The *COT2* Gene Is Required for Glucose-Dependent Divalent Cation Transport in *Saccharomyces cerevisiae*

DOUGLAS S. CONKLIN, CHING KUNG, AND MICHAEL R. CULBERTSON*

Laboratories of Molecular Biology and Genetics, University of Wisconsin—Madison,
1525 Linden Drive, Madison, Wisconsin 53706

Received 11 September 1992/Returned for modification 10 November 1992/Accepted 7 January 1993

Eleven cobalt-tolerant mutants were found to belong to a single complementation group, *cot2*. In addition to cobalt, the *cot2* mutants were found to tolerate increased levels of the divalent cations Zn^{2+} , Mn^{2+} , and Ni^{2+} as well. All of the *cot2* mutants exhibited a wiener-shaped cellular morphology that was exacerbated by the carbon and nitrogen source but was unaffected by metals. The rate of glucose-dependent transport of cobalt into cells was reduced in strains that carry mutations in the *COT2* gene. *COT2* is not essential for growth. Strains that carry a *COT2* allele conferring complete loss of function are viable and exhibit phenotypes similar to those of spontaneous *cot2* mutations. The sequence of the *COT2* gene shows that it is identical to *GRR1*, which encodes a protein required for glucose repression. The glucose dependence of the transport defect implies that *cot2* mutations affect the link between glucose metabolism and divalent cation active transport.

Trace metals play a major role as coenzymes or cofactors in many cellular processes. Several transition metals are required as catalysts in a variety of enzymatic reactions (14, 17). With the exception of iron, these metals are normally found at relatively low concentrations in the environment and must be actively accumulated. Although much is known about iron uptake (for a review, see reference 28), little is known regarding the accumulation of other trace metals.

In the yeast *Saccharomyces cerevisiae*, several metals of physiological importance are accumulated by an energy-dependent, low-specificity uptake system. The divalent cation transport system described by Fuhrmann and Rothstein (9) is responsible for the accumulation of Co^{2+} , Ni^{2+} , and Zn^{2+} ions. Competition experiments suggest that other metals are also accumulated with an affinity series of Mg^{2+} , Co^{2+} , $Zn^{2+} > Mn^{2+} > Ni^{2+} > Ca^{2+}$, and Sr^{2+} . The transporter has the highest affinity for Mg^{2+} , Co^{2+} , and Zn^{2+} ions, with K_m s for these metals in the range of 10 to 100 μM (9, 25). As is the case with most transport systems in *S. cerevisiae*, the activity of the divalent cation transport system is dependent upon glucose.

The *COT1* gene product has been shown to participate in the uptake of cobalt ions by yeast cells (5). The *COT1* gene encodes a 48-kDa membrane protein that is found in the mitochondrial membrane fraction of cells. *COT1* was isolated as a dosage-dependent suppressor of cobalt toxicity. Overexpression of the *COT1* gene confers increased tolerance to cobalt ions, whereas strains containing null alleles of *COT1* are viable yet more sensitive to cobalt than are wild-type strains. The increased tolerance of the high-copy-number *COT1* strains appears to result from the increased sequestration or compartmentalization of cobalt within the cell. Cobalt uptake is affected by the amount of *COT1* product present in cells yet not completely dependent upon it. Strains that carry the *COT1* gene on a high-copy-number plasmid accumulate more cobalt than do wild-type strains. Strains that contain a null allele of the *COT1* gene accumulate less cobalt than do wild-type strains, having an uptake rate about 55% of the wild-type rate. Cobalt uptake activity

is not abolished in *COT1* null mutants, suggesting that other cobalt accumulation mechanisms exist in *S. cerevisiae*. Although it is not known whether the *COT1* protein is involved in the uptake of other metals, increased tolerance is conferred only for the group 9 transition metals, cobalt and rhodium. Other gene products may be responsible for the uptake of other metals. The *ZRC1* gene, which was isolated as a high-copy-number zinc and cadmium toxicity suppressor (20), encodes a protein that specifically detoxifies the group 12 metals, Zn and Cd. The product of the *ZRC1* gene shows significant sequence similarity to the *COT1* protein (60% amino acid identity; 78% with conservative substitutions) and may also function in metal uptake.

In this study, we have taken advantage of the toxicity of relatively high concentrations of Co^{2+} ions on yeast growth to isolate a group of mutants that are defective in divalent metal transport. The transport defect appears to be due to a decrease in the glucose stimulation of divalent cation transport. Sequence analysis shows that the mutant gene is identical to *GRR1*, which is required for glucose repression in *S. cerevisiae*. We believe that these mutations affect the link between glucose metabolism and the activity of the divalent cation transport system.

MATERIALS AND METHODS

Genetic techniques. Yeast strains used in this study are listed in Table 1. Yeast transformations were performed by the method of Ito et al. (18). *COT2* was mapped to chromosome X by Southern blot hybridization to yeast chromosomes resolved by contour-clamped homogeneous electric field gel electrophoresis (4). Standard yeast genetic techniques were as described by Sherman et al. (38). *Escherichia coli* strains used were MC1066a (*leuB600 trpC9830 pyrF74::Tn5 Kan^r ara^r r_K⁻ m_K⁻ recA13*) (35) and DH5 α [*supE44* Δ *lacU169* (Φ 80 *lacZ* *DM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (12).

Media. Standard YPD was prepared as described elsewhere (38). Synthetic low-sulfate, low-phosphate (LSP) medium has been described previously (5). For plates containing proline, 80 mM proline was substituted for NH_4Cl ; 2% glucose is used as the carbon source unless indicated other-

* Corresponding author.

TABLE 1. Yeast strains

Strain	Genotype	Source
JC482a	<i>ura3-52 leu2-3,112 his4-539 MATa</i>	J. Cannon
CYP 484	<i>cot2-1 ura3-52 leu2-3,112 his4-539 MATa</i>	This study
CYP 520	<i>ura3-52 leu2-3,112 his4-539 MATa</i> (YcP351)	This study
CYP 521	<i>cot2-1 ura3-52 leu2-3,112 his4-539 MATa</i> (YcP351)	This study
CYP 522	<i>cot2-Δ1::LEU2 ura3-52 leu2-3,112 his4-539 MATa</i>	This study
CYX 379-7D	<i>cot2-1 ura3-52 leu2-3,112 trp1-Δ1 MATa</i>	This study
CYX 379-9D	<i>cot2-1 ura3-52 leu2-3,112 trp1-Δ1 MATa</i>	This study
CYP 490-2D	<i>cot2-Δ1::LEU2 ura3-52 leu2-3,112 MATa</i>	This study
CYP 490-17D	<i>cot2-Δ1::LEU2 ura3-52 leu2-3,112 trp1-Δ1 MATa</i>	This study
A141-37C	<i>leu2-3 met1 ade6 cdc11 pet17 his1 lys11 MATa</i>	L. Mathison
RTY315	<i>cyc8::URA3 ura3-52 leu2-3,112 his4-539 MATa</i>	R. Trumbly
AT18	<i>tup1-18 ade1 ade2 his7 gal1 ura1 lys2 tyr1 MATa</i>	R. Wickner
373	<i>ura3-52 his6 gal1 bar1 reg1-501 MATa</i>	M. Johnston

wise. Divalent cations were added from sterile stocks to media cooled to 65°C. Monovalent cations were added to growth media prior to autoclaving. Metal salts were added to growth test media at the following concentrations: MnCl₂, 15 and 20 mM; NiCl₂, 2.5 and 5 mM; CuSO₄, 0.3 and 1 mM; ZnSO₄, 15 and 20 mM; CdCl₂, 75 and 100 μM; LiCl, 100 and 200 mM; NaCl, 0.8 and 1.2 M; KCl, 2.0 and 2.5 M; and CsCl, 50 and 100 mM. Most metals were added to YPD. Zinc and copper were not soluble in YPD and were added to LSP plates.

Cloning and sequencing techniques. The glass bead method of Hoffman and Winston (16) was used for yeast plasmid rescue and the preparation of yeast genomic DNA. Cloning techniques were as described by Maniatis et al. (23). Nested deletions were constructed by using an Erase-a-Base sequencing kit from Promega. Double-stranded plasmid sequencing was done by using the Sequenase method (United States Biochemical Corp.) and [α -³⁵S]ATP (Amersham). Additional sequence data were obtained by using primers synthesized by the University of Wisconsin Biotechnology Center. The primers were 5'-GTGTCCAAGTAGCAGGG-3', which corresponds to *COT2* sequence positions -157 to -141, and 5'-CAACCAGAACTCTTGG-3', which is complementary to positions 497 to 514.

Nucleic acid hybridizations were carried out by the method of Southern as described by Maniatis et al. (23). Probes were purified following agarose gel electrophoresis by using a Gene Clean kit (Bio 101) and labeled with [α -³²P]CTP by using a random oligonucleotide priming kit (Pharmacia).

Plasmid constructions. Subclones of YcPWNR1-b43 were constructed by using standard techniques described by Maniatis et al. (23). YEpWNR100 and -101 contained different orientations of the 2.4-kb *EcoRI* fragment in the *EcoRI* site of YEp352 (15). YEpWNR102 and -103 contained the 1.2-kb *EcoRI* fragment in either orientation in the *EcoRI* site of YEp352. YEpWNR114 and YEpWNR120 were constructed from YEpWNR100, which contained an approximately 11.5-kb *BamHI* fragment in the *BamHI* site of YEp352. YEpWNR114 was constructed by digesting YEpWNR110 with *SalI* and religating. A clone that had lost the *SalI* fragment was chosen as YEpWNR114. YEpWNR120 was constructed in a similar manner except that YEpWNR110 was digested with *SmaI*.

A disruption plasmid of *COT2* was constructed by inserting the *LEU2* gene between the *BstXI* and *BstEII* sites of YEpWNR120. YEpWNR120 was digested with *BstXI* and *BstEII*, and the ends were made flush with Klenow fragment

of DNA polymerase I and all four deoxynucleoside triphosphates. The yeast *LEU2* gene was removed from plasmid JH-L1 (a gift of J. Hill) by digestion with *SmaII* and *HindIII*, and the *HindIII* ends were filled in with Klenow fragment. The two fragments were ligated together and transformed into MC1066a. Pyr⁺ Leu⁺ transformants were screened for the correct construction. The 2 μm circle DNA was removed from this construction by digestion with *SspI* and religation. The resulting plasmid containing the disrupted version of *COT2* was named pCOT2Δ and was digested with *PstI* prior to transplacement (33).

Transport assays. Cobalt uptake was measured by a rapid filtration technique (5). Briefly, cells were grown to mid-log phase in either 2% glucose or 2% ethanol containing LSP synthetic medium lacking leucine. The cells were harvested, washed twice, and resuspended in 50 mM Tris-succinate (pH 5.5). Suspensions of 10⁸ cells per ml were preincubated with or without 100 mM glucose, 100 mM 2-deoxyglucose, 100 mM fructose, 100 mM galactose, 100 mM maltose, 100 mM sucrose, or 2% ethanol for 30 min at room temperature. Approximately 1 μCi of ⁵⁷Co²⁺ (Amersham) was added with nonradioactive CoCl₂ to a final concentration of 100 μM cobalt. At each time point, 150 μl of sample was filtered onto a Millipore 0.45-μm-pore-size filter and washed three times with 15 ml of ice-cold wash buffer (50 mM Tris-succinate [pH 5.5], 20 mM MgSO₄, and 1 mM CoCl₂ with or without 100 mM glucose). For measurement of efflux, cultures were incubated with ⁵⁷Co²⁺ as described above for 60 min. At this point, the cultures were pelleted, washed once in 50 mM Tris-succinate (pH 5.5)-100 μM CoCl₂, and resuspended in 50 mM Tris-succinate (pH 5.5)-100 μM CoCl₂-100 mM glucose. Samples were removed as described above. ⁵⁷Co²⁺ contained on the filters was determined by counting in a Packard gamma ray counter on a ⁵⁷Co²⁺ channel. As judged by controls in which the carbon source or cells were omitted, nonspecific binding of ⁵⁷Co²⁺ to the external cell surface or filter was negligible. Calcium uptake was measured in the same manner, using ⁴⁵Ca²⁺ (New England Nuclear).

RESULTS

Isolation of *cot2* mutants. Ninety-eight independent mutants were isolated in a selection for cobalt-resistant yeast cells. Of these 98 mutants, 87 grew very well, forming colonies after 3 days of growth on 4 mM CoCl₂ YPD plates. Representatives of this fast-growing class did not show a 2:2 segregation pattern for cobalt resistance in crosses with a wild-type strain and were not investigated further. The other

FIG. 1. Divalent cation-related growth phenotypes of *cot2* mutants. Strains were transferred from master plates to the plates shown by replica plating. The YPD plate was photographed after 2 days of growth; the plates containing metals were photographed after 4 days. Strains used: *COT2*⁺, JC482; *cot2-1*, CYP 484; *cot2-Δ1*, CYP 522. Concentrations of metals: Co^{2+} , 5 mM CoCl_2 ; Zn^{2+} , 20 mM ZnCl_2 ; Ni^{2+} , 2.5 mM NiCl_2 ; Mn^{2+} , 20 mM MnCl_2 .

11 isolates formed colonies after 6 days of growth on 4 mM CoCl_2 YPD. All 11 were found to be recessive, as diploids produced in crosses with CYX 367-15C were not resistant to cobalt. Tetrads were dissected in four of these crosses, and in each case cobalt resistance segregated 2:2, indicating that the cobalt-tolerant phenotype was due to a single nuclear mutation. A segregant of one of these crosses, strain CYX 369-7D, was mated to all 11 cobalt-resistant isolates. The resulting diploids in all cases were capable of growth on 4 mM CoCl_2 YPD. Thus, all of the slowly growing mutants belonged to a single complementation group. CYX 369-7D was crossed to strain CYX 380-2C, which carries a *COT1* gene that has been disrupted by insertion of the yeast *URA3* gene. In this cross, the newly isolated mutation segregated independently from the *COT1-Δ1::URA3* mutation (4 parental ditype:3 nonparental ditype:16 tetratype asci; no linkage). This finding indicated that the 11 isolates represented the second locus that has been found to confer increased cobalt tolerance to *S. cerevisiae*. This locus has been designated *COT2*.

The growth of these strains on media containing other

metals was tested. As shown in Fig. 1, *cot2* mutant strains are capable of growth in media containing zinc, manganese, and nickel as well as cobalt. Resistance was not observed for sodium, potassium, lithium, cesium, copper, or calcium. The concentration of H^+ in the media had no effect on the mutant strains. The growth of *cot2* strains was no different from that of wild-type strains on YPD buffered with 100 mM succinate at pHs 3.5 and 4.5 or 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) at pH 7.5 (data not shown). From this result, it appears that the metals to which *cot2* mutant strains are tolerant are divalent cations with ionic radii near 0.075 nm. Tolerance is observed for metals of the following crystal ionic radii (in nm [35]): Co^{2+} (0.074), Ni^{2+} (0.069), Mn^{2+} (0.083), and Zn^{2+} (0.074) but not Ca^{2+} (0.100) or Cd^{2+} (0.095). Increased tolerance was not observed, however, for Mg^{2+} (0.072) and Cu^{2+} (0.073), which are divalent cations with ionic radii near 0.075 nm (see Discussion). The metals to which *cot2* mutant strains are tolerant are all transported by the divalent cation transport system described by Fuhrmann and Rothstein (9). Increased tolerance was not observed to the divalent cation transport system substrates Ca^{2+} and Mg^{2+} , which are relatively nontoxic, and Sr^{2+} , which is not soluble in yeast growth media.

Aberrant morphology of *cot2* mutants. During the genetic analysis of the cobalt-tolerant strains, it was noticed that the *cot2* isolates had an abnormal wiener-like morphology. Whereas unbudded haploid cells are generally spherical, the *cot2* mutants were oblong, being on average three times longer than wide (Fig. 2). Homozygous diploid *cot2* mutants were similarly elongated. This morphological phenotype was recessive and was characteristic of all 11 independent alleles. In the case of *cot2-1*, the aberrant morphology cosegregated with the cobalt tolerance in 59 of 59 tetrads. This result indicated that it was tightly linked to the cobalt tolerance gene and almost certainly due to the same mutation. This conclusion was confirmed by complementation of the mutant morphology of *cot2-1* with a clone of the *COT2* gene (see below).

FIG. 2. Morphological defects of *cot2* mutants. (A to D) Strains CYP 484 (*cot2-1*) and JC482 (*COT2*) were grown in YPD. Bud scars were stained with calcofluor as described previously (29) for panels C and D. (E) *cot2-1* cells from a culture grown overnight on YEP-potassium acetate. Photos were taken at a magnification of $\times 1,500$ on a Nikon Optiphot microscope.

The elongation of *cot2-1* cells occurred along the budding axis of the cell. From observations of populations of *cot2* cells, it was seen that greater than 95% of buds emerged immediately adjacent to the ends of wiener-shaped mother cells. Buds were never seen emerging from the middle of cells. Calcofluor staining of bud scars also showed that bud site selection in *cot2* mutants occurs exclusively at the ends of the elongated cells (Fig. 2D).

The elongation of *cot2* mutant cells occurs while the cells are budding. Large buds are elongate while still attached to mother cells and remain elongate (as do mother cells) throughout subsequent budding cycles. Although many *cot2* cells resemble shmooing cells, the abnormal morphology of strains carrying *cot2* alleles does not appear to be the result of G₁ cell cycle arrest, since *cot2* mutations cannot bypass the requirement for α -factor receptor during mating with a cells (data not shown).

Although the wiener morphology was most pronounced when the cells were growing exponentially on glucose-based media, strains carrying *cot2* alleles also displayed a germ tube-like process when grown on YEP-potassium acetate (Fig. 2E). These forms were also observed in populations of *cot2* mutants grown in synthetic media containing proline as the nitrogen source. The morphology of *COT2*⁺ strains was unaffected. Under these conditions, approximately 25% of the cells in a *cot2* population formed cells with germ tube-like processes. 4',6-Diamidino-2-phenylindole staining revealed that several of these cells contained a nucleus in the germ tube-like process, suggesting that these structures result from defective bud formation.

Analysis of the *COT2* gene. The recessive slow-growth phenotypes of *cot2* mutants were used to clone the *COT2* gene. Strain CYX 369-7D, which contains the *cot2-1* allele, was transformed with DNA from centromeric genomic libraries (32). The resulting uracil prototrophs were replica plated to YEpd, YEpd-1 M NH₄Cl, and YEP-8% glucose plates. Growth of replica-plated patches of wild-type strains on 1 M NH₄Cl-containing YEpd and 8% glucose plates was visible after 1 day. Growth of strains containing *cot2* mutations was visible after 2 to 3 days. Although the basis of these growth differences is unknown, the use of these media allowed for the rapid identification of clones conferring the Cot⁺ phenotype. Following incubation at 30°C for 1 day, morphologies of cells from fast-growing colonies were screened microscopically. Seven isolates from three different library pools exhibited both wild-type growth and wild-type morphology. All seven exhibited cosegregation of growth, morphology, and uracil prototrophy. In each case, plasmids were rescued from the Cot⁺ yeast strains and amplified in *E. coli*. Restriction analysis of these clones showed that each plasmid contained a distinct yet related insert.

Although the *cot2*-complementing clones were originally isolated on a centromere-based vector, the possibility existed that the inserts contained an extragenic suppressor of *cot2* mutations and not the *COT2* gene itself. This possibility was eliminated by inserting a copy of the cloned DNA that contained a *LEU2*⁺ gene in the yeast genome and observing linkage to the *COT2* locus. This procedure was carried out by using a disrupted copy of *COT2* and is described below.

Plasmid YCpWNR1-b43 contained a 13.5-kb insert in YCp50 (Fig. 3). Since this was the smallest of the seven isolates, it was subcloned to determine the extent of *cot2*-complementing activity. Fragments of YCpWNR1b43 were cloned into the multiple cloning site of YEp352. The region

FIG. 3. Restriction maps and in vivo complementation of the *cot2-1* allele data for genomic library isolate YCpWNR1-b43 and subclones. Plasmid constructions are described in Materials and Methods. CYP 484 transformants that carried the subclones were tested for growth on 5 mM CoCl₂ plates and wild-type morphology. The arrow indicates the position of the proposed *COT2* open reading frame.

of complementing activity was confined by the left end of plasmid YEpWNR114-Δ11 and the *SmaI* site (Fig. 3).

The *cot2*-complementing DNA was mapped to chromosome X by Southern blot hybridization to yeast chromosomes resolved by contour-clamped homogeneous electric field gel electrophoresis. Linearized YEpWNR100 was used as the probe. Hybridization to an ordered set of genomic yeast cosmids (27) showed that *COT2* mapped centromere distally to the *CDC11* locus. Strain CYP 484 was crossed to A141-37C, and linkage was observed between *cot2-1* and *cdc11* (8.3 centimorgans; 65 parental ditype:0 nonparental ditype:13 tetratype asci). This result suggests that the cloned DNA contains the *COT2* gene.

Sequence analysis of the *COT2* gene. Nested deletions of YEpWNR114, YEpWNR100, YEpWNR101, YEpWNR102, and YEpWNR103 were used as templates to sequence both strands of a 5.6-kb region. This region corresponds to DNA between the leftmost *EcoRI* site and the *SmaI* site of YCpWNR-b43 (Fig. 3) and contains a single long open reading frame of 3,453 bp. Since the region of *cot2*-complementing activity agrees with the location of this reading frame (Fig. 3), this is believed to be the *COT2* gene.

The sequence predicts an unmodified 132-kDa protein of 1,151 amino acid residues (assuming that translation initiates at the first ATG in the open reading frame). A codon bias index (2) of 0.02 suggests that *COT2* is not highly expressed. A hydropathy plot was generated by using the residue-specific hydrophobicity index of Kyte and Doolittle (21) and a window size of 19. The plot showed that the protein contains no stretches of hydrophobic residues large enough to be considered membrane-spanning domains (data not shown). From this finding, it seems unlikely that *COT2* is the structural gene for an integral membrane metal transporter.

The predicted *COT2* protein is relatively rich in asparagine (11.2%) and leucine (12.0%) residues. Tracts of asparagine-rich sequence are predominantly found at both amino and carboxy termini (Fig. 4A). YEpWNR114-Δ11, which encodes a truncated *COT2* gene product that is missing the carboxy-terminal polyasparagine tracts and is carried on a high-copy-number vector, complements the *cot2-1* allele (Fig. 3). Thus, the carboxy-terminal polyasparagine tracts are not essential for the function of the *COT2* protein.

A tandemly repeated structure is found between amino

FIG. 4. Structure of the *COT2* product. (A) Positions of the polyasparagine-containing sequences and weak repeats. (B) Alignment of the 13 weak repeats found in continuous sequence between amino acid residues 409 and 750. A consensus sequence for the repeats is shown below. Specific residues that occur in 6 or more of the 13 repeats are contained in open boxes; residue types that occur nonrandomly ($P > 0.95$) in a majority of the repeats are shaded. λ and β indicate hydrophilic and hydrophobic residues, respectively.

acid positions 409 and 750 in the *COT2* sequence (Fig. 4B). This motif is about 26 amino acid residues in length and is repeated 13 times. Hydrophobic residues (21) are found at positions 1, 2, 5, 8, 11, 13, 16, 19, and 24 in the consensus. Hydrophilic residues (21) are found at positions 6, 9, 10, 12, 17, 18, and 20. These repeats may assume a secondary structure that may be important for function. The nature of this secondary structure, however, cannot be readily deduced from the sequence.

A search of the NBRF, GenBank, and EMBL protein sequence libraries failed to find any sequences with significant homology to the *COT2* protein. A data base search performed by Mark Goebel found that the sequence of *COT2* was identical to the sequence of *GRR1*. *GRR1* was originally isolated in a search for glucose repression-defective mutants (1) and has been analyzed at the molecular level (7). Since the sequence and map position as well as mutant morphologies and slow-growth phenotypes are the same for the two genes, *COT2* and *GRR1* are allelic. To unify nomenclature, *COT2* will be referred to as *GRR1* in future reports, since this was the name first given to the locus (1, 7).

Loss of *COT2* gene function leads to metal tolerance. A null allele of *COT2* was introduced into wild-type diploid strain CYP 492 by transformation with *Pst*I-digested pCOT2Δ. Stable Leu⁺ transformants were analyzed by Southern hybridization and found to contain the expected disruption (data not shown). In this construction, the *LEU2* gene has replaced nucleotides -454 to 4737 of the *COT2* sequence. This alteration removes all but the last 63 residues of the *COT2* protein. In tetrads dissected from this strain, a 2:2 segregation pattern was observed for leucine prototrophy. This result indicated that the *COT2* gene is not essential for growth or germination. In each case, however, Leu⁺ spore clones were resistant to cobalt and displayed the wiener morphology. The disruption mutants exhibited the same range of metal resistances as did the original mutants (Fig.

FIG. 5. Tryptophan-related growth phenotypes of the *cot2-Δ1* allele. Strains were replica plated to YPD and tryptophan (4 g/liter)-containing YPD plates after overnight growth on a tryptophan (4 g/liter)-containing YPD plate. The strains used were CYX 367-15C (*COT2 trp1-Δ1*), CYX 490-2D (*cot2-Δ1 TRP1*), and CYX 490-17D (*cot2-Δ1 trp1-Δ1*).

1). Thus, the phenotypes of the original spontaneously arising mutants can be attributed to the loss of function of the *COT2* gene product.

To ensure that the cloned DNA originated from the *COT2* locus, a strain carrying the *cot2-Δ1::LEU2* allele was crossed to CYX 369-7D. The resulting diploid strain, CYP 490, exhibited the wiener morphology and metal resistances of the known *cot2* alleles. In tetrads dissected from a culture of CYP 490 that had undergone sporulation, no recombinant *COT2*⁺ spores were seen in 17 tetrads. This result confirms that the cloned DNA originated from the cobalt-tolerant locus and contains the *COT2* gene.

In general, the phenotypes of strains that carry the *cot2-Δ1::LEU2* mutation were indistinguishable from those of the original isolates. In crosses, however, it was noticed that strains that carry both the *trp1-Δ1* and *cot2-Δ1::LEU2* alleles grew very poorly. *trp1-Δ1* strains carrying original *cot2* alleles did not grow poorly. During the outgrowth of spores dissected from CYP 492, several spores formed very small colonies. Upon scoring, it was evident that each of the spore clones carried both *trp1-Δ1* and *cot2-Δ1::LEU2* alleles. As shown in Fig. 5, a *cot2-Δ1::LEU2 trp1-Δ1* strain grows very slowly on YPD medium, whereas *COT2 trp1-Δ1* and *cot2-Δ1::LEU2 TRP1* strains grow normally. This finding may mean that *cot2-Δ1::LEU2* strains do not utilize the tryptophan present in YPD medium as efficiently as do wild-type strains. This seems likely, as the addition of tryptophan to levels 100 times those normally present in growth media alleviates the slow growth of *cot2-Δ1::LEU2* strains (Fig. 5). The same slow-growth phenotype was observed when *cot2-Δ1::LEU2 trp1-Δ1* mutants were grown on synthetic media regardless of whether ammonium or proline was used as the nitrogen source (data not shown). Since expression of *GAP1* is derepressed on proline-based media, this finding indicates that the increased tryptophan requirement of *cot2-Δ1::LEU2 trp1-Δ1* mutants cannot be alleviated by the activity of the general amino acid permease which has been shown to transport tryptophan (31).

***cot2* mutants are defective in cobalt transport.** The metal-resistant phenotypes of the *cot2* strains suggested that mutations in *COT2* might alter the transport of divalent cations. To test this possibility, we assayed divalent cation transport by measuring ⁵⁷Co²⁺ and ⁴⁵Ca²⁺ uptake. Radioactive cobalt was chosen as a tracer because the *cot2* mutants were originally isolated as being tolerant to cobalt and because several of the other trace metals have only dangerous or

FIG. 6. $^{57}\text{Co}^{2+}$ accumulation by *COT2*, *cot2-1*, and *cot2-Δ1* strains. The strains used were CYP 520 (*COT2*⁺), CYP 521 (*cot2-1*), and CYP 522 (*cot2-Δ1*). Strains were grown and assayed in 100 mM glucose in panel A. Values for cobalt accumulated after 60 min of incubation were as follows: CYP 520, 18.0 nmol/10⁸ cells; CYP 521, 1.8 nmol/10⁸ cells; and CYP 522, 1.6 nmol/10⁸ cells. In panel B, 2% ethanol was used as the carbon source. Values for ethanol-dependent cobalt uptake at 60 min of incubation were about 3.4 nmol/10⁸ cells for each of the strains. Points in panels A and B represent the average of six experiments in each case; error bars indicate standard deviation. In panel C, the average of two experiments is shown. A strain carrying the *cot2-Δ1* allele was not tested.

prohibitively expensive radioactive isotopes. For the assays, the genetically closely related strains CYP 520, CYP 521, and CYP 522 were constructed. As shown in Fig. 6A, wild-type strain CYP 520 accumulates cobalt when incubated with glucose (18.0 nmol/10⁸ cells in 60 min). This uptake is both glucose and temperature dependent and represents active transport similar to that previously described by others (9, 25). Other fermentable sugars also supported significant divalent cation uptake in the wild-type strain (CYP 520). The values for cobalt uptake (expressed as nanomoles per 10⁸ cells at 60 min) were as follows: fructose, 18.7; galactose, 7.3; maltose, 7.1; and sucrose, 9.5. The nonmetabolizable glucose analog 2-deoxyglucose did not support cobalt uptake (data not shown).

Strains containing either the *cot2-1* or *cot2-Δ1::LEU2* allele, however, have significantly reduced levels of glucose-dependent cobalt uptake (Fig. 6A). The rate of uptake during the first 60 min is about 10% of that of the wild type for strains carrying either the *cot2-1* or *cot2-Δ1::LEU2* allele. The rate of cobalt uptake supported by fructose, galactose, maltose, and sucrose was also about 10% of that of the wild type (data not shown). There was no difference between wild-type and mutant cells in $^{45}\text{Ca}^{2+}$ uptake.

Since this assay measures only net uptake, the observed difference could be due to increased efflux of Co^{2+} ions. This possibility was ruled out by preloading cells with $^{57}\text{Co}^{2+}$ and finding that net cobalt efflux was negligible in both wild-type and mutant strains with or without glucose (Fig. 6C). Thus, decreased uptake of the toxic metals seems to be the most likely explanation for the observed resistance.

Since the activity of the divalent cation uptake system in wild-type cells is greatly stimulated by glucose (Fig. 6A) (9, 25) and other sugars, one possibility for the loss of transporter activity in *cot2* mutants is that a defect in some aspect of sugar metabolism exists. To test this possibility, cells were grown in YP-2% ethanol prior to the assay; 2% ethanol was used in place of glucose during the assay. The level of cobalt transport in wild-type cells is significantly lower when ethanol is used as an energy source than when glucose is used (Fig. 6A [open squares] versus Fig. 6B [open squares]). There was, however, no difference in the ethanol-supported accumulation of Co^{2+} between strains carrying mutant and nonmutant alleles of *COT2*. This finding might indicate that

the reduction in divalent cation transport exhibited by *cot2* strains is due to a defect in the coupling of the metabolism of glucose (or other sugars) to transport or in the metabolism of glucose itself. Alternatively, it may suggest that a second divalent cation transport system, which operates independently of glucose, exists in *S. cerevisiae*. If this is the case, *cot2* mutants, which are missing the glucose-dependent system, appear to have a normal glucose-independent system. This seems reasonable, since the level of cobalt accumulated by strains carrying mutant alleles of *COT2* is nearly the same on either glucose or ethanol.

Cobalt tolerance is not a consequence of a constitutive glucose derepressed response. The glucose repression-defective phenotype of *grr1* mutants suggests that this gene is required for the sensing of glucose (7). Although disruption of the *COT2/GRR1* locus results in both glucose repression and cobalt-tolerant phenotypes (7) (Fig. 1), it is not known whether the original *cot2* mutants, which were isolated as being tolerant to cobalt, also possess glucose repression defects. Nonetheless, a possible explanation for the defect seen in the glucose dependence of divalent cation transport of *cot2* cells exists in defective glucose repression. Strains that carry a mutation at the *COT2* locus do not sense glucose and therefore have no glucose-dependent cation transport and are tolerant to metals. This finding also suggests that other glucose repression mutants might also be resistant to metals in the media. To test this possibility, the cobalt tolerance of other glucose repression mutants was examined. As shown in Fig. 7, none of the other mutants tested were tolerant to elevated levels of cobalt. This finding suggests that the presumed loss of the glucose signal is specific to the loss of the *COT2* product and not a general phenomenon caused by loss of glucose repression. The glucose dependence of divalent cation uptake in yeast cells does not require the glucose repression pathway.

DISCUSSION

In this study, mutations that confer increased divalent cation tolerance upon yeast strains have been identified. The *cot2* locus is defined by 11 recessive, abnormally shaped mutants that were obtained in a selection for cobalt-tolerant yeast strains. In addition to cobalt, the strains were resistant

FIG. 7. Evidence that cobalt tolerance is not a general consequence of constitutive glucose derepression. Strains were transferred from master plates to the plates shown by replica plating and were photographed after 4 days of growth. The strains used were CYP 520 (wild type), CYP 521 (*cot2*), 373 (*reg1*), AT18 (*tup1*), and RTY315 (*cyc8*).

to zinc, manganese, and nickel ions. All of these metals have similar ionic radii of approximately 0.075 nm. Increased tolerance was not observed for any of the monovalent cations that we tested. From this finding, it appears that *cot2* mutants exhibit increased tolerance to a small group of ions of a specific size and charge rather than of a specific periodic group as is the case with *COT1* and *ZRC1* overexpression (5, 20).

The metals to which mutations in *COT2* confer tolerance are accumulated by the divalent cation transport system (9). That these mutants have decreased levels of cobalt uptake suggests that mutations in *COT2* compromise the activity of this system and may have defects in the uptake of Zn^{2+} , Mn^{2+} , and Ni^{2+} as well. Although magnesium and calcium are also transported by the divalent cation transport system, we did not detect increased tolerance to or increased dependence upon either of these metals. This result may be explained by the relatively nontoxic nature of calcium and magnesium ions. *S. cerevisiae* tolerates concentrations greater than 0.5 M of either metal in growth media. A ^{45}Ca uptake defect was not detected in the *cot2* mutants (data not shown), possibly because the divalent cation transport system is a rather minor entrance route for this ion into cells. The affinity of this system for Ca^{2+} is lower than its affinity for other metals (9). *cot2* mutations did not confer increased tolerance to Cu^{2+} ions, which are similar in size and charge to Co^{2+} ions. Cu^{2+} is not transported by the divalent cation transport system, however, and may gain access to the cell via another uptake pathway.

The results of cobalt transport experiments show that glucose-stimulated cobalt uptake (and, by extension, divalent cation transport activity) does not occur in *cot2* mutants. If the primary role of this system is to accumulate trace metals, it is expected that loss-of-function mutants should require higher concentrations of these metals for growth. We did not, however, observe that *cot2* mutants required higher concentrations of these metals for growth (data not shown). This suggests that other systems exist for the accumulation of divalent cations, which is not surprising given that *cot2* strains still exhibit about 10% of wild-type Co^{2+} uptake. The decreased entrance of toxic metals is, however, the most likely explanation for the increased metal

tolerance of *cot2* mutants. In this respect, mutations in *COT2* resemble other recessive drug resistance mutations such as *can1* (11) and *gap1* (34). Resistance in these cases is due to a decrease in the permeability of the plasma membrane to a toxin that is structurally related to the normally transported molecule. Unlike these cases, however, the *COT2* product does not appear to be the protein responsible for transport of the ions, since it has no membrane-spanning regions.

The primary structure of the *COT2* protein gives few clues to its actual function. The size of the 13 tandem structural repeats (Fig. 4B) and the spacing of the leucine or hydrophobic residues within them are similar to those found in Leu-Leu repeat proteins (8). The *COT2* repeat does not, however, strictly conform to the consensus sequence of the Leu-Leu motif proteins which are believed to mediate protein-protein interactions. The overall similarity, however, raises the possibility that the *COT2* protein is involved in some protein-protein interaction.

Since the loss of *COT2* function leads to increased metal tolerance, the role that the *COT2* protein plays in metal uptake is probably different from that of other proteins involved in metal resistance. The tolerance conferred by the *COT1*, *ZRC1*, and *CUP1* proteins is dosage dependent. It has been established for *CUP1* (19) and has been suggested for *COT1* and *ZRC1* (5) that direct interaction between these proteins and their respective toxic metals occurs during detoxification. *COT2* is likely to play a more general role in yeast physiology that indirectly controls the permeability of the membrane to metal ions or the driving force for the uptake of these metals. Such a role would fit with the pleiotropic nature of mutations in *COT2*. *COT2* might function in the global regulation or expression of plasma membrane transport proteins, for example. Alternatively, *COT2* might be involved in the glucose activation of the plasma membrane ATPase. The plasma membrane ATPase is believed to provide the energy for the transport of divalent metals and some amino acids into the cytoplasm (3, 30, 39). Either possibility could explain the observed defect in tryptophan utilization as well as the decreased cobalt uptake. It is interesting that cells that carry mutant alleles of genes that alter amino acid transport or plasma membrane ATPase activity also exhibit elongate cellular morphologies (10, 39).

Since cobalt uptake in cells grown on a nonfermentable carbon source by strains carrying *cot2* alleles was no different from that of wild-type strains, we suggest that the glucose dependency of the transporter has been impaired in the *cot2* mutants. The fact that *COT2* and *GRR1* are allelic underscores the involvement of this protein in glucose utilization yet gives few clues to which role in glucose utilization it performs. It is possible, for example, that the *COT2* product carries out some function required for the metabolism of glucose and other sugars. Since cobalt transport that is dependent upon other sugars is also affected in the *cot2* strains, we do not feel that divalent cation-glucose cotransport or glucose transport alone is responsible for the phenotypes of these mutants.

A clear cause-and-effect relationship does not exist between the glucose repression and metal transport defects of the *grr1/cot2* mutants. It is not likely that the transport defects are a result of defective glucose repression, since other glucose repression mutants such as *ssn6*, *tup1*, and *reg1* mutants do not exhibit increased cobalt tolerance. Nor is it likely that defective metal transport is responsible for the constitutive derepression exhibited by *grr1/cot2* mutants. If this were the case, it would be expected that metals

are somehow necessary for maintaining the repressed state. Increasing the concentration of divalent metals in growth media containing derepressing carbon sources, however, has the same effect on growth as it does in media containing repressing carbon sources (unpublished data). Thus, excess divalent cations are not sufficient to maintain the repressed state in yeast cells. It seems that both the glucose repression and metal transport defects are probably due to a more basic defect in the utilization of glucose or other fermentable carbon sources.

The wiener- and germ tube-like morphologies of strains carrying mutant *COT2* alleles presumably result from increased growth at the bud tip. Although morphogenesis and the establishment of cell polarity in microorganisms have been linked to ion transport (13), such a relationship could not be demonstrated in the *cot2* mutants. Directed growth is dependent upon the product of the *CDC24* gene, which encodes a Ca^{2+} -binding protein (24). Mutant alleles of this gene and of another gene involved in budding, *CDC43*, display Ca^{2+} -related phenotypes (24, 26), which suggests that Ca^{2+} ions are important for yeast morphogenesis (22). Although *cot2* mutants have defects in the metabolism of other divalent cations, Ca^{2+} metabolism appears to be normal. There was no effect of increased extracellular Ca^{2+} concentration on the morphologies of the *cot2* mutants, and no Ca^{2+} transport defects were observed. This suggests that the ion transport defects and the aberrant morphologies are independent manifestations of the loss of *COT2* function. It is possible that large increases in the concentration of Ca^{2+} ions in growth media may result in rather minor increases in the free ion concentration at the cell surface. It is also possible that a *COT2*-dependent Ca^{2+} flux related to budding occurs at a particular point in the cell cycle and represents only a small portion of total Ca^{2+} uptake in an unsynchronized population of cells. The most likely explanation is that, as in the cases of the glucose repression and metal transport defects, the morphological defect is caused by a basic physiological defect that is not yet understood.

ACKNOWLEDGMENTS

We thank J. Flick and M. Johnston for communicating results prior to publication, M. Goebel for pointing out the similarities to *GRR1* and the existence of weak internal repeats, and L. Riles for providing the ordered set of yeast cosmids and information regarding the position of the *COT2* clone.

This research was supported by Public Health Service grant GM37925 (to C.K. and M.R.C.), Public Health Service training grant GM07215 (to D.S.C.), and a grant from the Lucille P. Markey Charitable Trust.

REFERENCES

- Bailey, R. B., and A. Woodward. 1984. Isolation and characterization of a pleiotropic glucose repression resistant mutant of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **193**:507-512.
- Bennetzen, J. L., and B. D. Hall. 1982. Codon selection in yeast. *J. Biol. Chem.* **257**:3026-3031.
- Borst-Pauwels, G. W. F. H. 1981. Ion transport in yeast. *Biochim. Biophys. Acta* **650**:88-127.
- Chu, G., D. Vollrath, and R. W. Davis. 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science* **234**:1582-1585.
- Conklin, D. S., J. A. McMaster, M. R. Culbertson, and C. Kung. 1992. *COT1*, a gene involved in cobalt accumulation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **12**:3678-3688.
- Devereux, J., P. Haeblerli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387-395.
- Flick, J. S., and M. Johnston. 1991. *GRR1* of *Saccharomyces cerevisiae* is required for glucose repression and encodes a protein with leucine-rich repeats. *Mol. Cell. Biol.* **11**:5101-5112.
- Fresco, L. D., D. S. Harper, and J. D. Keene. 1991. Leucine periodicity of U2 small nuclear ribonucleoprotein particle (snRNP) A' protein is implicated in snRNP assembly via protein-protein interactions. *Mol. Cell. Biol.* **11**:1578-1589.
- Fuhrmann, G., and A. Rothstein. 1968. The transport of Zn^{2+} , Co^{2+} and Ni^{2+} into yeast cells. *Biochim. Biophys. Acta* **163**:325-330.
- Gimeno, C. J., P. O. Ljungdahl, C. A. Styles, and G. R. Fink. 1992. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. *Cell* **68**:1077-1090.
- Grenson, M., M. Mousset, J. M. Wiame, and J. Bechet. 1966. Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. I. Evidence for a specific arginine transporting system. *Biochim. Biophys. Acta* **127**:325.
- Hanahan, D. 1983. Studies on transformation of *E. coli* with plasmids. *J. Mol. Biol.* **166**:557.
- Harold, F. M. 1990. To shape a cell: an inquiry into the causes of morphogenesis of microorganisms. *Microbiol. Rev.* **54**:381-431.
- Hay, R. W. 1984. Bio-inorganic chemistry. John Wiley & Sons, New York.
- Hill, J. E., A. M. Meyers, T. J. Koerner, and A. Tsagoloff. 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* **2**:163-167.
- Hoffman, C. S., and F. Winston. 1987. A ten minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**:267-272.
- Hughes, M. N. 1981. The inorganic chemistry of biological processes. John Wiley & Sons, New York.
- Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**:163-168.
- Jeyaprasath, A., J. W. Welch, and S. Fogel. 1991. Multicopy *CUP1* plasmids enhance cadmium and copper resistance levels in yeast. *Mol. Gen. Genet.* **225**:363-368.
- Kamizono, A., M. Nishizawa, Y. Teranishi, K. Murata, and A. Kimura. 1989. Identification of a gene conferring resistance to zinc and cadmium ions in the yeast *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **219**:161-167.
- Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**:105-132.
- Madden, K., C. Costigan, and M. Snyder. 1992. Cell polarity and morphogenesis in *Saccharomyces cerevisiae*. *Trends Cell Biol.* **2**:22-29.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Miyamoto, S., Y. Ohya, Y. Ohsumi, and Y. Anraku. 1987. Nucleotide sequence of the *CLS4* (*CDC24*) gene of *Saccharomyces cerevisiae*. *Gene* **54**:125-132.
- Norris, P. R., and D. P. Kelly. 1977. Accumulation of cadmium and cobalt by *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **99**:317-324.
- Ohya, Y., Y. Ohsumi, and Y. Anraku. 1984. Genetic study of the role of calcium ions in the cell division cycle of *Saccharomyces cerevisiae*: a calcium-dependent mutant and its trifluoperazine-dependent pseudorevertants. *Mol. Gen. Genet.* **193**:389-394.
- Olson, M. V., J. E. Dutchik, M. Y. Graham, G. M. Brodeur, C. Helms, M. Frank, M. MacCollin, R. Scheinman, and T. Frank. 1986. Random-clone strategy for genomic restriction mapping in yeast. *Proc. Natl. Acad. Sci. USA* **83**:7826-7830.
- Ponka, P., H. M. Schulman, and R. C. Woodworth (ed.). 1990. Iron transport and storage. CRC Press, Boca Raton, Fla.
- Pringle, J. R. 1991. Staining of budscars and other cell wall chitin with calcofluor. *Methods Enzymol.* **194**:732-735.
- Roomans, G. M., A. P. R. Theuvsen, T. P. R. Van den Berg, and G. W. F. H. Borst-Pauwels. 1979. Kinetics of Ca^{2+} and Sr^{2+} uptake by yeast, effects of pH, cations and phosphate. *Biochim. Biophys. Acta* **551**:187-196.

31. Roon, R. J., G. M. Meyer, and F. S. Larimore. 1977. Evidence for a common component in kinetically distinct systems of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **158**:185.
32. Rose, M. D., P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink. 1987. A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene* **60**:237–243.
33. Rothstein, R. J. 1983. One-step gene disruption in yeast. *Methods Enzymol.* **101**:202–211.
34. Rytka, J. 1975. Positive selection of general amino acid permease mutants in *Saccharomyces cerevisiae*. *J. Bacteriol.* **121**:562–570.
35. Sandbaken, M. G., and M. R. Culbertson. 1988. Mutations in elongation factor EF1- α affect the frequency of frameshifting and amino acid misincorporation in *Saccharomyces cerevisiae*. *Genetics* **120**:923–934.
36. Shannon, R. D., and C. T. Prewitt. 1970. Revised values of effective ionic radii. *Acta Crystallogr.* **B26**:1046–1048.
37. Sherman, F. Getting started with yeast. 1991. *Methods Enzymol.* **194**:3–21.
38. Sherman, F., G. R. Fink, and C. W. Lawrence. 1979. *Methods in yeast genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. Vallejo, C. G., and R. Serrano. 1989. Physiology of mutants with reduced expression of plasma membrane H⁺-ATPase. *Yeast* **5**:307–319.