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In vivo specificity of Ure2 protection from heavy metal ion and oxidative cellular damage in *Saccharomyces cerevisiae*

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Abstract

The *S. cerevisiae* Ure2 protein is a prion precursor able to form large homopolymers with the characteristics of amyloid particles, a function largely restricted to its 90 N-terminal amino acids. The remaining C-terminal domain of Ure2 plays two important roles in cellular metabolism. First, it regulates nitrogen catabolic gene expression by forming a complex with the GATA transcription factor Gln3. This complex formation correlates with Gln3 being sequestered in the cytoplasm under conditions of excess nitrogen, where Gln3/Gat1-mediated transcription is minimal. Second, Ure2, which possesses structural homology with glutathione S-transferases and binds to xenobiotics and glutathione, has been recently shown to be required for Cd(II) and hydrogen peroxide detoxification. Present experiments demonstrate that Ure2 possesses a far broader protection specificity, being required to avoid the toxic effects of As(III), As(V), Cr(III), Cr(VI), Se(IV), as well as Cd(II) and Ni(II), and to varying lesser degrees Co(II), Cu(II), Fe(II), Ag(I), Hg(II), cumene and *t*-butyl hydroperoxides. In contrast, deletion of *URE2* greatly enhances a cell's ability to withstand toxic concentrations of Zn(II) and Mo(VI). In the case of Cd(II), Ure2 does not function to decrease intracellular Cd(II) levels or influence glutathione availability for glutathionation. In fact, *ure2* hypersensitivity to Cd(II) remains the same, even when glutathione is used as sole source of nitrogen for cell growth. These data suggest that Ure2 possesses a central role in metal ion detoxification, a role not demonstrably shared by either of the two known *S. cerevisiae* glutathione S-transferases, Gtt1 and Gtt2, or the two glutaredoxins, Grx1 and Grx2, that also possess glutathione S-transferase activity.

Keywords

Ure2; Gln3; glutathione S-transferase; heavy metal detoxification; peroxidase

Introduction

The Ure2 protein of *S. cerevisiae* has been the object of increasing study, due to its relation to two clinically important problems. First, Ure2 is a negative regulator of the GATA-family transcription activators, Gln3 and Gat1/Nil1. Gln3 intracellular localization and the resulting ability to function has become an important terminal reporter of the Tor1/2 signal

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transduction pathway and its inhibition by rapamycin. Rapamycin and its derivatives are being used clinically and in clinical trials to prevent rejection of transplanted organs and as potential antineoplastic agents, especially with respect to PTEN-associated tumours (Neshat *et al*., 2001; Podsypanina *et al*., 2001). Second, Ure2 is a prion precursor with significant potential as a model system to study the biochemistry and molecular biology of prion formation, which is an important causative agent in amyloidoses (Wickner *et al*., 2000; Perutz, 1999; Perutz and Windle, 2001).

Ure2 was discovered as a genetic locus whose product negatively regulates nitrogen catabolism, a process designated nitrogen catabolite repression (NCR) (Lacroute, 1968, 1971; Drillen and Lacroute, 1972). In the presence of good nitrogen sources (e.g. glutamine or in some strains ammonia) and a wild-type allele of *URE2*, nitrogen catabolic genes required for the transport and degradation of poor nitrogen sources are expressed at minimal levels (Hoffman-Bang, 1999; Magasanik, 1992; Cooper, 1982, 2002). In contrast, these genes are expressed at high levels when poor nitrogen sources (e.g. proline) are provided. This negative regulation is lost in *ure2* mutants, where nitrogen catabolic genes are expressed at high levels, irrespective of the nitrogen source provided. The transcriptional activators responsible for NCR-sensitive gene expression are Gln3 and Gat1 (Hoffman-Bang, 1999; Cooper, 2002). The regulation of Gln3 function is achieved through its localization in the cytoplasm under conditions of excess nitrogen and accumulation in the nucleus when the nitrogen supply is limiting (Cooper, 2002, 2004). One model of how this occurs is based on the observations that:

1. Treating cells with rapamycin, the inhibitor of the Tor1/2 kinases, increases NCR-sensitive gene expression (Beck and Hall, 1999; Cardenas *et al*., 1999; Hardwick *et al*., 1999; Bertram *et al*. 2000).
2. Nuclear accumulation of Gln3 occurs in both *ure2* cells and wild-type cells treated with rapamycin, the latter observation leading to the proposal that control of intracellular localization is regulated by the Tor1,2 protein kinases, the *in vivo* targets of rapamycin (Beck and Hall, 1999; Bertram *et al*., 2000).
3. Ure2 can form a complex with Gln3 and the amount of this complex diminishes under conditions where Gln3 is nuclear (Beck and Hall, 1999; Bertram *et al*., 2000).
4. Rapamycin-treatment results in dephosphorylation of Gln3 (Beck and Hall, 1999; Bertram *et al*., 2000).

According to this model, phosphorylated Gln3 forms a complex with Ure2 when Tor1,2 are inactivated. Either this complex, or the fact that it stabilizes the phosphorylated form of Gln3, have been posited to prevent its transport from cytoplasm to nucleus (Beck and Hall, 1999; Bertram *et al*., 2000). Although there is broad agreement on the observations upon which the model is based, the cause–effect events responsible for NCR-regulated Gln3 intracellular localization remain controversial. This is due in part to the finding that detectable Gln3 phosphorylation/dephosphorylation do not correlate with nuclear Gln3 localization, except at early times following the onset of rapamycin treatment (Cox *et al*., 2004), and the results of a more thorough characterization of *tap42* mutants (Wang *et al*.,

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2003). Further, there is no correlation of detected phosphorylation levels with the nature of the nitrogen source provided or the cellular localization of Gln3 following nitrogen and carbon starvation (Cox *et al*., 2004).

In addition to its regulatory role in gene expression, the fact that some *ure2* mutant alleles segregated in a non-Mendelian fashion, and the genetic analysis of this phenomenon, led to the finding that Ure2 is a prion precursor (Wickner, 1994). Domain mapping of Ure2 localized prion formation to the asparagine-rich, N-terminal portion of the protein (ca. 90 amino acids) and the nitrogen regulatory function to the remaining C-terminal portion (Masison *et al*., 1997; Maddelein and Wickner, 1999). Structural analysis of Ure2 demonstrated that it shares primary sequence and three-dimensional homology with glutathione S-transferases (Umland *et al*., 2001; Bousset *et al*., 2001). Furthermore, crystal structures with glutathione or xenobiotics bound to the protein have been reported (Bousset *et al*., 2001b). Together, these data raised the possibility of a third Ure2 function, that of a glutathione S-transferase. Consistent with this possibility, recent *in vivo* data demonstrated a high requirement of Ure2 for protection against Cd(II) and Ni(II) ions and the cellular oxidant hydrogen peroxide (Rai *et al*., 2003).

Data showing that Ure2 is required for detoxification of Cd(II) raised the question of whether these observations derived from Ure2 possessing a broadly applicable function in heavy metal metabolism, or alternatively from an indirect effect of Ure2 on Cd(II) uptake and intracellular accumulation. To distinguish these possibilities, we have determined the breadth of specificity of Ure2 participation in heavy metal metabolism and intracellular Cd(II) accumulation. *In vivo* data reported here indicate that Ure2 is required for detoxification of a wide variety of transition metal ions, albeit with some surprising exceptions, and multiple hydroperoxides. Hypersensitivity of *ure2* mutants to Cd(II) does not derive from increased levels of intracellular Cd(II) accumulation, because wild-type cells accumulate higher concentrations of Cd(II) than a *ure2* . Further, Cd(II) toxicity does not decrease in the presence of exogenously added glutathione, or when cells are growing with glutathione as the sole nitrogen source. We were unable to find evidence that Gtt1/2, *S. cerevisiae* glutathione S-transferases (Choi *et al*., 1998) and proteins to which Ure2 exhibits greatest homology are required for metal ion detoxification. Similarly, mutants lacking glutaredoxins Grx1 and Grx2, reported to possess glutathione S-transferase activity and overlap in function with Gtt1/2 (Collinson *et al*., 2002; Collinson and Grant, 2003) or a quadruple *gtt1,gtt2,grx1,grx2* mutant, were no more sensitive than wild-type to Cd(II). This suggests that these glutathione S-transferases do not demonstrably participate in heavy metal ion detoxification or functionally overlap with Ure2 in this process.

Materials and methods

The *S. cerevisiae* strains we used are listed in Table 1. Rich medium was YEPD, and minimal medium was (0.17%) Difco yeast nitrogen base (YNB) without amino acids or ammonium sulphate, to which were added 2% glucose and the indicated nitrogen source at 0.1%, unless otherwise indicated. Further additions of metal ions or other compounds and their concentrations are indicated in the captions to figures. Standard auxotrophic supplements were added where necessary. Cells were grown at 30°C. Although the

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photographs are mostly of cells at a single concentration of the compound tested, in most cases we collected images at multiple (4–8) concentrations and have presented the ones in which differences between wild-type and mutant were greatest. We have also presented images at multiple times. This yields a better appreciation of the time course of growth. This was particularly useful when growth differences between wild-type and mutant were subtle and/or when mutant cells continued to grow after the wild-type reached saturation (rate of increase in colony diameter slowed late in growth). Pictures of untreated wild-type control cells appear in the CoCl2 and CuSO4 experiments. Similar controls were performed in all cases, with similar results.

$^{109}CdCl_2$ accumulation assays

Uptake of $^{109}CdCl_2$ was performed as described previously for ^{14}C-methylamine (Tate and Cooper, 2003). The final concentration of radioactive $^{109}CdCl_2$ was 0.05 mM.

Results

Spectrum of metal ions to which *ure2* cells are hypersensitive

The necessity of a wild-type *URE2* allele for growth of *S. cerevisiae* in the presence of Cd(II) ($CdCl_2$), Ni(II) ($NiSO_4$) and hydrogen peroxide prompted us to determine the breadth of Ure2 involvement in heavy metal and hydroperoxide detoxification. We first studied the affects of arsenic and chromium ions on cell growth. These transition metals stably exist in multiple oxidation states and, in some cases, the oxidation state of the metal is altered prior to detoxification (Rosen, 2002; Eide, 1998). Arsenic ions exist as As(III) (As_2O_3) and As(V) (Na2HAsO4), which are taken into the cell by Fps1 and the phosphate transporters, respectively (Rosen, 2002). Although Ure2 was required for growth in the presence of both ions, mutant cells exhibited a clearer phenotype with As(III) than As(V) (Figure 1 right panels). Further, the phenotype with As(III) was similar in both YNB–ammonia and glutamate media. In contrast, differences in the sensitivity of wild-type and *ure2* to As(V) were clearer in YNB–ammonia than glutamate medium, especially following extended incubation (Figure 1, right panels).

Chromium ions, which are reported to be transported along with Zn(II) and probably Fe(II) by Zrt1 and Fet4, respectively (Dix *et al*., 1994; Gitan *et al*., 2003), similarly exist in two oxidation states, Cr(III) ($CrCl_3$) and Cr(VI) (CrO_3). As with arsenic, the more reduced ion, Cr(III), generated the clearer *ure2* phenotype (Figure 1, left panels). Cr(VI) overall was toxic to both wild-type and *ure2* cells at lower concentrations than Cr(III), but there was less difference between the two strains' sensitivity to Cr(VI) ions. When the above experiments were repeated in YEPD, the medium most often used for such studies, the growth responses to the metal ions were quite different. The *ure2* mutant was still sensitive to As(III) but not Cr(III) (Figure 2, upper right panels). These different responses, however, are likely due to the reaction of $CrCl_3$ with compounds in the medium, as demonstrated by marked and progressive changes in the $CrCl_3$ absorption spectrum 30–90 s following its addition to YEPD medium.

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Among the remaining metal ions tested, Se(IV) (Na_2SeO_3) was the one to which the *ure2* was most sensitive. Here, the hypersensitive phenotype was just as clear as with As(III), Cr(III), Cd(II) or Ni(II) (cf. Figure 2, left top panels, with Figure 1, this paper and Figure 1 in Rai *et al*., 2003). Greater sensitivity was observed in YNB–glutamate than in YNB–ammonia, but this difference was also observed in the wild-type strain. Further, as growth progressed, the centres of wild-type Se(IV)-treated colonies developed a strong reddish colour that was not present at the colony margins and was nonexistent in the *ure2* (data not shown). Co(II) ($CoCl_2$), Cu(II) ($CuSO_4$), Fe(II) ($FeSO_4$) and Ag(I) ($AgNO_3$), were slightly to moderately toxic in *ure2* cells early in growth (Figures 3 and 4, left panels). Fe(II) generated a phenotype in the *ure2* that was similar to Se(IV), but only at high concentration (Figure 4, left bottom panels). In two cases, Co(II) and Cu(II), after growth of the wild-type approached saturation (i.e. the rate of increase in colony diameter slowed), mutant cells continued to grow slowly to the point that differences between wild-type and mutant colonies were less clear than earlier in the experiment (Figure 3). As observed with Se(IV), wild-type colonies grown in the presence of Cu(II) developed an increasingly strong olive-brown colour that was absent in colonies of the *ure2* (data not shown). These would be the expected results if wild-type cells accumulated more Se(IV) and Cu(II) than the *ure2* . The last two metal ions evaluated, Zn(II) ($ZnSO_4$) and Mo(VI) (Na_2MoO_4), behaved just the opposite to all the others. Wild-type cells were far more sensitive to these metal ions than the *ure2* (Figure 4, right panels).

Mn(II) ($MnCl_2$), Pb(II) ($Pb \cdot C_2H_3O_2)_2$-2Pb $(OH)_2$) and Sb(III) ($SbCl_3$) did not demonstrably effect growth of the wild-type or *ure2* mutant, even at concentrations of 20, 5, and 0.5 mM, respectively (data not shown). Hg(II) ($HgCl_2$), on the other hand, inhibited growth of the wild-type with glutamate as nitrogen source, even at the lowest concentrations (0.0001 and 0.0002 mM) we used (Figure 2 right bottom panels). There was, however, a slight requirement of Ure2 for growth with ammonia as nitrogen source at 0.0004 mM$HgCl_2$ (Figure 2, bottom right panels).

It was conceivable that sensitivity of *ure2* mutants to heavy metals and their resistance to Zn(II) and Mo(VI) derived indirectly as a result of increased Gln3- and Gat1-mediated transcription that occurs in the absence of Ure2. We evaluated this possibility by comparing the metal ion sensitivity and resistance of cells growing under conditions of high and low Gln3-/Gat1-mediated transcription, i.e. with proline or glutamine provided as sole nitrogen source, respectively. Although cells grew more slowly when provided with the poor nitrogen source, proline, both proline- and glutamine-grown cells remained sensitive to As(III), Cr(III) and As(VI), as well as resistant to Zn(II) and Mo(VI) [Figure 5, and data not shown for As(VI)].

Gtt1 and Gtt2 are *S. cerevisiae* glutathione S-transferases and the proteins to which Ure2 is most homologous (Choi *et al*., 1998). Therefore, we tested *gtt1, gtt2* and *gtt1gtt2* double mutants (JC101, JC102, JC103) for metal ion sensitivity, but did not observe it (data not shown). Ni(II) and Cd(II) were the ions tested. Two additional proteins have recently been reported to possess glutathione S-transferase activity and to overlap functionally with Gtt1 and Gtt2, the glutaredoxins Grx1 and Grx2 (Collinson *et al*., 2002; Collinson and Grant, 2003). Therefore, it was possible that the presence of the Grx proteins masked the

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requirement of Gtt1 and Gtt2 for heavy metal detoxification. To test this possibility, we assayed the Cd(II) sensitivity of *grx1grx2* double and *gtt1gtt2grx1grx2* quadruple mutants. As shown in Figure 6, both mutants were no more sensitive to Cd(II) than the wild-type.

Glutathione S-transferases in some instances also participate in protecting cells from the deleterious effects of hydroperoxides. Since *ure2* cells are hypersensitive to hydrogen peroxide (Rai *et al*., 2003), we determined whether Ure2 was required for protection against other hydroperoxides. As shown in Figure 2, left bottom panels), *ure2* was sensitive to both cumene and *t*-butyl hydroperoxide. The *ure2* phenotype with cumene was not quite as strong as with hydrogen peroxide (Figure 2 in Rai *et al*., 2003), but was seen equally in ammonia- and glutamate-grown cells. The phenotype with *t*-butyl hydroperoxide was less strong, being similar to that observed with diamide and CDNB (Figures 5 and 7 in Rai *et al*., 2003), and was clearer with ammonia than glutamate.

Intracellular Cd(II) accumulation in a *ure2*

It was recently reported that *ure2* cells are more sensitive than wild-type to aluminium ions (Basu *et al*., 2004). This hypersensitivity correlated with, and was concluded to derive from, increased intracellular accumulation of aluminum ions in the mutant (Basu *et al*., 2004). Although Al(III) sensitivity was less than we observed for Cd(II), we determined whether hypersensitivity of the *ure2* to Cd(II) was similarly derived by comparing $^{109}CdCl_2$ accumulation in wild-type and *ure2* strains. It is important to note that $^{109}CdCl_2$ was provided at a concentration (0.05 mM) previously shown to be toxic to *ure2* cells (Rai *et al*., 2003). As shown in Figure 7A, $^{109}CdCl_2$ accumulations in wild-type and mutant cells were indistinguishable during the first 20 min of the experiment. Thereafter, a small but reproducible decrease in $^{109}CdCl_2$ accumulation was observed in the *ure2* mutant. In the long term there was less $^{109}CdCl_2$ accumulation in the *ure2* than in wild-type. When accumulation was corrected for A_{600nm} values determined immediately following collection of the final radioactive samples (wild-type = 0.78; *ure2* = 0.55), wild-type cells accumulated 24% more $^{109}CdCl_2$ than the *ure2*. These data argue that Cd(II) hypersensitivity of the *ure2* is unlikely to derive from increased Cd(II) uptake and/or accumulation.

Exogenously provided glutathione partially alleviates, but does not reverse, heavy metal ion toxicity in *ure2* cells

Another possible explanation for the observed metal ion hypersensitivity of *ure2* strains was glutathione deficiency. Expression of *CIS2*, the gene encoding the glutathione-degrading enzyme γ-glutamyl transpeptidase, is regulated by GATA-factors Gln3, Gat1/Nil1 and Dal80/Gzf3 (Springael and Penninckx, 2003). Ure2 downregulates Gln3-and Gat1-mediated transcription by inhibiting nuclear accumulation of these transcription factors when cells are grown in the presence of good nitrogen sources, such as glutamine, or ammonia as well as glutamate, a somewhat poorer, but still repressive, nitrogen source (Tate and Cooper, 2003; Rai *et al*., 2004). Therefore, one could reasonably hypothesize that deletion of *URE2* would cause increased γ-glutamyl transpeptidase production, decreased intracellular glutathione, and hence increased metal ion sensitivity. The situation would be

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similar to the increased Cd(II) sensitivity that occurs in a *gsh1*, which is unable to synthesize glutathione (Dormer *et al*., 2000).

Consistent with this possibility, *CIS2–lacZ* expression and γ-glutamyl transpeptidase activity are two- and three-fold higher, respectively, in ammonia-grown *ure2* cells relative to wild-type (Springael and Penninckx, 2003). Although this is not a dramatic increase in transpeptidase activity, it might still be enough to create glutathione deficiency in the presence of Cd(II) ions. Therefore, we measured the effect of adding 2 mM reduced glutathione to YNB–ammonia and glutamate media containing Cd(II) or As(III) and monitored growth of wild-type and *ure2* cells. Glutathione addition had no effect on Cd(II) sensitivity of a *ure2* in YNB– ammonia medium, even after extended incubation (Figure 8, left two columns). Growth of *ure2* increased a little when glutathione was added to YNB–glutamate medium containing Cd(II), but hypersensitivity again clearly remained (Figure 8, right two columns). On the other hand, glutathione partially alleviated *ure2* hypersensitivity to As(III) in ammonia medium (Figure 9, left two columns). The mutant growth phenotype was least clear in the presence of added glutathione after extended incubation, but this derives in part from the fact that the wild-type cells reached saturation early on, while mutant cells continued to grow, albeit slowly. In spite of this problem, the wild-type colonies remained larger than those of the mutant. With glutamate as the nitrogen source, addition of glutathione had no effect on *ure2* hypersensitivity to As(III) (Figure 9, right two columns).

To more directly test whether increased *CIS2* (encoding γ-glutamyl transpeptidase) expression that occurs in a *ure2* was responsible for increased heavy metal ion and hydroperoxide sensitivity in *ure2* cells, we constructed a *ure2*, *cis2* double deletion and compared its sensitivities to those of a *ure2* mutant. As shown in Fig. 10, *ure2* and *ure2*, *cis2* strains were equally sensitive to Cd(II), irrespective of the nitrogen source. There was a little improvement in the ability of the double mutant to protect itself against hydrogen peroxide toxicity relative to *ure2*, but less than would be required to explain the *ure2* phenotype. This is not a surprising observation, given the number of other proteins that participate in glutathione-dependent detoxification of hydroperoxides.

Although γ-glutamyl transpeptidase is the main enzyme associated with glutathione degradation, it has been suggested there might be a second unknown enzyme that degrades glutathione. Therefore, we determined whether the *ure2* pheno-type remained intact under conditions where cells were provided with glutathione as sole nitrogen source. This experiment was predicated on the observations that glutathione (a tripeptide, glutamate–cysteine–glycine) is transported into the cell by Hgt1 (Bourbouloux *et al*., 2000) and can be degraded by *S. cerevisiae* when provided as sole sulphur source (Kumar *et al*., 2003). Further, the dry weight of wild-type cells increases three-fold in 24 h following the onset of nitrogen starvation, whereas those lacking γ-glutamyl transpeptidase increase only 1.6-fold (Mehdi and Penninckx, 1997). We cultured wild-type and *ure2* cells in YNB–glutathione medium alone or containing either 0.05 mM $CdCl_2$ or 0.2 mM As_2O_3. Although growth was both slow and limited, even in wild-type, metal-free cultures, the *ure2* remained sensitive to Cd(II) and As(III) (Figure 11, top three panels). In the above experiment, we used a glutathione concentration similar to that used by others in the field studying metal ion

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metabolism. Although a nitrogen source provided at 2 mM is sufficient for growth, it is a lower concentration than we would normally use. Therefore, we repeated the experiment but increased the glutathione concentration to 0.2%. To our surprise, glutathione at this higher concentration would not serve as a nitrogen source for the *ure2* strain grown in metal-free medium (Figure 11, cf. images in the left-most column). This occurred even though at 0.2%, glutathione was present at only 3.5-fold greater concentration than when it was provided at 2 mM. One explanation for this result was that increased γ-glutamyl transpeptidase production in the *ure2* mutant permitted toxic by-product(s) of glutathione catabolism to accumulate faster than the cells could divide and metabolize or dilute it/them out. Consistent with this suggestion was the fact that there was more growth of a *ure2* culture whose medium contained 0.05 mM $CdCl_2$ after 140 h of incubation than in wild-type (Figure 11, centre panel, bottom row). This growth continued (173 h) in Cd(II)-treated but not untreated cells, suggesting that Cd(II) was slightly facilitating the use of glutathione as sole nitrogen source. One way of explaining these data is to suggest that Cd(II) decreases intracellular glutathione, which would also decrease any toxic byproduct(s) of its catabolism. Cd(II) glutathionation occurs at a slow rate in the absence of enzyme catalysis (Li *et al*., 1997). Alternatively, Gtt1/2 and Grx1/2 (Collinson and Grant, 2003), which possess glutathione S-transferase activity with CDNB as substrate, might account for a very slow gluthionation-mediated diminution of glutathione levels, even if Cd(II) ions were not the normal substrate of these proteins.

Discussion

Data presented above demonstrate that Ure2 is broadly required for detoxification of many heavy metal ions, particularly those in the transition portion of the periodic table. This requirement is greatest for As(III), As(V), Cr(III), Cr(VI), Se(IV) and Fe(II), but is also observed to varying lesser degrees for Co(II), Cu(II), Ag(I) and Hg(II). These results add to the previously reported requirement for Ni(II) and Cd(II) detoxification (Rai *et al*., 2003). Additionally, Ure2 was required for detoxification of cumene hydroperoxide and *t*-butyl hydroperoxide, although to a lesser degree than observed for hydrogen peroxide. These are characteristics of the glutathione S-transferase family of enzymes and, while it is tempting to suggest that Ure2 might possess transferase activity, that conclusion is not presently justified. It is, however, important to note the four known *S. cerevisiae* proteins that can be demonstrated to possess glutathione S-transferase activity *in vitro*, Gtt1, Gtt2, Grx1 and Grx2, do not appear to participate in heavy metal ion detoxification. This conclusion is based on the observation that *gtt1gtt2, grx1grx2* and *gtt1gtt2grx1grx2* double and quadruple mutants are no more sensitive to heavy metal ions than isogenic parent strains. Although we and others have so far been unable to demonstrate that Ure2 possesses glutathione S-transferase enzyme activity with heavy metals as substrates, Bai *et al*. have recently reported in prepublication that purified Ure2 does possess peroxidase activity such as that seen in glutathione S-transferases and glutaredoxins (Bai *et al*., 2004; Choi *et al*. 1998; Collinson *et al*., 2002; Collinson and Grant, 2003). The *in vivo* requirement of Ure2 for protection of cells against oxidative damage generated by cumene, *t*-butyl hydroperoxide and hydrogen peroxide support the conclusion of that work (Rai *et al*., 2003; present work). We have also been able to demonstrate that purified (ca 90%) Ure2 is able to synthesize a product whose

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molecular weight is greater than that of free cadmium ions, in a protein-, glutathione- and temperature-dependent manner. However, since we are as yet unable to identify the product, we cannot conclude that Ure2 is in fact capable of glutathionating cadmium ions (T.G. Cooper, unpublished observations). Therefore, although present evidence continues to support the idea that Ure2 may participate directly in heavy metal ion metabolism, the question must remain open until the enzyme activity is demonstrated and characterized biochemically *in vitro*.

On the other hand, Ure2 possesses the potential, as a negative transcriptional regulator, for generating indirect effects. Experiments presented above demonstrate this potential in at least two cases. Although deletion of *URE2* greatly increases sensitivity to Cd(II), Ni(II), As(III), As(V), Cr(III), Cr(VI), Se(IV) and Fe(II), and less so for Co(II), Cu(II), Ag(I) and Hg(II), it has just the opposite phenotype for Zn(II) and Mo(VI). The *ure2* is much more resistant than wild-type to high concentrations of both ions; not the expected phenotype of a glutathione S-transferase required for detoxification of these heavy metals. This is surprising for Zn(II), because the coordination chemistry of Zn(II) and Cd(II) are very similar, and several proteins participate in both Cd(II) and Zn(II) metabolism. Overproduction of Zrc1, a protein required for Zn(II) accumulation in isolated vacuolar membrane vesicles, confers resistance to both Zn(II) and Cd(II) (Kamizono *et al*., 1989; Conklin *et al*., 1992). Zrt1, the high-affinity Zn transporter (Zhao and Eide, 1996), also becomes a major route of Cd(II) uptake in Zn(II)-limited cells. Hypersensitivity to Cd(II) is inversely related to Zrt1 activity, i.e. inactivation of Zrt1 results in Cd(II) resistance, whereas inability to inactivate Zrt1 causes sensitivity to 50 nM Cd(II) (Gomes *et al*., 2002; Gitan *et al*., 2003). Finally, incubation of cultures with Zn(II) increases *URE2* expression 2.8-fold (Lyons *et al*., 2000).

Hypersensitivity of a *ure2* to Al(III) is another example of Ure2 indirectly influencing metal ion metabolism and one that is pertinent to the evaluation of Cd(II) sensitivity. Mutant *ure2* cells are hypersensitive to Al(III) and intracellular oxidation levels increase two- to three-fold in response to its addition to the culture medium (Basu *et al*., 2004). This addition also results in a nearly twofold increase in *URE2* expression, as occurs with Zn(II) addition. However, irrespective of whether good (ammonia) or poor (proline) nitrogen sources are provided, Al(III) sensitivity is the same. This is a somewhat surprising observation, because the most thoroughly documented function of Ure2 is its down regulation of GATA-factor-mediated transcription when good nitrogen sources are provided in the medium (Cooper, 2004). Al(III) sensitivity in *ure2* cells correlates with, and is concluded to result from, seven- to eight-fold increased Al(III) accumulation. However, a similar increase in Al(III) accumulation also occurs in a *phgpx1* /2 /3 triple mutant (Basu *et al*., 2004). Although events that connect loss of Ure2 and phospholipid hydroperoxide glutathione peroxidase activities with increased Al(III) accumulation remain unclear, hypersensitivity of a *ure2* to Cd(II) cannot be explained in the same way. This is because the *ure2* mutant accumulated only 80% as much $^{109}CdCl_2$ as wild-type. By reasoning similar to that with Al(III), we would have expected to see increased resistance to Cd(II), as occurred with Zn(II) and Mo(VI). Further supporting this interpretation, *ure2* cells incubated with Cu(II) or Se(IV) did not become coloured, as did the wild-type. Such colouration occurs with the

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accumulation of metal ion complexes, again arguing against the possibility that *ure2* hypersensitivity to these metal ions derives from increased accumulation.

If the broad specificity of Ure2 in metal ion detoxification is indirect, it is not easily explained by a single mechanism. As noted above, Zn(II) and Cd(II) exhibit significant chemical similarity, as do As(III) and Sb(III). Yet different responses to members of each pair of ions were seen in the *ure2* . Indirect mechanisms of metal ion hypersensitivity and resistance are common. However, when this occurs, all of the ions involved in the growth phenotype usually share specific transport or other components of metabolism and/or detoxification that account for the altered sensitivity (Eide, 1998); the converse is also true. The *ure2* is sensitive to many metal ions, but correlations between all of the pertinent ions/compounds and cellular constituents needed to make credible cause–effect explanations of the *ure2* phenotype are not apparent. It is clear from data presented here and by others that Ure2 plays central roles in the detoxification and metabolism of a wide variety of metal ions, and appears to do so by indirect as well as potentially direct mechanisms. The challenge that remains is to elucidate the molecular mechanisms associated with each of these newly identified roles.

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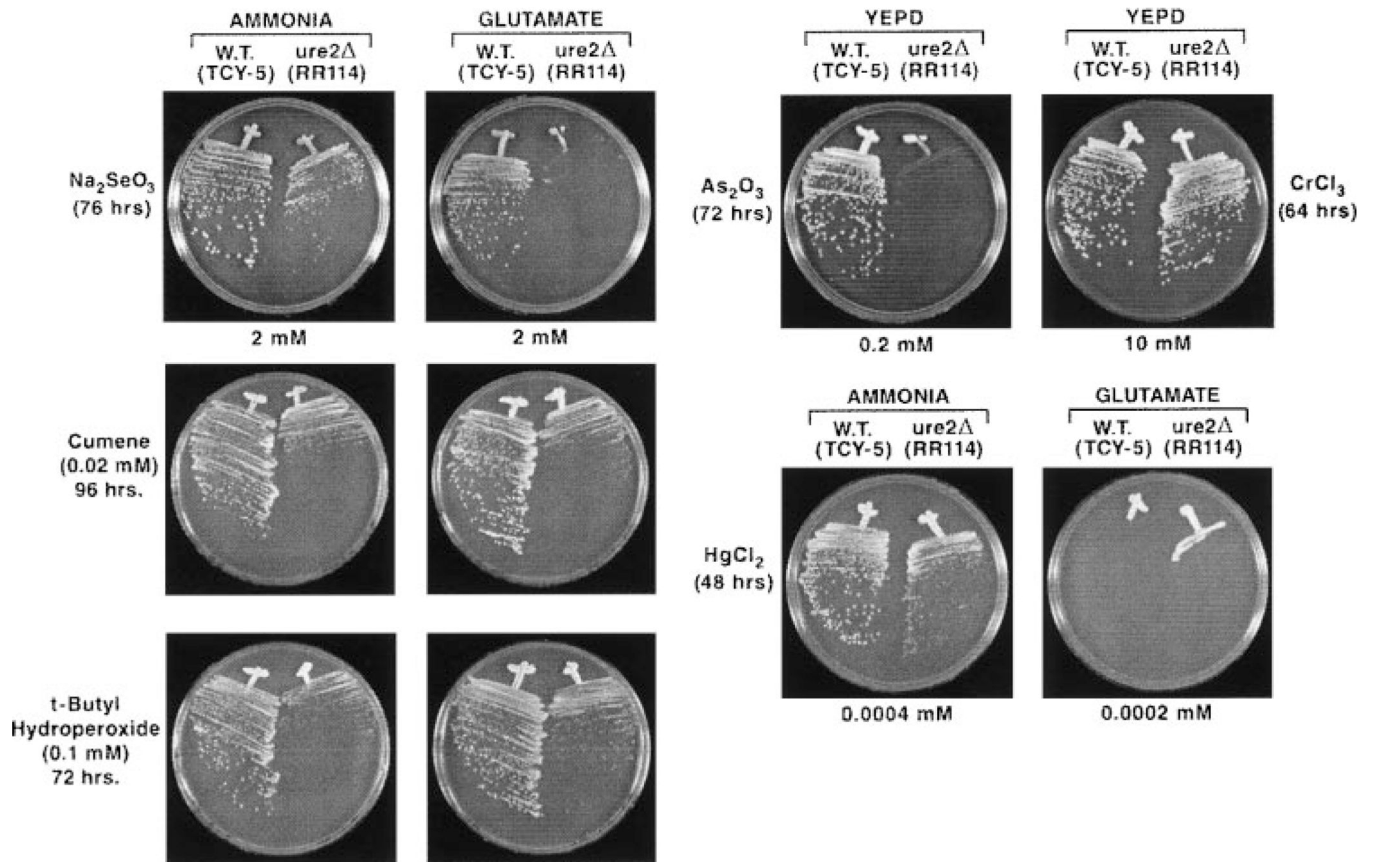

Figure 1.

Growth of wild-type (TCY5) and *ure2* (RR114) strains in minimal YNB-ammonia and glutamate media containing the indicated final concentrations of $CrCl_3$, CrO_3, As_2O_3 and Na_2HAsO_4. The time of incubation and metal ion concentrations are indicated adjacent to and below the images, respectively

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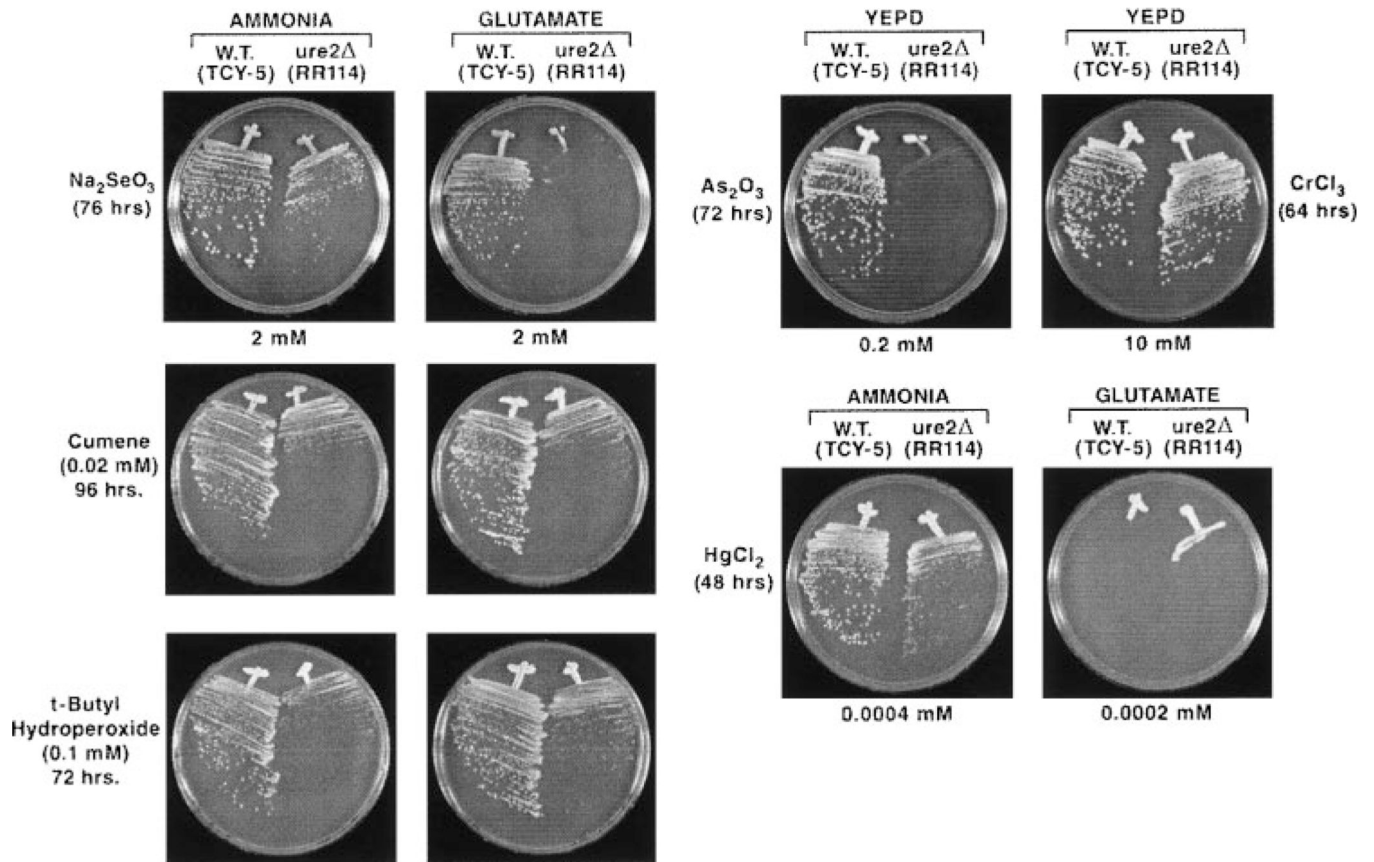

Figure 2.

Growth of wild-type and *ure2* strains in YNB-ammonia, YNB-glutamate or YEPD medium containing either heavy metal ions (Na_2SeO_3, As_2O_3, $HgCl_2$) or hydroperoxides (cumene, *t*-butyl hydroperoxide). Organization and labelling are as in Figure 1

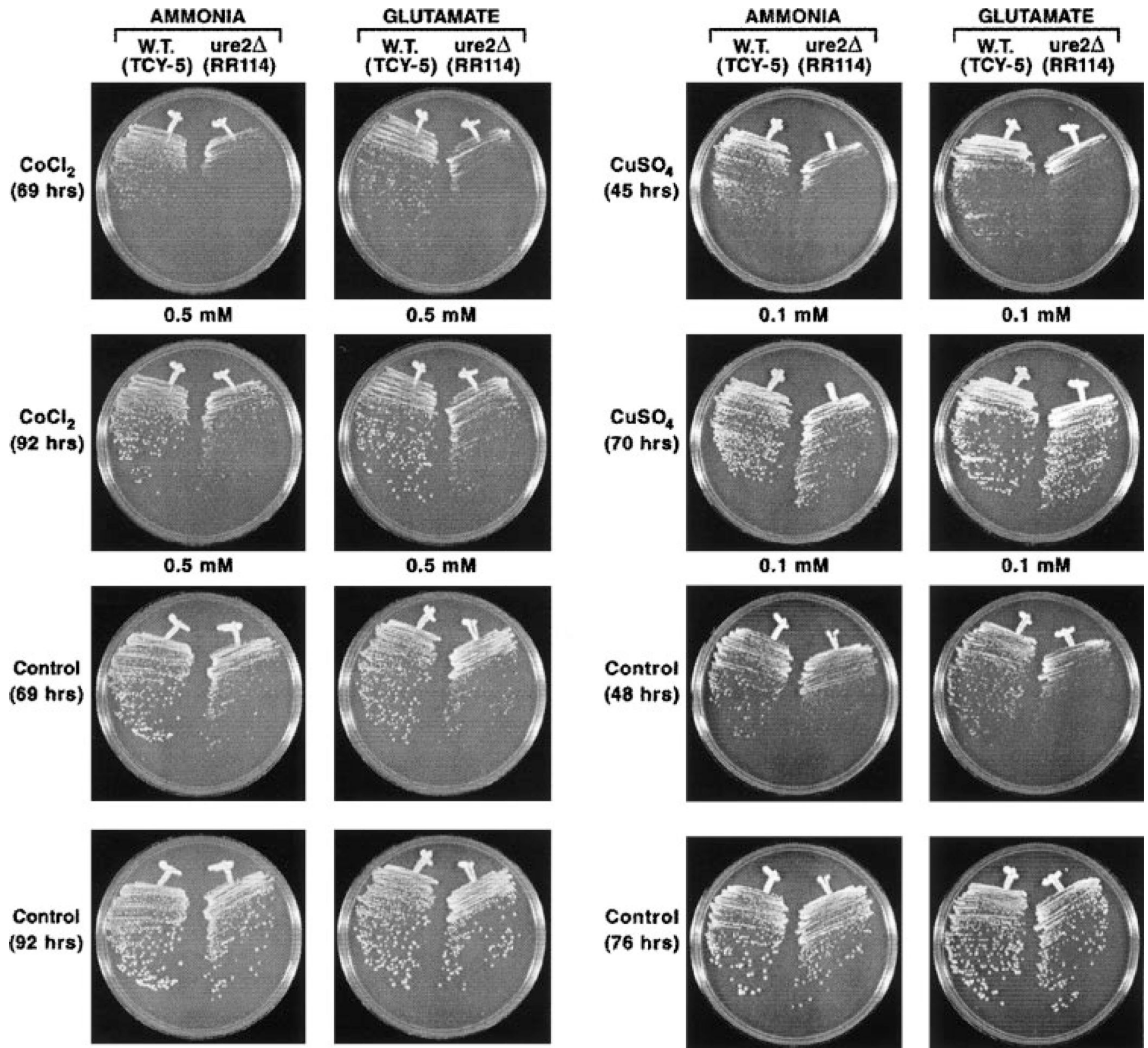

Figure 3.

Growth of wild-type and *ure2* strains in the presence or absence (Control) of $CoCl_2$ and $CuSO_4$. Organization and labelling are as in Figure 1

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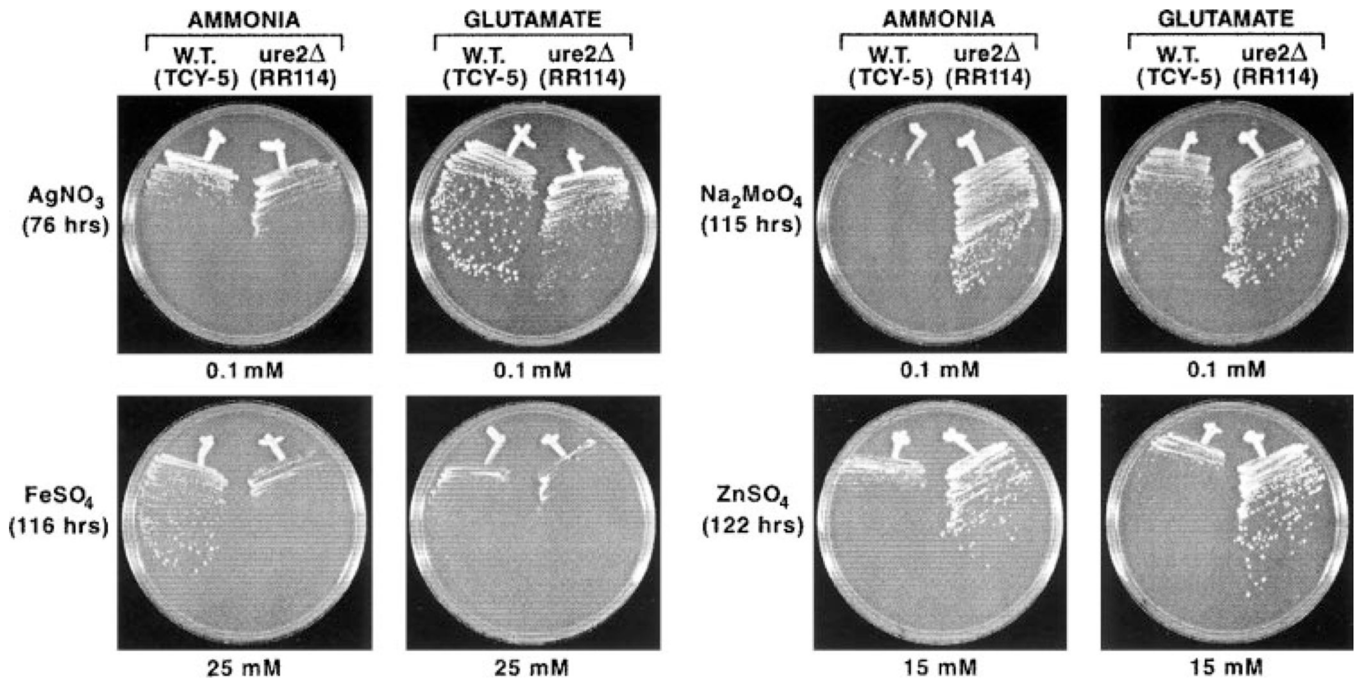

Figure 4.

Growth of wild-type and *ure2* strains in medium containing $AgNO_3$, $FeSO_4$, Na_2MoO_4 or$ZnSO_4$

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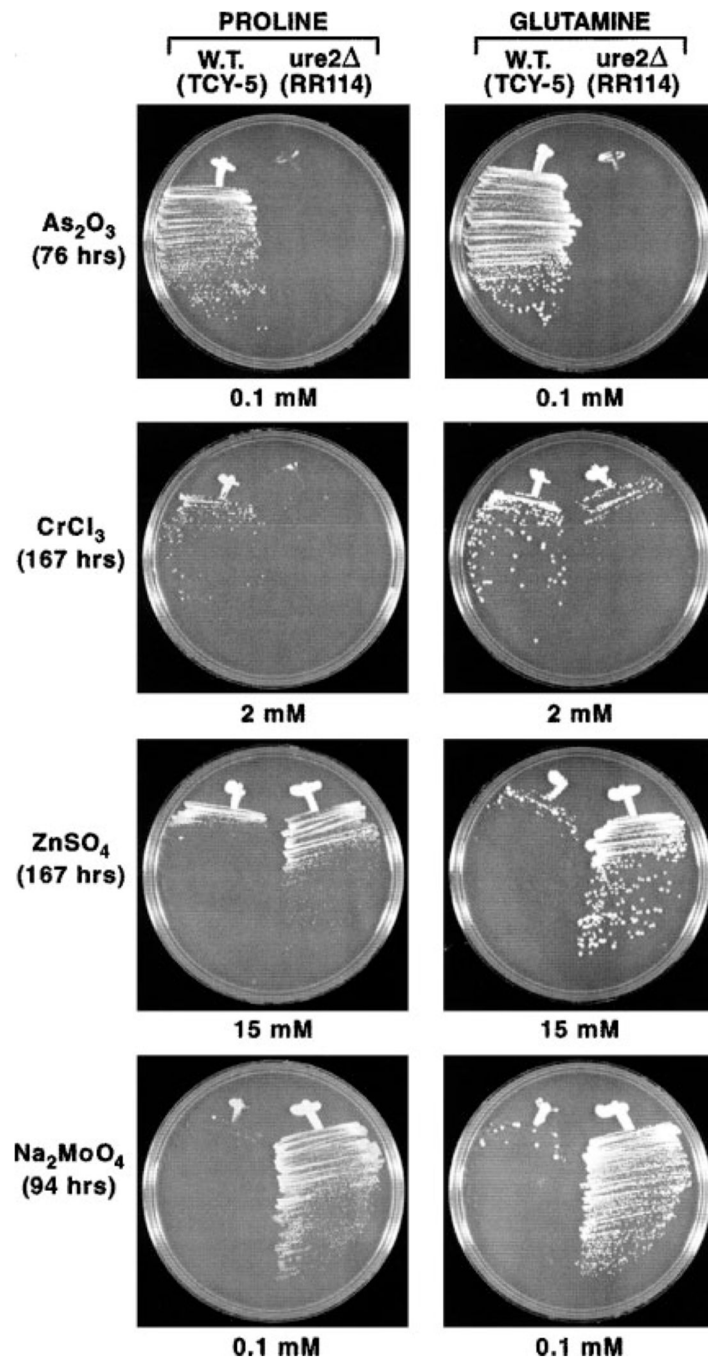

Figure 5.

Growth of wild-type (TCY5) and *ure2* (RR114) strains in the presence of $CrCl_3$, As_2O_3, $ZnSO_4$ or Na_2MoO_4 as in Figures 1–4, except that the nitrogen sources were 0.1% proline or glutamine

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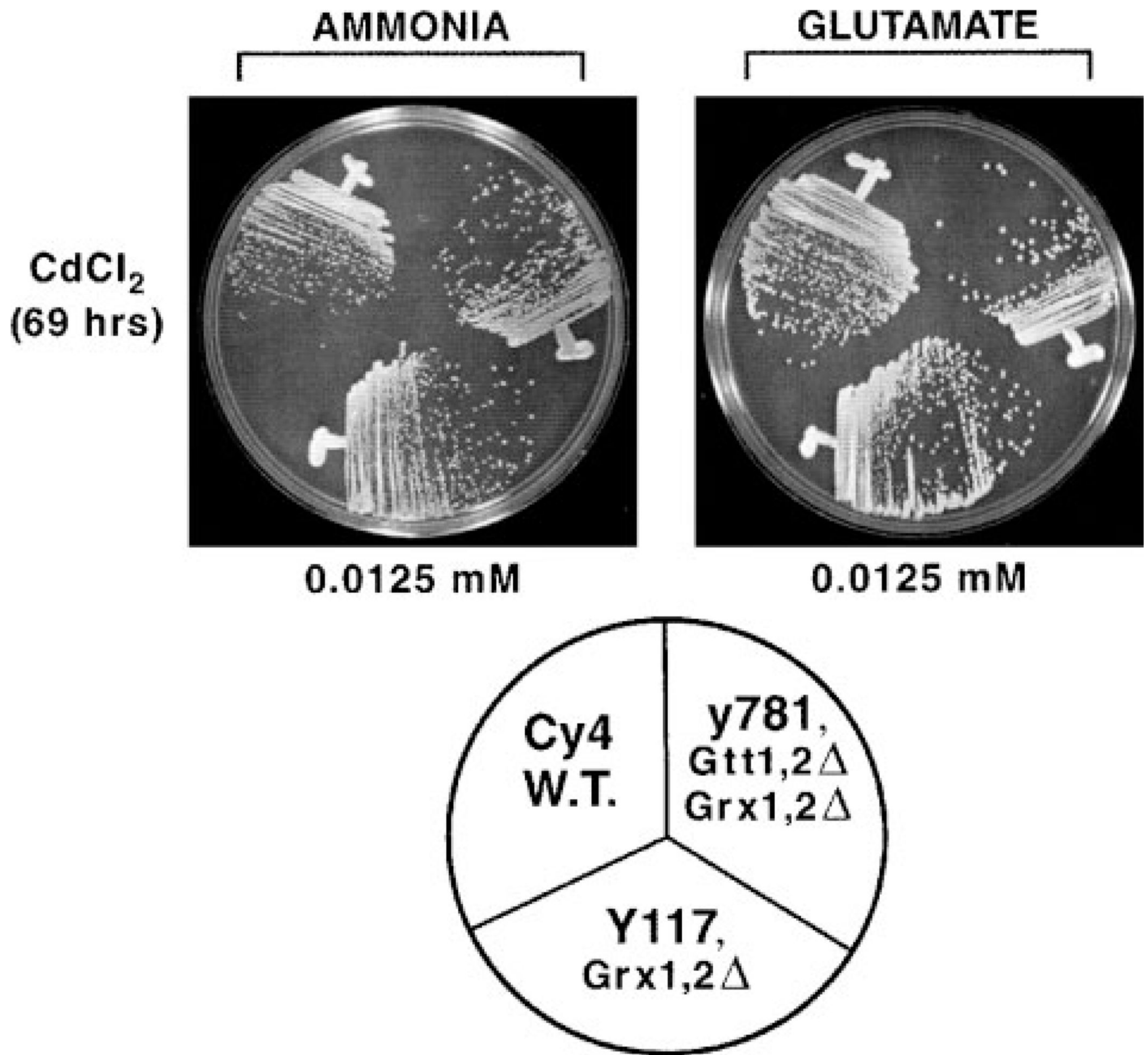

Figure 6.
Growth of wild-type (CY4), *grx1grx2* (Y117) and *gtt1gtt2grx1grx2* (Y781) in the presence of 0.0125 mM $CdCl_2$. A lower concentration of $CdCl_2$ was used in this experiment because the wild-type strain used here is more sensitive than TCY5 to $CdCl_2$

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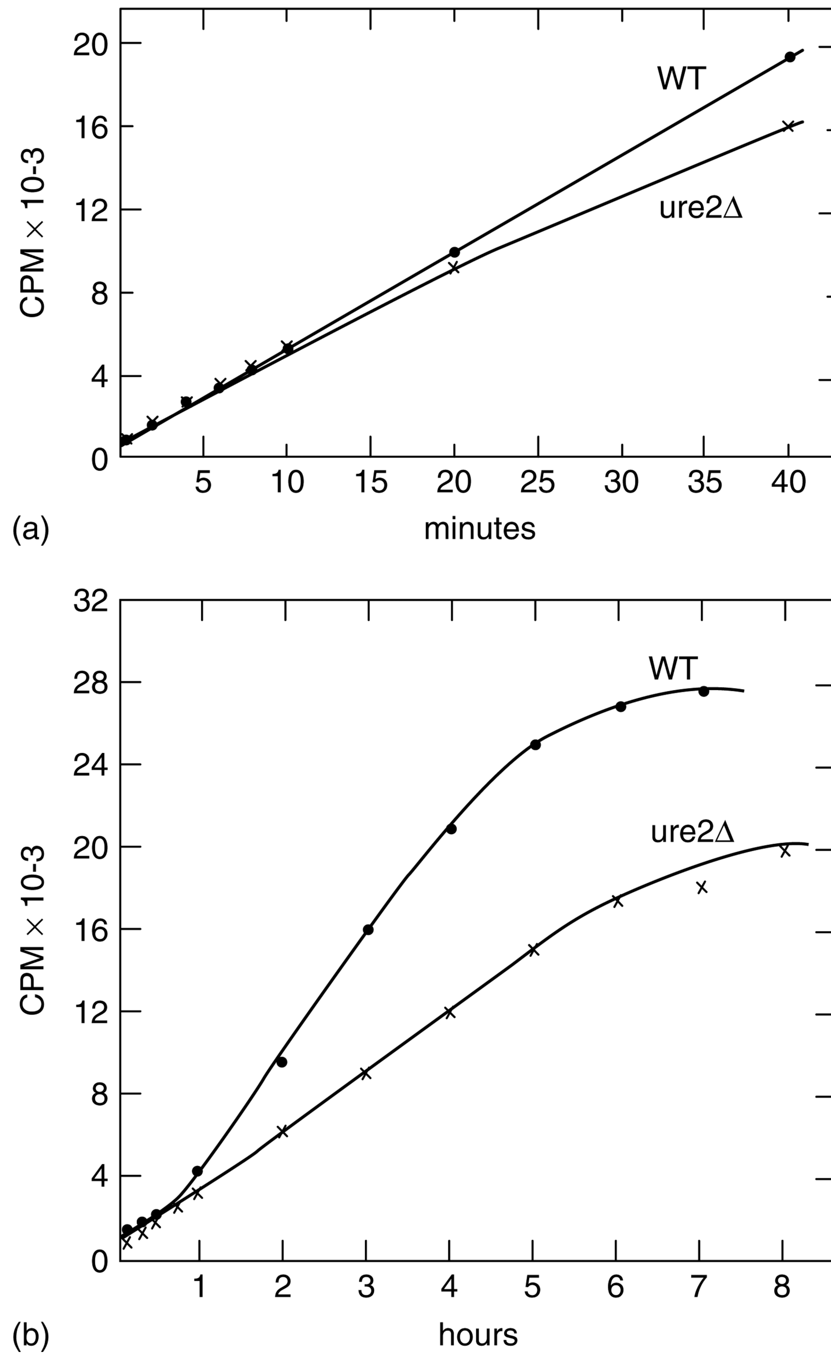

Figure 7.

Accumulation of $^{109}CdCl_2$ by wild-type and *ure2* mutant cells grown in YNB-ammonia medium. $^{109}CdCl_2$ was added to yield a final concentration of 0.05 mM, and 1.0 ml samples of the cultures harvested by filtration at the indicated times. Filters were processed as described in Methods. CPM indicates counts per minute of radioactivity

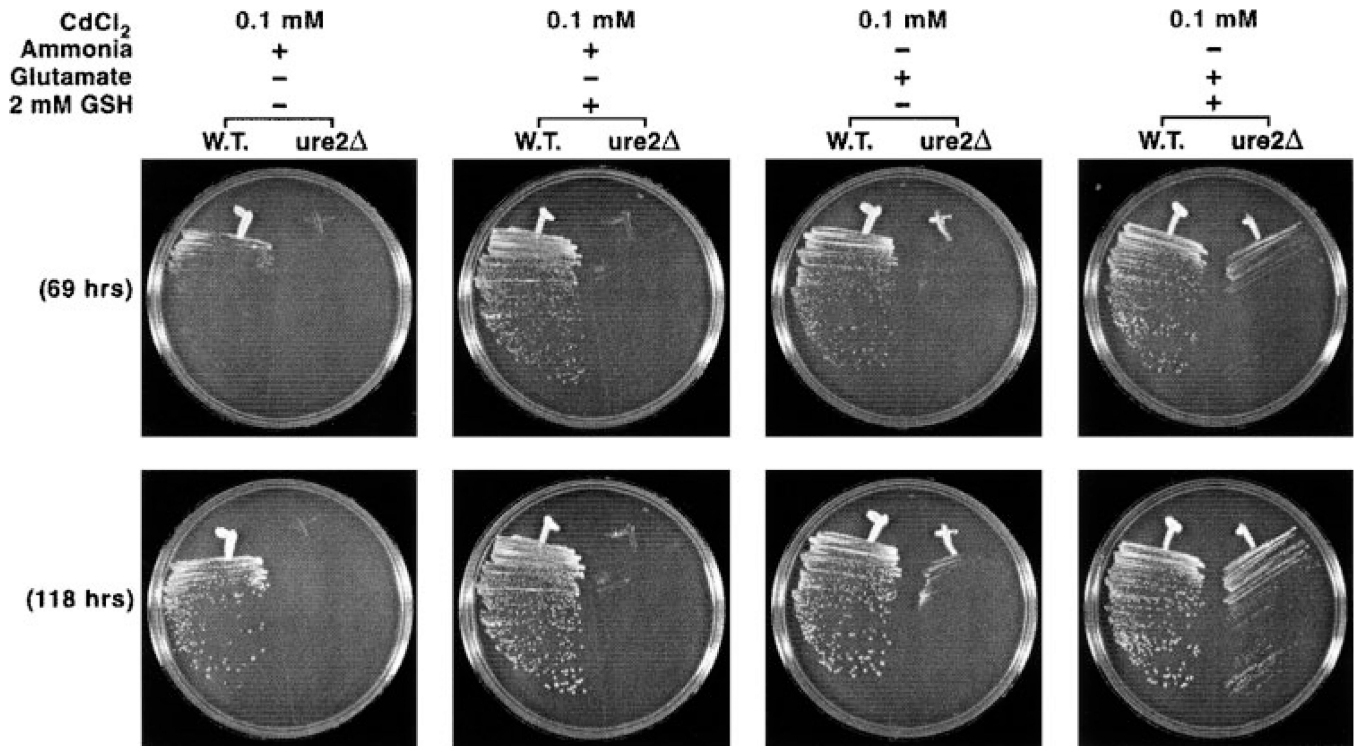

Figure 8.

The effect of exogenously added glutathione on the growth of wild-type and *ure2* strains in the presence or absence of $CdCl_2$. Strains were grown in YNB-ammonia or YNB-glutamate medium in the presence (+) or absence (−) of 2 mM reduced glutathione

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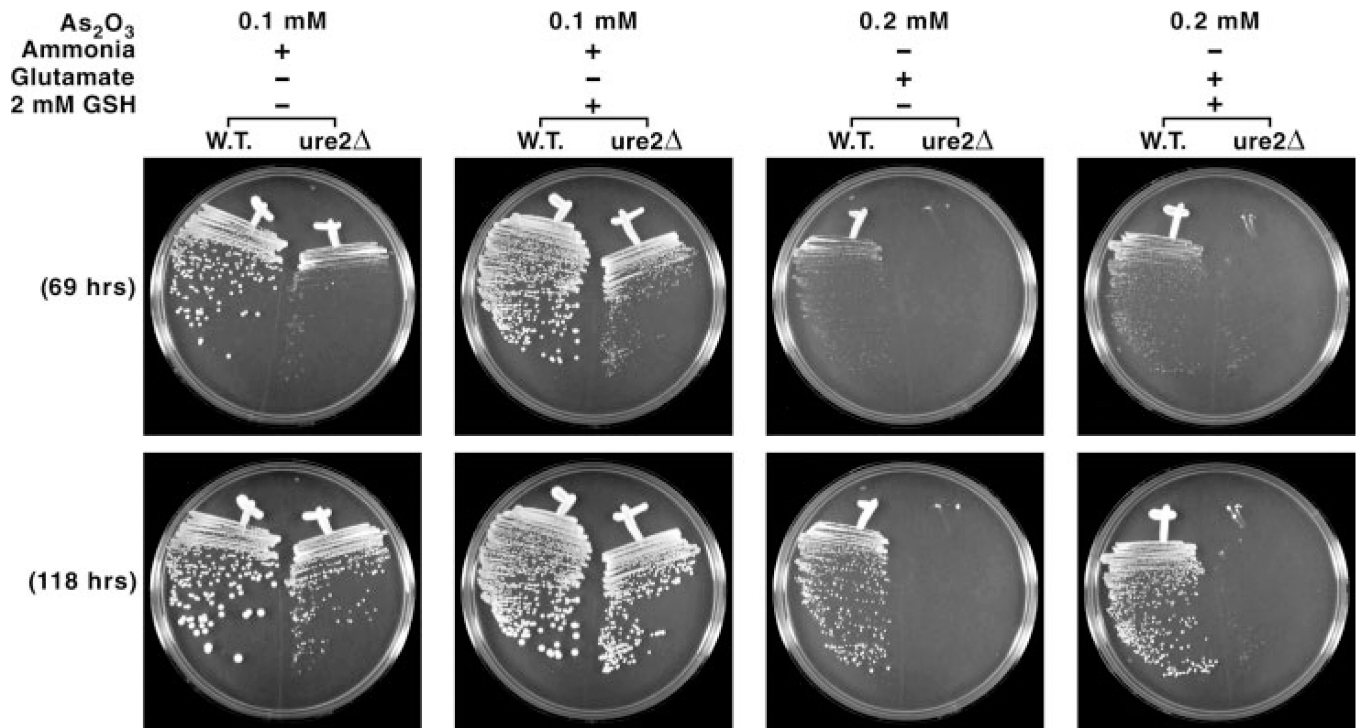

Figure 9.

The effect of exogenously added glutathione on the growth of wild-type and *ure2* strains in the presence or absence of As_2O_3. Strains were grown in YNB-ammonia or YNB-glutamate medium in the presence (+) or absence (–) of 2 mM reduced glutathione

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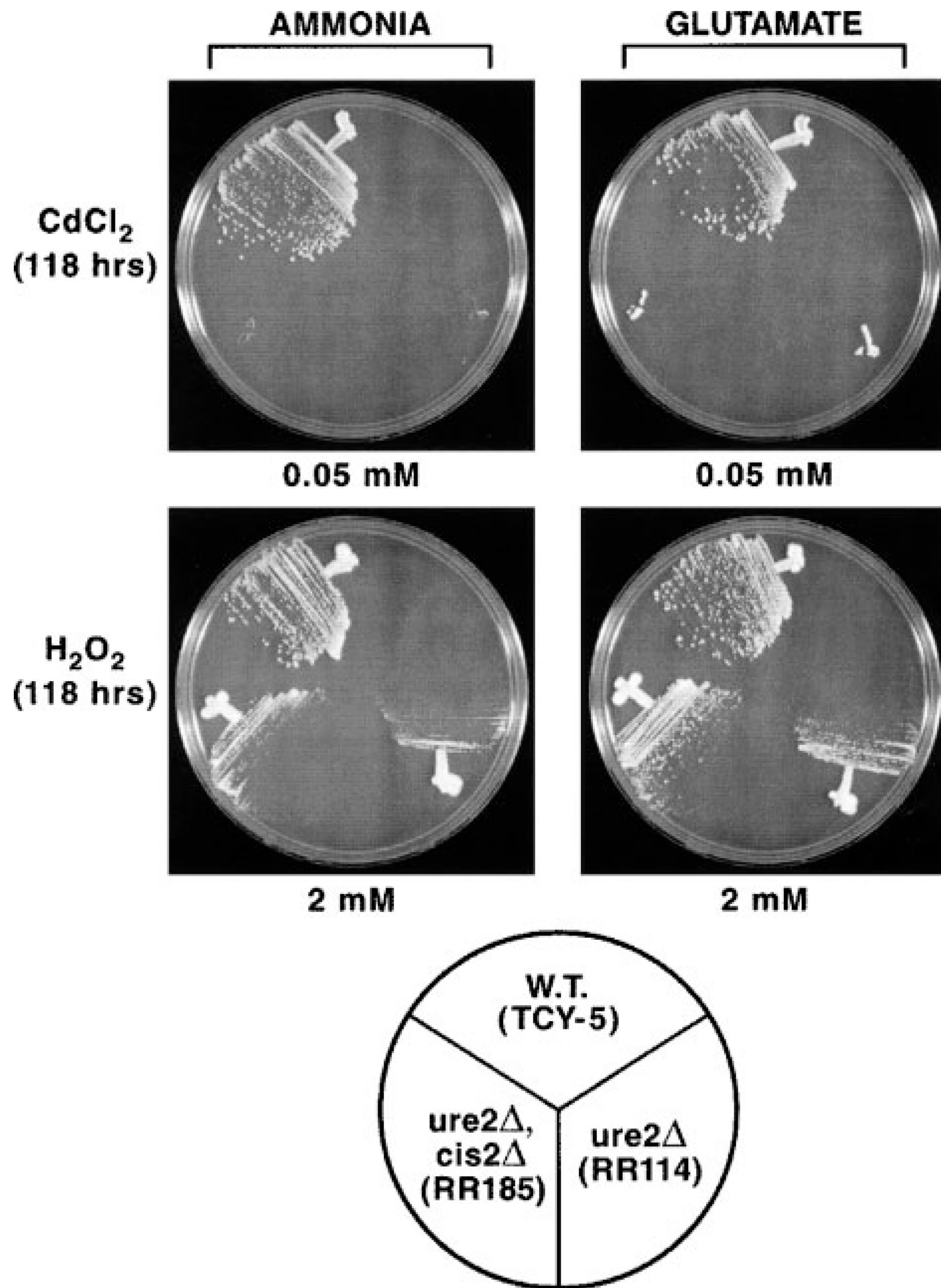

Figure 10.

Growth of wild-type (TCY-5), *ure2* (RR114) and *ure2 cis2* (RR185) double mutant strains in YNB-ammonia or YNB-glutamate containing $CdCl_2$ or hydrogen peroxide

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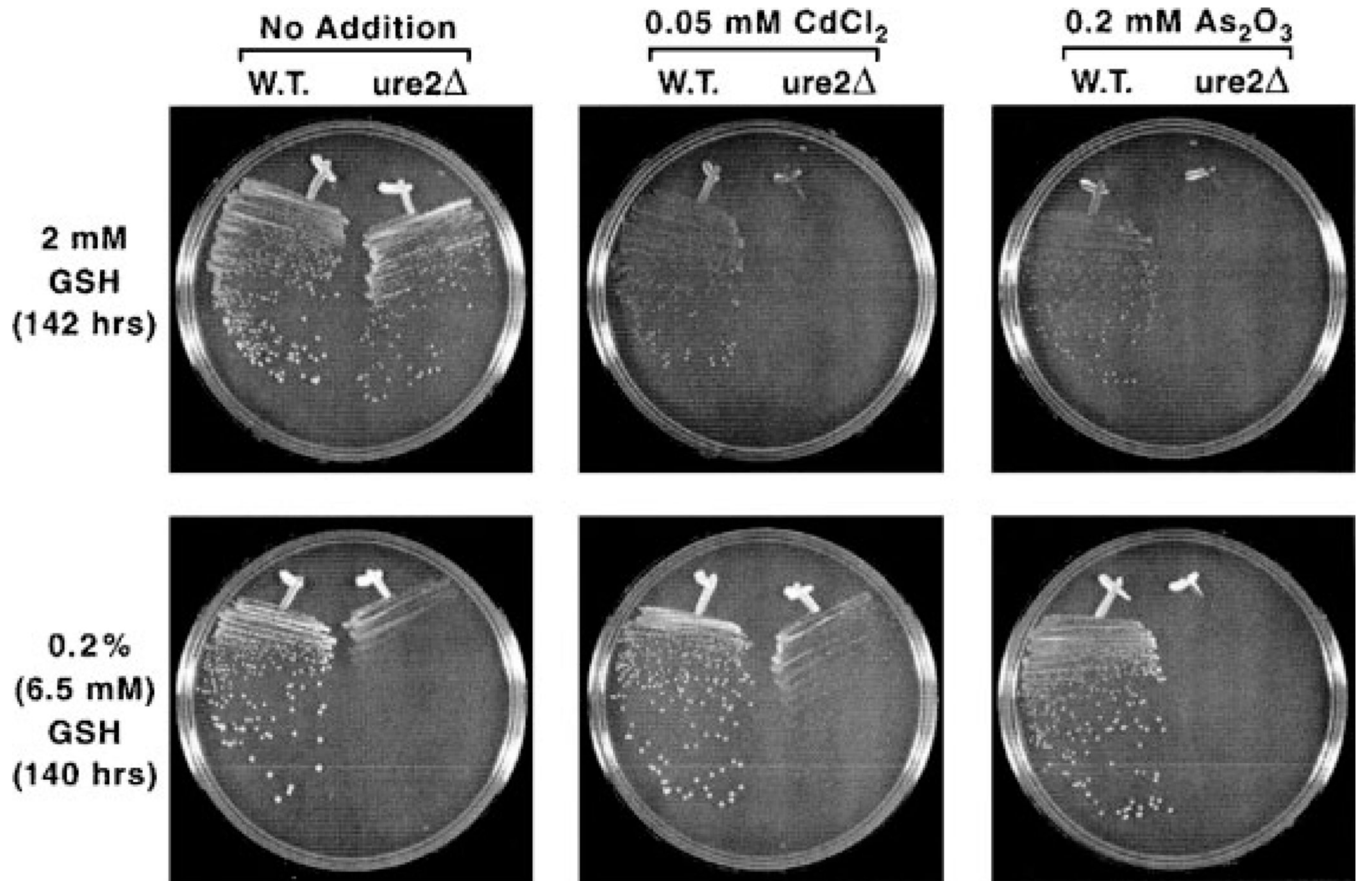

Figure 11.

Effect of $CdCl_2$ or As_2O_3 on the growth of wild-type and *ure2* strains in YNB medium containing reduced glutathione (2 mM or 0.2%, 6.5 mM) as sole nitrogen source

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Table 1

S. cerevisiae strains used in this work

Strain	Genotype
TCY5	*MATα lys2 ura3–52 trp1*
RR114	*MATα lys2 ura3–52 trp1 ure2::TRP1*
RR185	*MATα lys2 ura3–52 trp1 ure2::TRP1 cis2::KnKX4*
W303-1a	*Matαade2-1 his3–11, 15 leu2–3, 112 trp1-1 ura3-1 ssd1-d2 can1–100*
JC101	W303-1a *gtt1::URA3*
JC102	W303-1a *gtt2::URA3*
JC103	W303-1a *gtt1::TRP1 gtt2::URA3*
CY4	*Matαade2–1 his3–11 leu2–3, 112 trp1-1 ura3–52 can1–100*
Y117	CY4 *grx1::LEU2 grx2::HIS3*
Y781	CY4 *grx1::LEU2 grx2::HIS3 gtt1::TRP1 gtt2::URA3*