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Research Article

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Selection and Isolation of a Mutant Yeast Strain Tolerant to Multiple Targeted Heavy Metals

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Abstract

BACKGROUND: This study was performed for selecting yeast mutants with a high tolerance for targeted metals, and determining whether yeasts strains tolerant to multiple heavy metals could be induced by sequential adaptations.
METHODS AND RESULTS: A mutant yeast strain tolerant to the heavy metals cadmium (Cd), copper (Cu), nickel (Ni), and zinc (Zn) was selected by sequential elevated exposures to each metal with intermittent mutant isolation steps. A Cd-tolerant mutant was isolated by growing yeast cells in media containing $CdCl_2$ concentrations that were gradually increased to 1 mM. Then the Cd-tolerant mutant was gradually exposed to increasing levels of $CuCl_2$ in growth media until a concentration of 7 mM was reached, thus generating a strain tolerant to both Cd and Cu. In the subsequent steps, this mutant was exposed to $NiCl_2$ (up to 8 mM), and a resultant isolate was further exposed to $ZnCl_2$ (up to 60 mM), allowing the derivation of a yeast mutant that was simultaneously tolerant to Cd, Cu, Ni, and Zn.
CONCLUSION: This method of inducing tolerance to multiple targeted heavy metals in yeast will be useful in the bioremediation of heavy metals.

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Introduction

Nonessential heavy metals such as cadmium (Cd), lead (Pb), and mercury (Hg) are toxic to living organisms. Therefore, these metals must be removed from contaminated soils and water sources. A variety of effective physical and chemical approaches have been developed for removing heavy metals from soil and water; however, these methods are only effective in cases where the concentration of heavy metals is high. Furthermore, these methods demand high costs and are not applicable in the wide range of areas with low concentrations of heavy metals. Therefore, bioremediation, a technique using living organisms to decontaminate toxic materials, has been developed as an alternative approach (Gaur *et al.*, 2014).

Studies on heavy metal bioremediation using microorganisms have progressed rapidly and have focused on the development of organisms with increased tolerance and uptake of heavy metals. Previous studies have shown that bacteria pre-exposed to heavy metals have a greater tolerance than bacteria that were not pre-exposed (Díaz-Raviña and Bååth, 2001) and that heavy metal-tolerant microorganisms may be isolated from areas polluted with heavy

metals (Villegas *et al.*, 2005; Zafar *et al.*, 2007; Rehman *et al.*, 2008). Moreover, tolerant microorganisms may be artificially induced by exposing them to increasing levels of heavy metals (Díaz-Raviña and Bååth, 1996). Another study showed that heavy metals such as Cd have mutagenic effects (Serero *et al.*, 2008); thus, they can induce tolerance through a selective process. Induction of copper tolerance in yeast was also found to induce tolerance to other heavy metals such as zinc (Zn), Hg, nickel (Ni), and Cd (Rehman *et al.*, 2008). However, it remains to be determined whether the development of cross-tolerance to heavy metals in microorganisms such as yeast is a broad phenomenon, or if it only occurs in isolated cases.

Microorganisms can be used in the bioremediation of either wastewaters or soils contaminated with heavy metals (Gad *et al.*, 2010). Yeasts strains such as *Saccharomyces cerevisiae* are capable of removing a wide range of metals (namely Cd, Cu, Ni, and Zn) by biosorption, can aggregate into multicellular masses, and settle rapidly in suspension media. These properties make yeasts useful in the bioremediation of heavy metals (Soares and Soares, 2012). However, the isolation of mutant yeast strains that are both hyper-accumulating and tolerant to heavy metals has proven difficult (Ruta *et al.*, 2010). Thus, the derivation of microorganisms that thrive in the presence of high heavy metal concentrations is more amenable to bioremediation than developing microorganisms with a capacity to uptake heavy metals at lower concentrations.

Therefore, this study was focused on selecting yeast mutants with a high tolerance for targeted metals, and determining whether yeasts strains tolerant to multiple heavy metals could be induced by sequential adaptations. Little is known regarding the feasibility of sequentially adapting microorganisms to multiple targeted heavy metals, and the development of such methodology should be widely useful in the bioremediation of targeted heavy metals.

Materials and Methods

Saccharomyces cerevisiae strain INVsc1 (*his3△1/his3△1 leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52*) (Invitrogen, Carlsbad, CA) transformed with the pYES2 vector (Invitrogen) was used for both induction and isolation of heavy metal-tolerant mutants. Yeast cells were grown in yeast nitrogen base supplemented with appropriate amino acids and 2% glucose without uracil (Lee and Kim, 2010). A Cd-tolerant mutant (designated CdR1) was isolated by growing yeast cells in media containing $CdCl_2$ concentrations that were gradually increased up to 1 mM $CdCl_2$. Next, the CdR1 mutant was grown in the presence of gradually increasing $CuCl_2$ concentrations up to 7 mM, resulting in the derivation of CdR1-CuR7. Subsequently, this mutant was grown in the presence of progressively increasing $NiCl_2$ concentrations up to 8 mM $NiCl_2$ to generate the CdR1-CuR7-NiR8 strain. Finally, the CdR1-CuR7-NiR8 mutant strain was exposed to increasing concentration of $ZnCl_2$ that peaked at 60 mM $ZnCl_2$, resulting in the generation of CdR1-CuR7-NiR8-ZnR60, a strain that tolerated high concentrations of Cd, Cu, Ni, and Zn.

To assay for sensitivity to heavy metals, yeast cells were grown for 24 h at 30 C in the presence of various metal concentrations, after which cell densities were measured spectrophotometrically at 600 nm.

Results and Discussion

The isolated CdR1 mutant showed markedly higher tolerance to Cd than did control cells (Fig. 1). Specifically, control cells showed almost complete inhibition of growth at 250 μM Cd. However, CdR1 cells showed only slight growth retardation at 250 μM

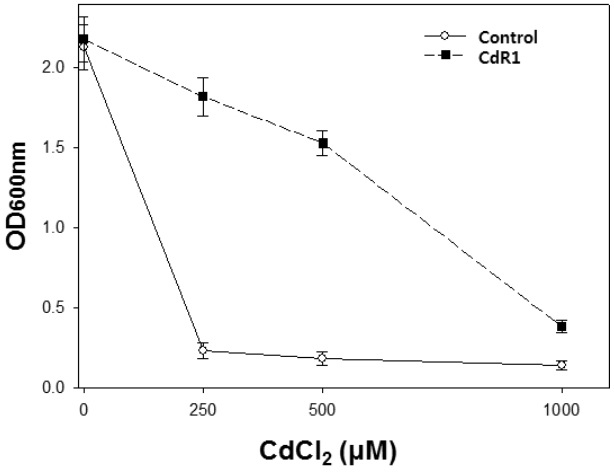

Fig. 1. Cd sensitivities in control and CdR1 yeast cells. Both control and CdR1 cells were grown for 24 h at 30 C in liquid media containing the indicated Cd concentrations. Cell densities were then measured spectrophotometrically at 600 nm. Values shown are the means ± SE of three replicate experiments. Control INVsc1 yeast cells were *S. cerevisiae* yeast transformed with the pYES2 vector, whereas the Cd-resistant yeast mutant CdR1 was isolated from adapted control yeast cells after exposure to 1 mM $CdCl_2$ stress

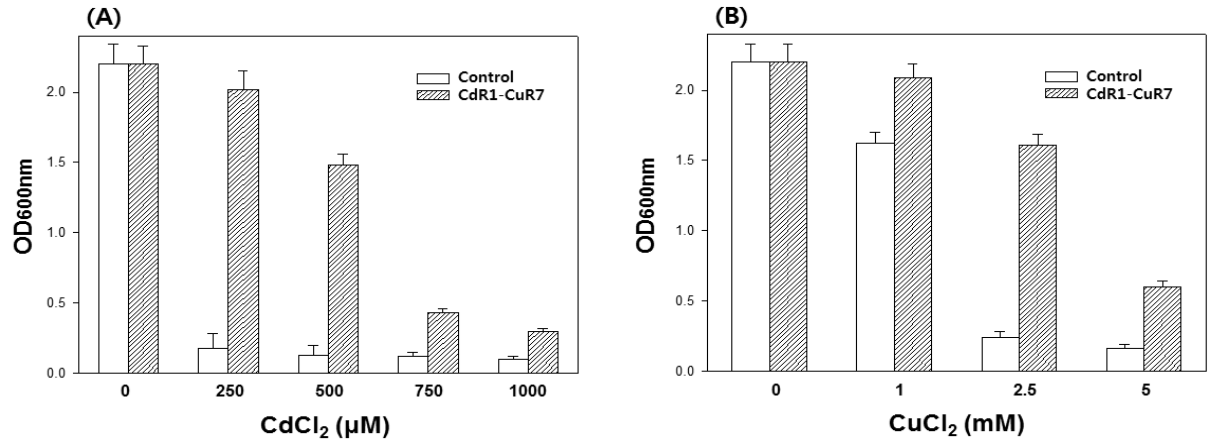

Fig. 2. Cd and Cu sensitivities in control and CdR1-CuR7 yeast cells. Both control and CdR1-CuR7 cells were grown for 24 h at 30 C in liquid media containing the indicated concentrations of (A) $CdCl_2$ and (B) $CuCl_2$. Cell densities were then measured spectrophotometrically at 600 nm. Values shown are the means ± SE of three replicates experiments. INVsc1 yeast cells containing the pYES2 vector were used as a control. The Cd+Cu-resistant yeast mutant CdR1-CuR7 was isolated from adapted CdR1 cells after exposure to 7 mM $CuCl_2$ stress

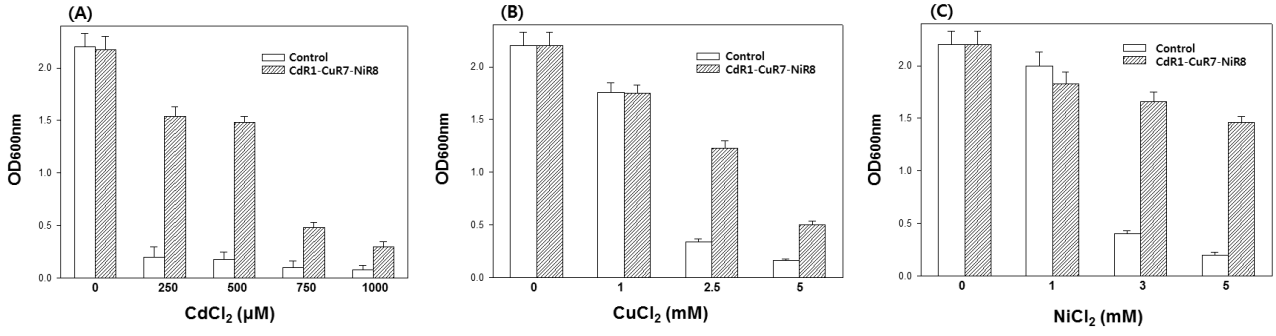

Fig. 3. Cd, Cu, and Ni sensitivities in control and CdR1-CuR7-NiR8 cells. Both control and CdR1-CuR7- NiR8 were grown for 24 h at 30 C in liquid media containing the indicated concentrations of (A) Cd, (B) Cu, and (C) Ni. Cell densities were then measured spectrophotometrically at 600 nm. Values shown are the means ± SE of three replicate experiments. INVsc1 yeast cells containing the pYES2 vector were used as a control. The Cd+Cu+Ni-resistant yeast mutant CdR1-CuR7-NiR8 was isolated from adapted CdR1-CuR7 cells after exposure to 8 mM $NiCl_2$ stress

Cd, although the growth was almost completely inhibited at 1,000 μM Cd. It is well known that most heavy metals, including Cd, are mutagenic (Jin *et al.*, 2003); Giagnis *et al.*, 2006). Microorganisms can acquire tolerance to heavy metals through a combination of both physiological and genetic adaptation to metals stress (Duxbury and Bicknell, 1983; Díaz-Raviña and Bååth, 1996). CdR1 cells survived exposure to 1,000 μM Cd during the induction period, although they could not grow well at 1,000 μM Cd after removing physiological adaptation by several rounds of subculture without Cd stress. Nonetheless, the observation that the CdR1 mutant showed stable tolerance to 500 μM Cd after removing physiological adaptation (as did mutants resistant to Cd and other heavy metals) suggests that the resistant strains underwent selective mutations to acquire Cd tolerance.

Next is to derive a yeast strain that was tolerant to both Cd and Cu by growing CdR1 cells in media containing $CuCl_2$, the concentration of which was gradually increased up to 7 mM. A resulting isolate (CdR1-CuR7) showed higher tolerance to Cd as CdR1 did when compared to control cells (Fig. 2A). CdR1-CuR7 cells also showed higher tolerance to Cu compared to control cells. The CdR1-CuR7 mutant showed only slightly inhibited growth at 2.5 mM Cu, while the growth of control cells was almost blocked at this Cu concentration (Fig. 2B).

To derive a yeast strain that was simultaneously

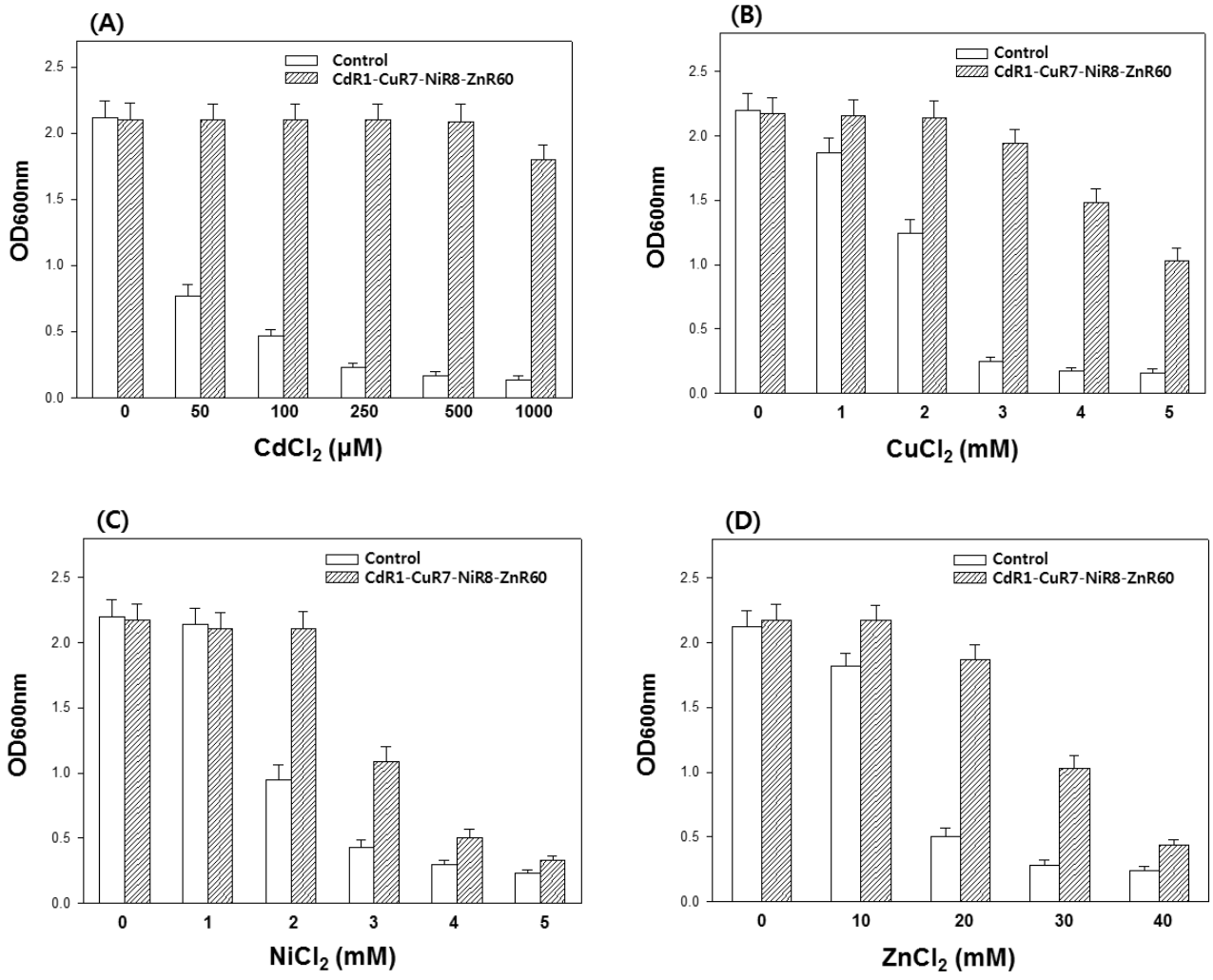

Fig. 4. Cd, Cu, Ni and Zn sensitivities in control and CdR1-CuR7-NiR8-ZnR60 cells. Both control and CdR1-CuR7-NiR8-ZnR60 were grown for 24 h at 30 C in liquid media containing the indicated concentrations of (A) Cd, (B) Cu, (C) Ni, and (D) Zn. Cell densities were then measured spectrophotometrically at 600 nm. Values shown are the means ± SE of three replicate experiments. INVsc1 yeast cells containing the pYES2 vector were used as a control. The Cd+Cu+Ni+Zn-resistant yeast mutant CdR1-CuR7-NiR8-ZnR60 was isolated from adapted CdR1-CuR7-NiR8 cells after exposure to 60 mM $ZnCl_2$ stress

tolerant to Cd, Cu, and Ni, CdR1-CuR7 cells were grown in media containing $NiCl_2$ concentrations that were gradually increased to 8 mM. The resulting mutant CdR1-CuR7-NiR8 showed still higher tolerance to Cd when compared to control cells (Fig. 3A). CdR1-CuR7-NiR8 cells were also resistant to Cu compared to control cells, even though their tolerance was slightly lower than that of CdR1-CuR7 cells (Fig. 3B). These results suggest that the increased tolerance to one metal results in increased sensitivity to other metal(s). Consistent with this finding, a previous study showed that heterologous expression of a Cd-resistance gene in yeast leads to increased tolerance to Cd with a concomitantly increased sensitivity to Cu (Lee and Kim, 2010). CdR1-CuR7-NiR8 cells showed higher tolerance to Ni, as demonstrated by slightly inhibited growth at 5 mM Ni, whereas the growth of control cells was almost inhibited at that Ni concentration (Fig. 3C).

Finally, CdR1-CuR7-NiR8 cells were exposed to increasing concentrations of $ZnCl_2$ (up to 60 mM) in growth media to produce a mutant strain tolerant to Cd, Cu, Ni, and Zn at the same time. This mutant, designated CdR1-CuR7-NiR8-ZnR60, showed even higher tolerance to Cd than did any of the CdR1, CdR1-CuR7, or CdR1-CuR7-NiR8 strains, when compared to control cells. Moreover, CdR1-CuR7-NiR8-ZnR60 cells were almost completely resistant to growth

retardation even at 1000 μM Cd (Fig. 4A). This result implies that an increased tolerance for one metal can lead to an increased tolerance toward another metal(s) (Rehman *et al.*, 2008). Similarly, CdR1-CuR7-NiR8-ZnR60 cells are more tolerant to Cu than are control, CdR1, CdR1-CuR7, and CdR1-CuR7-NiR8 cells (Fig. 4B). CdR1-CuR7-NiR8-ZnR60 cells showed increased tolerance to Ni compared to control cells, although their tolerance was slightly diminished in comparison to CdR1-CuR7-NiR8 cells (Fig. 4C). Similar to the data shown in Fig. 3B, this result suggests that increased tolerance to one metal can result in an increased sensitivity with respect to other metal(s). CdR1-CuR7-NiR8-ZnR60 cells also showed increased tolerance to Zn when compared to control cells (Fig. 4D).

Conclusion

This study shows that tolerance of multiple targeted heavy metals in yeast can be induced by sequential adaptation to metals stress, and the results presented herein will be useful in bioremediation of targeted heavy metals using microorganisms.

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