J. C., McGovern, K., and Beckwith, J. (1991) *Cell* **67**, 581–589
- Berkmen, M., Boyd, D., and Beckwith, J. (2005) *J. Biol. Chem.*
- Missiakas, D., Georgopoulos, C., and Raina, S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 7084–7088
- Watarai, M., Tobe, T., Yoshikawa, M., and Sasakawa, C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 4927–4931
- Zapun, A., Missiakas, D., Raina, S., and Creighton, T. E. (1995) *Biochemistry* **34**, 5075–5089
- Bader, M. W., Xie, T., Yu, C. A., and Bardwell, J. C. (2000) *J. Biol. Chem.* **275**, 26082–26088
- Kadokura, H., Tian, H., Zander, T., Bardwell, J. C., and Beckwith, J. (2004) *Science* **303**, 534–537
- Rietsch, A., Belin, D., Martin, N., and Beckwith, J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 13048–13053
- Bader, M. W., Hiniker, A., Regeimbal, J., Goldstone, D., Haebel, P. W., Riemer, J., Metcalf, P., and Bardwell, J. C. (2001) *EMBO J.* **20**, 1555–1562
- Riddles, P. W., Blakeley, R. L., and Zerner, B. (1983) *Methods Enzymol.* **91**, 49–60
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Missiakas, D., Georgopoulos, C., and Raina, S. (1994) *EMBO J.* **13**, 2013–2020
- Missiakas, D., Schwager, F., and Raina, S. (1995) *EMBO J.* **14**, 3415–3424
- Rietsch, A., Bessette, P., Georgiou, G., and Beckwith, J. (1997) *J. Bacteriol.* **179**, 6602–6608
- Porat, A., Cho, S. H., and Beckwith, J. (2004) *Res. Microbiol.* **155**, 617–622
- Metheringham, R., Tyson, K. L., Crooke, H., Missiakas, D., Raina, S., and Cole, J. A. (1996) *Mol. Gen. Genet.* **253**, 95–102
- Bessette, P. H., Cotto, J. J., Gilbert, H. F., and Georgiou, G. (1999) *J. Biol. Chem.* **274**, 7784–7792
- Rensing, C., and Grass, G. (2003) *FEMS Microbiol. Rev.* **27**, 197–213
- Roberts, S. A., Weichsel, A., Grass, G., Thakali, K., Hazzard, J. T., Tollin, G., Rensing, C., and Montfort, W. R. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 2766–2771
- Kachur, A. V., Koch, C. J., and Biaglow, J. E. (1999) *Free Radic. Res.* **31**, 23–34
- Matsui Lee, I. S., Suzuki, M., Hayashi, N., Hu, J., Van Eldik, L. J., Titani, K., and Nishikimi, M. (2000) *Arch. Biochem. Biophys.* **374**, 137–141
- Dailey, F. E., and Berg, H. C. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 1043–1047
- Silhavy, T. J., Casadaban, M. J., Shuman, H. A., and Beckwith, J. R. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 3423–3427
- Tian, H., Boyd, D., and Beckwith, J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 4730–4735
- Wedemeyer, W. J., Welker, E., Narayan, M., and Scheraga, H. A. (2000) *Biochemistry* **39**, 7032
- Cecconi, I., Moroni, M., Vilaro, P. G., Dal Monte, M., Borella, P., Rastelli, G., Costantino, L., Garland, D., Carper, D., Petrash, J. M., Del Corso, A., and Mura, U. (1998) *Biochemistry* **37**, 14167–14174
- Takemoto, L. J., and Hansen, J. S. (1982) *Invest. Ophthalmol. Vis. Sci.* **22**, 336–342
- Alksne, L. E., Keeney, D., and Rasmussen, B. A. (1995) *J. Bacteriol.* **177**, 462–464
- Sone, M., Akiyama, Y., and Ito, K. (1997) *J. Biol. Chem.* **272**, 10349–10352
- Kershaw, C. J., Brown, N. L., Constantinidou, C., Patel, M. D., and Hobman, J. L. (2005) *Microbiology* **151**, 1187–1198
- Pogliano, J., Lynch, A. S., Belin, D., Lin, E. C., and Beckwith, J. (1997) *Genes Dev.* **11**, 1169–1182
- Dartigalongue, C., Missiakas, D., and Raina, S. (2001) *J. Biol. Chem.* **276**, 20866–20875
- Zheng, M., Wang, X., Doan, B., Lewis, K. A., Schneider, T. D., and Storz, G. (2001) *J. Bacteriol.* **183**, 4571–4579