The Multidrug Resistance-associated Protein (MRP) Subfamily (Yrs1/Yor1) of Saccharomyces cerevisiae Is Important for the Tolerance to a Broad Range of Organic Anions*

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The ATP-binding cassette (ABC) superfamily of transporter proteins is involved in the tolerance of yeasts to a wide diversity of cytotoxic agents. Multiple drug resistance (MDR) proteins (P-glycoprotein) from mammals (1, 2) decrease the toxicity of a variety of anti-tumorogenic drugs. Similarly, the ABC transporters of the yeast Saccharomyces cerevisiae, such as Yor1/Ydr1 and Snq2, are involved in pleiotropic drug resistance (3–6). Members of the ABC protein superfamily are characterized by the presence of a highly conserved nucleotide binding domain that is associated with a more variable region capable of spanning the membrane multiple times. This superfamily was divided into two major subfamilies, according to the alignment analysis of amino acid residues that comprise the nucleotide-binding fold (NBF) (7). One subgroup consists of human MRP (multidrug resistance-associated protein) (7), the CFTRs (cystic fibrosis transmembrane conductance regulator) (8), and Leishmania IgA (9). The other consists of human MDR, the bacterial exporters HlyB and LktB (10, 11), and the yeast mating factor exporter Ste6 (12). Recently, a S. cerevisiae gene encoding the cadmium factor Ycf1, which shares a strong sequence similarity with MRP and CFTR, was identified (13).

Yor1/Ydr1 and Snq2, are involved in pleiotropic drug resistance (MDR) in wild-type S. cerevisiae and have been divided into two subfamilies, according to the alignment analysis of amino acid residues that comprise the nucleotide-binding fold (NBF) (7). One subgroup consists of human MRP (multidrug resistance-associated protein) (7), the CFTRs (cystic fibrosis transmembrane conductance regulator) (8), and Leishmania IgA (9). The other consists of human MDR, the bacterial exporters HlyB and LktB (10, 11), and the yeast mating factor exporter Ste6 (12). Recently, a S. cerevisiae gene encoding the cadmium factor Ycf1, which shares a strong sequence similarity with MRP and CFTR, was identified (13).

Roveromycin A is an anionic drug that inhibits the cell division cycle of mammalian cells arresting at G1 phase (14). In the present study, we identified a novel yeast gene YRS1 that complements the phenotype of a reveromycin A-sensitive mutation (yrs1-1). This gene encodes an ABC transporter required for tolerance to various organic anions including reveromycin A but not to none of other type of the drugs examined. Based upon the primary structural features of NBF, Yrs1 is classified as a member of a subfamily of ABC transporter proteins typified by human MRP.

MATERIALS AND METHODS
Yeast Strains and Growth Media

The isogenic S. cerevisiae strains WHU-2d (MAT a trpl1au2 HIS3 ura3 ade2 can1-100) and WHU-2a (MAT a trpl1au2 his3 URA3 ade2 can1-100, and MLC26 (Mat a trpl1au2 HIS3 ura3 ade2 can1-100 yrs1-1:HIS3) derivatives of W303 were used in this study. Yeast strains were grown on YPD medium (5) at 28°C. Roveromycin A-containing solid YPD medium was prepared by adding an appropriate amount of roveromycin A from a stock solution of the drug (1 mg/ml in water) to autoclaved YPD-agar medium that was adjusted at pH 4.5 using 1 N HCl before autoclaving.

Sensitivity Test

The sensitivity of yeast strains to various drugs, metallic ions, and other compounds was assessed in two ways.

Solid Medium Assay—Yeast cells suspended in water (2 × 10⁷ cells/ml) were applied using a cell applicator (about 4 × 10⁶ cells/salt) on YPD solid medium (adjusted to pH 4.5 or unadjusted, as indicated) containing various concentrations of drugs or other compounds. The plates were incubated at 28°C for 3 days.

Liquid Culture Assay—Cells were inoculated to fresh YPD liquid medium (pH 4.5) containing various concentrations of reveromycin A at a cell concentration of 5 × 10⁵ cells/ml, grown at 28°C for 20 h with shaking, and their A₆₅₀ values were measured.

Isolation of Reveromycin A-sensitive Mutants

Wild type strain WHU-2d was treated with ethylmethanesulfonate as described by Sherman et al. (15). Mutagenized cells were plated on YPD plate (200 colonies/plate), and reveromycin A-sensitive mutants were screened by replica plating onto a YPD plate containing 1 μg/ml reveromycin A.

Gene Cloning and Sequence Analysis

The reveromycin A hypersensitive mutant yrs1-1 was transformed with a Ycp50-based yeast genomic library constructed with DNA from the W303-1A strain. Ura⁻ transformants were replica plated onto a YPD plate containing 1 μg/ml reveromycin A. After 3 days of incubation at 28°C, transformants that could grow on the drug plate were isolated.
with the wild type strain WHU-2a. The resulting diploids in each case showed a level of reveromycin A sensitivity similar to that of the wild type diploid strain, suggesting that they were all recessive mutations. Phenotypic complementation analysis among the mutant strains indicated that all of these mutations belong to a single complementation group, which was designated yrs1 (for yeast mutant with reveromycin A sensitivity).

Cloning the Gene by Complementation—Gene cloning was performed by complementation of reveromycin A sensitivity of a yrs1 mutant (yrs1-1), using a YEp50 plasmid bank of the yeast genome. The yrs1 mutant transformed with the bank was selected on the plate containing 1 μg/ml reveromycin A. Five plasmids that complemented the yrs1 mutation were identified. These clones contained a common sequence as indicated by restriction and Southern blot analyses (data not shown).

DNA Sequence Analysis Reveals That YRS1 Encodes an ABC Superfamily Transporter Protein—A total of 6,040 nucleotides that extend from the BstEII site to 466 base pairs downstream from the EcoRI site was sequenced. Within this region, a long open reading frame (4,431 nucleotides) that is predicted to encode a protein of 1,477 amino acids was identified (data not shown). A homology search of the deduced amino acid sequence of this open reading frame revealed significant similarities to several transport proteins that belong to the ABC superfamily. The highest similarities were to human MRPs (7) (30.7% identity in a 1,421-residue overlap) and recently reported yeast Ycf1, required for cadmium resistance (13) (31.1% identity in a 1,259-residue overlap). The similarity of Yrs1 to MDRs of human (MDR1) and yeast (Pdr5) were less extensive (23.1 and 15.5% in a 550- and 297-residue overlap, respectively). Homology analysis of Yrs1 with MRPs and Ycf1 showed an extensive similarity in their carboxyl-terminal halves (Fig. 1A). The hydropathy profile of Yrs1 predicted the presence of two putative transmembrane regions (TM1 and TM2), each consisting of several potential transmembrane domains and two NBFs (NBF1 and NBF2), disposed in the order of TM1, NBF1, TM2, and NBF2 from the amino terminus (Fig. 1B). The overall similarity of NBF1 (residues 604–752) of Yrs1 with those of MRPs, HCFTR, Ycf1, and MDR1 was 48.3, 38.7, 44.3, and 28.9%, respectively. The ABC superfamily proteins have been classified into two major subgroups (7). One consists of the cluster that contains MRPs (7), CFTRs (8), Leishmania Pgp1 (9), and Ycf1 (13). The other group contains MDR1 and the yeast mating factor exporter (Ste6). These results demonstrated that YRS1 encodes a member of the subfamily of ABC transporter proteins typified by MRPs and Ycf1. When these analyses were completed, we found that the amino acid sequences of Yrs1 were completely identical to those of Yor1 required for the resistance to oligomycin (20).

The Yeast YRS1 Gene Is Not Essential for Viability but Is Required for the Resistance to Reveromycin A and Various Organic Anions—To investigate the physiological roles of the Yrs1 transporter, we constructed a strain carrying a chromosomal deletion of this gene. The 3,958-base pair XbaI-BamHI fragment within the open reading frame was replaced with the 1,8-kb XbaI-BamHI fragment containing the HIS3 gene. The chimeric gene was excised by MluI and introduced to an isogenic diploid strain W303. Tetrad analysis of 13 tetrads derived from the YRS1/Δyrs1::HIS3 heterozygous diploid were carried out upon sporulation. The resulting Δyrs1 strain was viable and grew at a slightly slower rate compared with the YRS1 strain in each of the tetrad, demonstrating that YRS1 is not essential for growth but may be required to support normal growth. The Δyrs1 cells exhibited hypersensitivity to reveromycin A, indicating that the endogenous YRS1 gene is an important determinant of growth in the presence of the drug (Fig. 2A).
Yeast ABC Transporter Important for Tolerance to Organic Anions

To learn about drug specificity of the YRS1 gene, we compared the sensitivity of Δyrs1 strain to various drugs with those of null mutants (Δpdr5 and Δsnq2) of the previously characterized ABC-type multidrug resistance genes (Fig. 2A). The Δyrs1 strain exhibited increased sensitivity to reveromycin A but not to cycloheximide (0.1 μg/ml), fluphenazine (30 μg/ml), and cerulenin (0.3 μg/ml), to which the Δpdr5 strain exhibited hypersensitivity (5). Further, the Δyrs1 strain was not hypersensitive to 4-nitroquinoline (0.1 μg/ml) to which the Δsnq2 strain exhibited hypersensitivity (6). Moreover, the sensitivities of Δpdr5 and Δsnq2 strains to reveromycin A were similar to that of wild type strain. When present in multicopies, YRS1 caused an elevation in the resistance to reveromycin A but not to cycloheximide, fluphenazine, and 4-nitroquinoline (data not shown). These results demonstrated that the drug specificity of Yrs1 was clearly distinct from those of the previously characterized multidrug resistance transport proteins of yeast.

In addition to reveromycin A, the Δyrs1 strain exhibited increased sensitivity to the drugs containing carboxyl group(s), which included tautomycin (21) and leptomycin B (22) (Fig. 2B). The maleic anhydride group of tautomycin A should be femaleic acid at the pH values of regular medium. In fact, the activity as determined by the growth-inhibiting activity of yeast was 20 times more potent at pH 4.5 than at pH 6.4. Surprisingly, in addition to these drugs, Δyrs1 disruptant exhibited increased sensitivity to the carboxylic acids such as acetic, propionic, and benzoic acids (Fig. 2B). These results suggested that YRS1 was required for the resistance to a wide range of the compounds with carboxyl group(s). An increase in the sensitivity of Δyrs1 cells to tautomycin was also observed at pH 6.4 (using a 20-fold higher drug concentrations than that at pH 4.5), indicating that the low pH of the medium is not important for Yrs1 function (Fig. 2B). Consistent with the phenotypes of the disruption mutant, a high dosage of YRS1 conferred increased resistance to reveromycin A, tautomycin, and leptomycin B (Fig. 2B). However, no significant increase in the resistance levels of the transformant to acetic acid, propionic acid, and benzoic acid was observed. The reason for this is unknown.

Yrs1 Shares Specificity with Ycf1 in the Resistance to Cadmium—Since the primary structure of Yrs1 was clearly related to Ycf1 which is required for cadmium resistance, we tested the possibility that Yrs1 is functionally redundant with Ycf1 by examining cadmium sensitivity of the Δyrs1 strain. The Δyrs1 strain was more sensitive to cadmium than wild type strain (Fig. 2B). Further, overexpression of YRS1 resulted in increased resistance to cadmium (Fig. 2B). The YRS1 gene was not responsible for the resistance to other metals examined, which included sodium, lithium, potassium, calcium, manganese, copper, and zinc (data not shown). These results indicated that Ycf1 and Yrs1 have an overlapping substrate specificity in eliminating the toxicity of cadmium. The drug specificity of YCF1 still remains to be established.

The Defect of YRS1 Results in Increased Cellular Content of the Anionic Drug—To evaluate the effect of YRS1 disruption on the cellular content of externally added organic anion, we
used an anionic fluorescent compound rhodamine B as probe. The growth of yeast was not severely affected by rhodamine B (100 μg/ml) during 24 h of cultivation in SD medium (pH 4.5). Under these conditions, the Δyrs1 strain grew at a slightly slower rate than wild type strain, indicating that the cellular content of rhodamine B is regulated in a manner similar to that of other toxic anionic compounds (data not shown). The cells (Δyrs1 and wild type strains) were cultivated in SD medium (pH 4.5) containing 100 μg/ml rhodamine B for 20 h, and the cellular content of the fluorescent compound was measured by flow cytometry (Fig. 3). The intensities of endogenous fluorescence were similar in both strains. The incubation of the Δyrs1 cells with rhodamine B resulted in a significant increase in fluorescence intensity. An 80% increase in mean fluorescence intensity was observed with Δyrs1 cells, whereas only a 21% increase was observed with wild type cells. The cell size was not significantly influenced by the presence of rhodamine B (data not shown). These results suggested that Yrs1 is responsible for the decrease of the cellular content of organic anions, rather than for the sequestration of the drugs to organelles.

The Expression Levels of the YRS1 Gene Respond to Reveromycin A—We investigated if the expression of YRS1 can be induced by reveromycin A. The cells of wild type and the transformant with the high copy plasmid YEpyRS1 were cultivated in the presence of a sublethal concentration (1 μg/ml) of reveromycin A, and the mRNA levels after various periods of incubation were measured by Northern blot analysis (Fig. 4). The expression level of YRS1 gene in wild type cells was normally very low and induced strikingly by reveromycin A. The basal YRS1 mRNA level of the cells containing the YEpyRS1 plasmid was higher than that of wild type cells, and the mRNA level further increased during incubation with reveromycin A (Fig. 4).

**DISCUSSION**

Yeast gene YRS1, which encodes an ABC superfamily protein implicated in the alleviation of the deleterious effect of reveromycin A, was cloned and characterized. Alignment analysis of the amino acid residues that comprise the NBFs of the ABC transporter proteins indicated that Yrs1 is a member of the subfamily of MRP, CFTR, and Ycf1, as contrasted with the subfamily of MDR1 and Ste6. The Yrs1 transporter was required for the resistance to a range of the toxic xenobiotics containing a carboxyl group(s). Thus, the Yrs1-mediated resistance mechanism seems important in alleviating the deleterious effect of various toxic organic anions.

In addition to these anions, Yrs1 was specifically required, among various toxic metallic ions tested, for the resistance to cadmium, indicating the functional redundancy of Yrs1 and Ycf1 in cadmium detoxification (13). It is tempting to speculate that cadmium is detoxified by Yd1 and Yrs1 as an anionic conjugate to the endogenous compound, such as glutathione. Cellular glutathione has been suggested to be important against cadmium toxicity in animals, plants, and the yeast *Schizosaccharomyces pombe* (23). YOR1/YRS1 was recently isolated as the gene that confers oligomycin resistance when present in multicopies (20). However, to our surprise, oligomycin does not contain anionic groups in its structure. The involvement of YRS1 in the tolerance to oligomycin was confirmed (data not shown). Oligomycin might be modified in vivo to generate a carboxyl group (e.g. by the hydrolysis of an ester bond in the macroride ring) before reaching to the target site located in the mitochondria. Alternatively, oligomycin might be pumped out as a conjugate to an anionic compound such as glutathione.

It has been demonstrated that animals eliminate various exogenous and endogenous substances as anionic conjugates of glutathione using ATP and membrane potential-dependent transport mechanism (24, 25). Similarly, in an in vitro system of *S. cerevisiae*, it was recently demonstrated that the yeast secretory vesicles contain ATP-dependent transport activities that result in intravesicular accumulation of the anionic compounds such as bile acids and glutathione conjugates (26). Whether the transport of organic anions observed with the yeast vesicles involves the Yrs1 (and possibly Ycf1) transport proteins still remains to be clarified.

Several explanations may be possible for the mechanism by which Yrs1 functions to alleviate the toxic effect of various organic anions. First, Yrs1 may participate directly in the active transport of toxic organic anions through the plasma membranes or organelar membranes as a multispecific organic anion transporter, to export out or compartmentalize the toxic molecules. Alternatively, Yrs1 may participate in resistance by influencing the uptake or subcellular compartmentalization of organic anions indirectly by transporting certain ions.
that affect intracellular pH. If Yrs1 affects intracellular pH, its disruption and overexpression could alter cytoplasmic or intracellular pH. This may result in altered uptake or sequestration of toxic organic anions. These different possibilities cannot be experimentally distinguished at present. To understand how Yrs1 functions in drug detoxification, total cellular drug accumulation and its subcellular localization by YRS1 disruption and overexpression need to be established. Our results suggested that the defect of Yrs1 results in the increased level of cellular drug concentration. Further analyses will be required to establish the Yrs1 intracellular localization and the precise biochemical mechanism whereby Yrs1 functions in drug resistance.

The expression of YRS1 was induced dramatically by the drugs that are relevant to the resistance mediated by the Yrs1 protein. Further, a high gene dosage of YRS1 resulted in increased mRNA level, conferring resistance to the anionic drugs. Thus, the Yrs1-mediated resistance seems an important protection mechanism to decrease the cytoplasmic concentrations of a range of anionic toxic environmental molecules. The Yrs1-mediated resistance mechanism would provide an interesting model system for the study of the mammalian MRP system of cancer cells.

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