Note

Identification and Characterization of a Gene Encoding an ABC Transporter Expressed in theDicarboxylic Acid-Producing Yeast Candida maltosa

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A gene, CmCDR1, encoding an ABC transporter of the dicarboxylic acid (DCA)-producing yeast Candida maltosa was cloned. Transcription of CmCDR1 was upregulated in a DCA-hyper-producing mutant of C. maltosa in a later phase of culture on n-dodecanec, but not in its parental strain. CmCDR1 expression was significantly induced by the longer-chained DCA in this mutant.

Key words: Candida maltosa; n-alkane; dicarboxylic acid; ABC transporter

Long-chain dicarboxylic acids (DCAs) are valuable materials in the synthesis of various chemicals, including polymers and perfumes, in many industrial fields. Chemical synthesis of long-chain DCAs of certain carbon numbers is an established technique, but for DCAs of other carbon numbers, fermentative production from alkanes by microbes is a promising method. The n-alkane-assimilating yeast Candida maltosa strain 1098 has been isolated as a microbe that produces dodecanedioic acid from n-dodecane.1,2 It produced 1.4 g/L of brassylic acid, a DCA of 13 carbons, from n-tridecane, when cultivated in a flask. By repeated mutagenesis, a mutant strain, C. maltosa M2030, that produced 125 g/L of brassylic acid in the media has been established.1,2 In n-alkane-assimilating yeasts, n-alkanes are hydroxylated to fatty alcohols in the endoplasmic reticulum (ER) membrane by cytochromes P450ALK belonging to the CYP52 family.3–10 Fatty alcohols are sequentially oxidized to fatty acids, which are used in the synthesis of membrane or storage lipids or are degraded by β-oxidation in the peroxisome. n-Alkanes and/or fatty acids are also ω-hydroxylated by certain P450ALKs with ω-hydroxylation activities and converted to DCAs.1,11 To improve the productivity of DCAs from n-alkanes, it is necessary to reduce fatty acid consumption through β-oxidation. Indeed, DCA-hyper-producing mutant M2030 exhibited reduced protein levels of acyl-CoA oxidase and 3-ketoacyl-CoA thiolase, which are involved in the β-oxidation of fatty acids, as compared to the parental strain.1,12 In addition, proliferation of peroxisomes, where fatty acid β-oxidation occurs, was scarcely observed in the M2030 strain cultured on n-alkane.

For higher productivity of DCA, it is assumed that the intracellular DCA concentration must be kept at a low level, since DCA accumulation can be toxic to cells and since DCA production can be decreased by product inhibition or feedback regulation caused by accumulated DCA. Thus it is possible that a DCA-hyper-producing mutant can excrete DCA efficiently into the culture medium. In addition, further enhancement of the DCA excretion ability by genetic engineering might lead to improvements in DCA productivity. ABC transporters are widely conserved membrane proteins containing two highly conserved nucleotide-binding domains (NBDs). They function in the efflux of a wide variety of hydrophobic compounds in various organisms, including yeasts.13,14 In the present study, we isolated a gene encoding an ABC transporter of C. maltosa and analyzed its expression in a DCA-hyper-producing mutant.

In the NBDs of ABC transporters, the Walker A, Walker B, and ABC signature motifs, which are important for the hydrolysis of ATP, are well conserved.15 From the amino acid sequences of these motifs and the surrounding regions of yeast ABC transporters, Cdr1p to Cdr4p of Candida albicans,16–19 Snq2p, Pdr5p, and Pdr12p of Saccharomyces cerevisiae,20–22 we designed degenerate primers 5'-TGYTCNACHHTT-NITTRAARAC-3' and 5'-CKYTTWCKYTCCHCCCDGA-3', taking the codon usage of C. maltosa into consideration. We amplified DNA fragments with these primers by PCR from the total DNA of wild-type C. maltosa strain IAM12247. We obtained two 353-bp DNA fragments, 5-1 and 5-2, the deduced amino acid sequences of which exhibited 88% identity to that of C. albicans Cdr1p and 81% identity to that of C. albicans Cdr4p respectively. With DNA fragment 5-1 as probe, a DNA fragment was obtained from a genomic DNA library of the C. maltosa IAM12247 strain by colony hybridization by means of the ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare). The nucleotide sequence of this clone contains part of the coding region of a protein highly homologous to C. albicans Cdr1p and the 5'-non-coding region. By a second round of colony hybridization with the probe DNA amplified from the DNA clone obtained with primers 5'-GTACCATCATCCTCCACTCC-3' and
5'-GAGATGAGATGTAAACAACCTG-3', a DNA fragment was obtained that contained the rest of the coding region and the 3'-non-coding region. We determined the 6,133-bp nucleotide sequence of both fragments, and found an open reading frame (ORF) of 4,503 bp that encoded a 1,500 amino acid protein, which exhibited 83%, 84%, and 83% identity to the Cdr1ps of *C. albicans*, *Candida dubliniensis*, and *Candida tropicalis* respectively (DDBJ accession no. AB830574). Cdr1p of *C. albicans* is involved in cycloheximide resistance.23) The amino acid sequences of Walker A motifs, Q-loops, ABC signature motifs, Walker B motifs, and H-loops in the two putative NBDs are completely conserved.13,14) We named this gene *CmCDR1*.

Next we analyzed the expression of *CmCDR1* in the mutant M2030 and parental strain 1098. Strains M2030 and 1098 precultured in SD medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate (Becton Dickinson), 0.5% ammonium sulfate, nitrogen base without amino acids and ammonium and 10% precultured in SD medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate (Becton Dickinson), 0.5% ammonium sulfate, nitrogen base without amino acids and ammonium and 10% precultured in SD medium or in those at the early phase of culture condition, transcripts of *CmCDR1* were detected in the cells precultured in SD medium or in those at the early phase of culture condition, but not in strain 1098. However, the expression of *CmCDR1* was much higher in strain M2030 at the later phase of culture, but not in strain 1098.

Next we analyzed whether transcription of *CmCDR1* would be upregulated by DCA added to the culture medium (Fig. 2). Strains M2030 and 1098 were cultured in SD medium in the presence of DCAs of 10 to 20 carbons at concentrations of 1% for 2 h. In this culture condition, transcripts of *CmCDR1* were detected in both strains, and their levels were highly increased in strain M2030 in the presence of longer carbon chain DCAs. More *CmCDR1* transcripts were detected in strain M2030 as the length of the DCA increased from C14 to C18. These results imply that transcription of *CmCDR1* was activated particularly in strain M2030 by the DCAs in the culture medium, although it is not clear whether the DCAs were sensed inside or outside the cells.

In *C. albicans*, transcription of *CDR1* and *CDR2*, which encode ABC transporters involved in drug resistance, is induced by the addition of drugs.24,25) We analyzed to determine whether transcription of *CmCDR1* would be upregulated by the addition of drugs (Fig. 3A). Strains M2030 and 1098 were cultured in SD medium containing miconazole, cycloheximide, 4-nitroquinoline 1-oxide, or ketoconazole for 1 h. Transcripts of *CmCDR1* were significantly induced by cyclohex-
imide in both strains. In *C. maltosa*, transcription of a gene encoding an L41 ribosomal protein responsible for the inducible cycloheximide-resistant phenotype is activated by the addition of cycloheximide, and C-Gcn4p, a *C. maltosa* ortholog of transcription activator Gcn4p of *S. cerevisiae*, is involved in this activation,\(^2\)\(^6\),\(^2\)\(^7\) but the transcription activation of CmCDR1 by cycloheximide was observed in the deletion mutant of C-GCN4 of a *C. maltosa* strain of a different genetic background (data not shown), suggesting that the transcription of CmCDR1 is activated by a distinct mechanism in response to cycloheximide. Strain M2030 was more resistant to cycloheximide than 1098 under these culture conditions (Fig. 1), these results raise the possibility that CmCdr1p, the expression of which is induced by DCA produced in the n-dodecane-containing medium, confers cycloheximide resistance on strain M2030.

The results of this study suggest that CmCDR1 encodes an ABC transporter that is involved in the excretion of DCA and cycloheximide. We analyzed CmCDR1 from wild-type *C. maltosa* strain IAM12247, but the nucleotide sequences of CmCDR1 of strains M2030 and 1098 remain to be determined. It would be of interest to isolate and characterize CmCDR1 from strains M2030 and 1098, since strain M2030 might have obtained mutations in the promoter of CmCDR1 that increase its transcription or mutations in the ORF that potentiate the excretion of DCAs. In addition, our results suggest the possibility that the production of DCAs can be improved by overexpression of the genes encoding the transporters involved in the excretion of DCAs in *C. maltosa* and other DCA-producing yeasts.

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**References**