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Short Communication

Metalloadsorption by *Saccharomyces cerevisiae* cells expressing invertase-metallothionein (Suc2-Cup1) fusion protein localized to the cell surface

Harushi Nakajima,* Taro Iwasaki, and Katsuhiko Kitamoto

Department of Biotechnology, The University of Tokyo, Tokyo 113–8657, Japan

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In recent years, the decontamination of widespread heavy metal pollution in the aqueous environment by the exploitation of microorganisms has achieved growing attention. A wide variety of fungi, algae, and bacteria are now under study or in use as biosolvents for heavy metal remediation (Gadd, 1992; Volesky and Holan, 1995). We recently reported the selection of three *Candida* strains exhibiting a higher Ni^{2+} uptake ability (Kambe-Honjoh et al., 1997) and proposed a novel bioassay method for the screening of heavy-metal-adsorbing microorganisms (Kambe-Honjoh et al., 1998).

The in vivo expression of metal-binding peptides is a new approach for obtaining potent metal-adsorbing microorganisms. It has been reported that the enhancement of metal-adsorption capability of *Escherichia coli* cells results from the introduction of histidine clusters or metallothioneins on the outer membrane proteins (Sousa et al., 1996, 1998). We previously reported the expression of a fusion protein composed of TAA (Taka-amylase A of *Aspergillus oryzae*) containing a hexahistidine repeat and C-terminal GPI-anchoring domain of Cwp1 on *Saccharomyces cerevisiae* cell surface and enhancement of the metal adsorption capacity of the cells (Kambe-Honjoh et al., 2000).

In *S. cerevisiae*, several secretory enzymes are known to localize mainly on the cell surface without being released into the medium (de Nobel and Barnett, 1991). Invertase (Suc2) encoded by the *SUC2* gene is a heavily glycosylated enzyme and is mainly retained in the periplasmic space as homo-octamer (Esmon et al., 1987).

Metallothioneins are highly conserved low-molecular-weight Cys-rich proteins that bind metal ions (e.g., Cu^{2+}, Ni^{2+}, Cd^{2+}, Hg^{2+}, and Ag^{+}) and sequester them in a biologically inactive form (Butt and Ecker, 1987; Hamer, 1986). *S. cerevisiae* metallothionein (Cup1), encoded by the *CUP1* gene, is a 53 amino acid protein containing 12 cysteine residues that originate eight binding sites for monovalent and four binding sites for divalent metal ions (Winge et al., 1985).

To utilize *S. cerevisiae* Suc2 as a carrier and anchor for the expression of Cup1 in the cell surface, we introduced restriction enzyme sites on the N-terminal and C-terminal of the *SUC2* gene. In general, the intracellular expression of metallothioneins is difficult to detect, since they may be quickly degraded by host proteases (Derman et al., 1993; Silhavy et al., 1984). To avoid an expression of Cup1 fusion proteins in the cytoplasm, the second methionine residue codon of the *SUC2* gene, the initiation codon of the cytoplasmic form of Suc2, was changed to create an *Xho*I site for

* Address reprint requests to: Dr. Harushi Nakajima, Department of Biotechnolgy, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan.

E-mail: ahnakaji@mail.ecc.u-tokyo.ac.jp

the insertion of foreign sequences. We also changed the termination codon of the *SUC2* gene for introducing the *Bgl*II site (Fig. 1, a and b). The modified *SUC2* gene was constructed with two-step PCR. Two terminal primers corresponding to the 700-bp upstream region of the *SUC2* initiation codon (primer 1: 5′-AGCTGTCGAAGATATCCATTTTATCATG-3′; the *Eco*RV site is underlined) and the modified C-terminal of the Suc2 coding region (primer 4: 5′-GGGGCATGCCCCGGGGGAGATCTTTACTTCCCTTACTTGGAACTT-3′; the *Sph*I and *Bgl*II sites are underlined), respectively, and two primers for modification of the N-terminal of the Suc2 (primer 2: 5′-GCTAGTTTCGTTTGCTCGAGATGCAGATAT-3′ and primer 3: 5′-ATATCTGCATCTCGAGCAAACGAAACTAGC-3′; the *Xho*I sites are underlined) were designed. PCR reactions using primers 1 and 2 and primers 3 and 4 were performed with pRB58 (Carlson and Botstein, 1982) as templates. The amplified fragments were mixed and subjected to the second PCR with the external primers 1 and 4. The final amplified fragment was digested with *Eco*RV and *Sph*I and ligated to a yeast high-copy vector pYES2 (Invitrogen BV, Leek, Netherlands), which was digested with *Ssp*I and *Sph*I for the deletion of the *GAL1* promoter region and designed as pSU2. The *CUP1* gene was amplified by PCR (primers, 5′-GGGGATCCGTCGACAAATGTTCAGCGAATTAATTAAC-3′ and 5′-GGGTCGACCGGAGATCTTTTTCCCAGAGCAGCATGACTTCTT-3′; *Bam*HI, *Sal*I, *Sal*I, and *Bgl*II sites are indicated by underlines, respectively) with genomic DNA from *S. cerevisiae* as template. The amplified fragment was digested with *Sal*I or *Bam*HI and inserted into the *Xho*I or *Bgl*II site of pSU2, respectively, and designated pCU-S2 and pS2-CU (Fig. 1c).

To confirm the expression and cell surface localization of Suc2 fusion proteins, *S. cerevisiae* SEY6210 (*MATα suc2-Δ9 ura3 leu2 trp1 his3 lys2*) (Robinson et al., 1988) strains carrying pYES2, pSU2 (carrier Suc2), pCU-S2 (Cup1-Suc2), or pS2-CU (Suc2-Cup1) were precultured in YEP7D medium (1% yeast extract, 2% polypeptone, and 7% glucose) at 30°C for 18 h, then cultured in YP2S medium (1% yeast extract, 2%

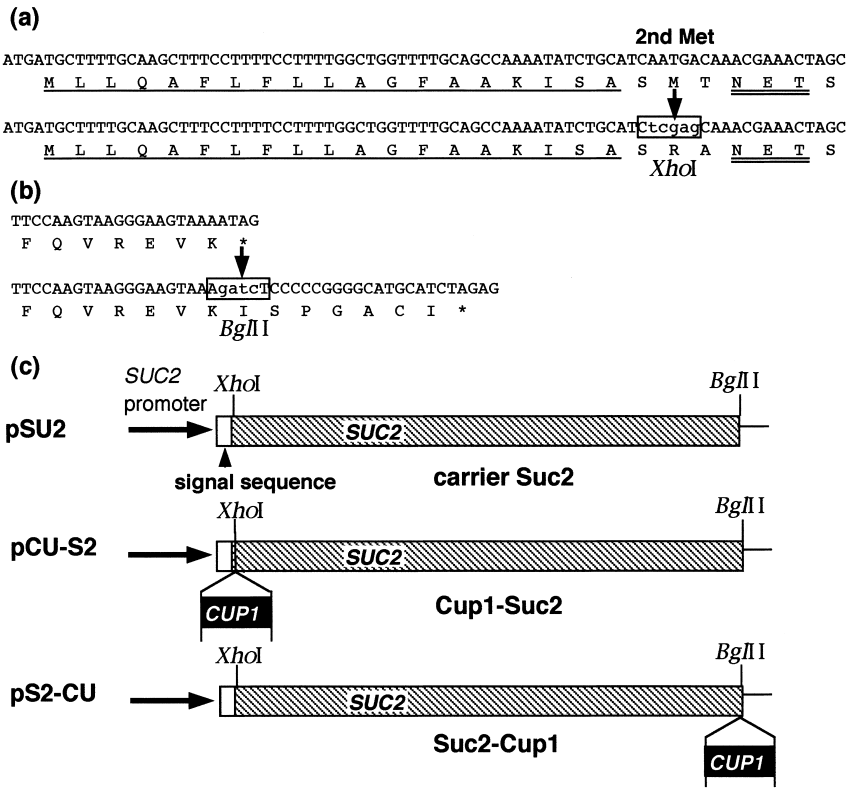

Fig. 1. Construction of Suc2 fusion gene.

Modification of the N-terminal (a) and C-terminal (b) of *SUC2* gene. The signal sequence and a potential N-linked glycosylation site are indicated by an underline and a double underline, respectively. Created restriction enzyme sites are indicated as boxes. (c) Construction of pCU-S2 and pS2-CU encoding the fusion protein Cup1-Suc2 and Suc2-Cup1, respectively.

polypeptone, and 2% sucrose) at 30°C for 2 h for induction of the *SUC2* promoter (Carlson and Botstein, 1982). The protein contents of the cells were measured by the Lowry method. The cells were washed and incubated in Tris/HCl (pH 8.0) buffer for 2 h with shaking. They were then separated from the buffer by centrifugation, and the activities of invertase released into the buffer and retained on the cells were quantified by measuring the production of reducing sugar form sucrose (Bernfeld, 1955). Table 1 shows the invertase activities in the buffer and on the cells, respectively. A similar level of invertase activity, which remained in the cell body, was observed in both strains expressing Suc2-Cup1 and carrier Suc2. Furthermore, in the strains expressing carrier Suc2 and Suc2-Cup1, over 99% of invertase activity remained in the cell body. This indicates that the carrier Suc2 and Suc2-Cup1 were substantially anchored to the cell surface and formed correct conformation with enzymatic activity. Suc2-Cup1 fusion may form octamer similar to intact Suc2 and retain it in the cell wall. Only 15% of invertase activity was detected in the Cup1-Suc2 expressing strain compared with the carrier Suc2 strain. The SEY6210 strain carrying pCU-S2 also showed slightly slow growth on YP2S medium. It is suggested that Cup1-Suc2 fusion protein folds inadequate structure and affects the growth of the host cells.

To investigate the cellular localization of the fusions, SEY6210 cells carrying pYES2, pRB58 (intact Suc2), pSU2 (carrier Suc2), pCU-S2 (Cup1-Suc2), or pS2-CU (Suc2-Cup1) were cultured with invertase induction. The cells were fractionated into cell wall and cytoplasmic fractions and analyzed on non-denaturing polyacrylamide gel (Kaiser et al., 1987). Invertase fusions localized in the gel were visualized by staining with 2,3,5-triphenyltetrazolium chloride (Goldstein and Lampen, 1979). From intact Suc2 expressing cells, a non-glycosylated form of invertase was detected in the intracellular fraction, and a highly glycosylated form was observed in the cell wall fraction (Fig. 2, lanes 3 and 4). Carrier Suc2 and Suc2-Cup1 expressing cells produced no such cytoplasmic form of invertase. It is shown that a highly glycosylated form of Suc2 fusions were localized in the cell surface (Fig. 2, lanes 5, 6, 9, and 10). This indicates that carrier Suc2 and Suc2-Cup1 were expressed in the cell wall with highly glycosylated modification. Cup1-Suc2 fusion was detected in the intracellular and cell wall fractions as a low molecular weight band (Fig. 2, lanes 7 and 8). Cup1-Suc2 may form an abnormal conformation in the secretory pathway and be degraded as unfolded proteins. It suggests that the insert position of functional peptide is important for an efficient expression of Suc2 fusion protein in the cell surface.

Table 1. Expression of Suc2 fusion proteins.

Plasmid	Invertase activity (unit/mg protein)	
	Intact cells	Buffer
pYES2	2.2	0.02
pSU2	50.5	0.46
pCU-S2	7.2	0.04
pS2-CU	53.0	0.29

S. cerevisiae SEY6210 cells carrying pYES2 (vector), pSU2 (carrier Suc2), pCU-S2 (Cup1-Suc2), or pS2-CU (Suc2-Cup1) were cultured with invertase induction, and the protein contents of the cells were measured. The cells were incubated in 20 mM Tris/HCl (pH 8.0) for 2 h. They were separated from the buffer by centrifugation; then the activities of invertase released into the buffer and retained on the cells were quantified.

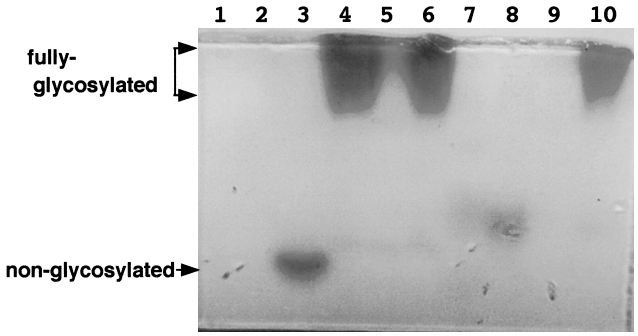

Fig. 2. Cell surface localization of invertase fusion proteins. *S. cerevisiae* SEY6210 cells carrying pYES2 (lanes 1 and 2), pRB58 (lanes 3 and 4), pSU2 (lanes 5 and 6), pCU-S2 (lanes 7 and 8), or pS2-CU (lanes 9 and 10) were spheroplasted to fractionate intracellular (lanes 1, 3, 5, 7, and 9) and periplasmic (lanes 2, 4, 6, 8, and 10) pools. Invertase fusions localized in the gel were stained for visualizing the invertase activity. Non-glycosylated (single arrow) and fully glycosylated (double arrow) invertase correspond to 60 and 110–150 kDa, respectively, in molecular mass.

The ability of yeast cells expressing Suc2 fusion to remove heavy metal ions from an aqueous solution was measured by the adsorption capacities of yeast cells carrying pSU2 and pS2-CU against Ni^{2+} and Cu^{2+}. *S. cerevisiae* SEY6210 cells carrying pSU2 (control) or pS2-CU were precultured in YP7D medium at 30°C for 18 h, then cultured in YP2S medium at

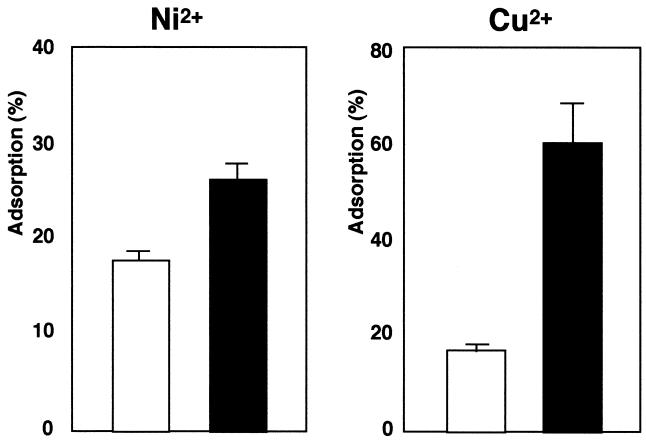

Fig. 3. Metal adsorption by the cells of *S. cerevisiae* SEY6210 harboring the plasmids pSU2 (open bar) or pS2-CU (closed bar).

The adsorption of metal ions by yeast cells was calculated from the concentrations of metal ions left in the supernatants. The initial concentrations of metal ions (2 ppm each) are 34.1 μM Ni^{2+} (left) and 31.5 μM Cu^{2+} (right). Experiments were replicated four times.

30°C for 2 h. The cells were harvested by centrifugation and washed with 20 mM Tris/HCl (pH 4.5) and 20 mM Tris/HCl (pH 8.0) sequentially. Approximately 6×10^9 cells were suspended in 10 ml of 20 mM Tris/HCl (pH 8.0) containing 2 ppm of divalent metal cation: Cu^{2+} or Ni^{2+} derived from dissolved $CuSO_4$ and $NiCl_2$. After incubation with gentle shaking at 25°C for 2 h, the cells were removed from the solution and concentrations of metal ions remaining in the supernatants were measured by an atomic absorption spectrometer (Hitachi Z-8100).

It was observed that the *S. cerevisiae* SEY6210 (pS2-CU) cells respectively showed 1.6-fold and 3.5-fold increases in Ni^{2+} and Cu^{2+} ion adsorbing capacity when compared with the control SEY6210 (pSU2) cells (Fig. 3). The enhanced capability of metal adsorption by SEY6210 (pS2-CU) cells was due to the expression of Cup1 on the cell surface. It is calculated that approximately 2.7×10^6 of Ni^{2+} ions or 1.07×10^7 of Cu^{2+} ions were adsorbed per Suc2-Cup1 expressing yeast cell. This is the first report of an expression of metallothionein on the yeast cell surface for increasing metal-adsorption capacity.

In this study, we showed the expression of Suc2 and Cup1 fusion protein in the yeast cell surface and used Suc2 as the cell surface carrier and anchor for an expression of functional protein. The Suc2-Cup1 fusion protein was efficiently expressed and localized in the cell wall without being released into the medium. It is therefore suggested that Suc2 is an available carrier for the expression of functional peptides on yeast cell surface. It was reported that the treatment of intact cells with β-mercaptoethanol caused a release of invertase dimer (Esmon et al., 1987). It suggests that Suc2 fusion proteins will be able to recover from the cells by β-mercaptoethanol treatment, when necessary. We expect that these molecular breeding techniques are useful for an expression of functional domains on the yeast cell surface and that they enhance the heavy metal bioremediation capability of the yeast strains.

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