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New insights into the pyrimidine salvage pathway of *Saccharomyces cerevisiae*: requirement of six genes for cytidine metabolism

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Abstract Cytidine metabolism in the yeast *Saccharomyces cerevisiae* was analyzed by genetic and biochemical approaches. Disruption of a unique ORF (Genbank accession No. U 20865) bearing homology with eucaryotic or bacterial cytidine deaminases abolished cytidine deaminase activity and resulted in 5-fluorocytidine resistance. The gene encoding cytidine deaminase will be referred to as *CDD1* (Genbank accession number AF080089). The ability to isolate mutants resistant to 5-fluorocytidine which mapped to five other loci demonstrated the existence of a complex cytidine metabolic network. Deciphering this network revealed several original features:

(1) cytidine entry is mediated by the purine-cytosine transporter (Fcy2p),
(2) cytidine is cleaved into cytosine by the uridine nucleosidase (Urh1p),
(3) cytidine is phosphorylated into CMP by the uridine kinase (Urk1p),
(4) a block in cytosine deaminase (Fcy1p), but not in cytidine deaminase (Cdd1p), constitutes a limiting step in cytidine utilisation as a UMP precursor.

Key words Pyrimidine salvage pathway · Cytidine deaminase · Cytidine metabolism · *CDD1* · Uridine/cytidine kinase

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Introduction

The pyrimidine salvage pathway in *Saccharomyces cerevisiae* is well documented for pyrimidine bases (Grenson 1969; Jund and Lacroute 1970). Specific transporters mediate active transport of exogenously supplied pyrimidines across the cell membrane to restore the growth of strains with a deficiency in the pyrimidine de novo biosynthetic pathway (Jund et al. 1977). The purine/cytosine transporter encoded by *FCY2* is responsible for the intracellular entry of cytosine (Chevallier et al. 1975) and 5-fluorocytosine (5-FC), whereas uracil and 5-fluorouracil (5-FU) enter the cell by the cognate permease encoded by *FUR4* (Jund et al. 1988). Cytosine deaminase (Fcy1p, EC 3.5.4.1), the product of *FCY1*, deaminates cytosine to uracil (Erbs et al. 1996). Uracil phosphoribosyltransferase (Fur1p, EC 2.4.2.9), encoded by *FUR1* which controls the salvage pathway linked to the regulation of de novo pyrimidine biosynthesis, catalyses the conversion of uracil and 5-phosphoribosyl-1-pyrophosphate (5-PRPP) into uridine 5′ monophosphate (UMP) (Kern et al. 1991). However, little is known about cytidine utilisation in *S. cerevisiae* and there is no report to-date about the genetics of this metabolism. The aim of the present work was to further investigate the relationship between cytidine metabolism and the salvage pathway of pyrimidines. We report the selection of mutants impaired in cytidine metabolism, the disruption and characterisation of *CDD1* encoding the *S. cerevisiae* cytidine deaminase (Cdd1p, EC 3.5.4.5), as well as the phenotypic and biochemical analysis of several mutants in cytidine metabolism.

Materials and methods

Strains, plasmids, media and genetic methods. All of the *S. cerevisiae* strains used in this study were isogenic to Fl100 and are listed in Table 1. Strains bearing point mutations in *fcy2-3*, *fcy1-1*, *fur1-8* and *urk1-1* display equivalent enzymatic and phenotypic properties to the corresponding null mutations obtained by deletion in other

Table 1 *S. cerevisiae* strains

Name	Genotype	Origin
Fl100	Wild-type	F. Lacroute
RJ202-1B	*MAT* **a** *leu 2-1 ura2-9, 15, 30 trp1-4*	Lab collection
RJ200	*MAT* **a** *leu2-1 ura2-9, 15, 30 trp1-4 fcy1-1*	Lab collection
IRC100	*MAT* **a** *leu 2-1 ura2-9, 15, 30 trp1-4 cdd1::TRP1*	This work
IRC101-1B	*MAT* **a** *leu2-1 cdd1::TRP1*	This work
IRC101-1C	*MAT* **a** *leu2-1 cdd1::TRP1 fcy1-1*	This work
IRC102-1A	*MAT* **a** *leu2-1 ura2-9, 15, 30 cdd1::TRP1 fcy1-1*	This work
IRC103-4C	*MAT* **a** *leu2-1 ura2-9, 15, 30 cdd1::TRP1 fcy1-1 urk1-1*	This work
IRC103-8C	*MAT* α *leu2-1 ura2-9, 15, 30 cdd1::TRP1 fcy1-1 urh1-2 urk1-1*	This work
IRC103-9C	*MAT* α *leu2-1 ura2-9, 15, 30 cdd1::TRP1 fcy1-1 urh1-2*	This work
RJ16-1A	*MAT* **a** *urh1-2*	Lab collection
Fl442-2D	*MAT* **a** *fcy1-1*	Lab collection
Fl570-1A	*MAT* α *fur1-8*	Lab collection
Fl480-1B	*MAT* **a** *fcy2-3*	Lab collection
Fl483-1A	*MAT* **a** *fur4-7*	Lab collection
RJ32-1A	*MAT* **a** *urk1-1*	Lab collection
IRC108	*MAT* **a** *leu2-1 cdd1::TRP1* [pRS 415-CDD1]	This work

backgrounds (Kern 1990; Weber et al. 1990; Erbs et al. 1996). *Escherichia coli* DH5α was used for the transformation and propagation of plasmids. Plasmids pFL35, p414GPD and p415GPD were described by Bonneaud et al. (1991), and Mumberg et al. (1995). Complete YPG (1% yeast extract, 1% peptone, 2% glucose) or minimal YNB (0.67% yeast nitrogen base Difco, 2% glucose) media with the appropriate supplements (L-leucine 20 μg/ml, L-tryptophan 20 μg/ml, uracil 40 μg/ml) were used to support yeast growth. Yeast genetic methods were as described by Mortimer and Hawthorne (1966).

Nucleic acid manipulations and molecular cloning. Standard methods for DNA manipulations were employed (Sambrook et al. 1982). *E. coli* and yeast strains were transformed by electroporation (Dower et al. 1988; Erbs et al. 1996). Large-scale plasmid DNA was prepared using the Wizard *plus* Maxiprep purification system (Promega). Genomic DNA of *S. cerevisiae* was extracted with the Wizard genomic DNA purification kit as described by the supplier (Promega). DNA amplification by PCR was performed with a MJ Research thermo Minicycler using *Pfu* polymerase (Stratagene). The putative *CDD1* gene was amplified with primers sccddn (5′ CGGGATCCATGAAAGTAGGTGGC3′) and sccddc (5′ CGGAATTCCTAGTTTAAATGAGATGG3′) supplied by Eurogentec. Amplification conditions were as follows: 2 min denaturation at 94 °C; 10 cycles of 1 min at 92 °C, 1 min at 55 °C, 2 min 30 s at 72 °C, followed by 20 cycles of 1 min at 94 °C, 1 min at 65 °C, 2 min 30 s at 72 °C; and 10 min of final elongation at 72 °C. The PCR product was purified by electrophoresis in low-melting 1.2% agarose gel (NuSieve "GTG" FMC), and the expected 446-bp fragment was recovered.

DNA sequencing. Double-stranded DNA was sequenced by the dideoxy chain-termination method (Sanger et al. 1977) using modified T7 DNA polymerase and [α-^{35}S] dATP. DNA and protein sequences were analysed using the UWGCG programs (Devereux et al. 1984).

Disruption of the CDD1 gene. We searched the genome of *S. cerevisiae* for sequences that are homologous to conserved regions of cytosine/cytidine and CMP deaminase genes. Alongside the known cytosine and CMP deaminase sequences, we found a putative ORF (Genbank accession number U20865) bearing the selected motif (Fig. 1). A 446-bp fragment containing the putative ORF (*CDD1* gene) was amplified from genomic DNA by PCR. Restriction sites for *Bam*HI and *Eco*RI endonucleases were inserted in the sccddn and sccddc primers. The PCR product was digested with *Bam*HI and *Eco*RI enzymes and inserted into the *Bam*HI *Eco*RI sites of the integrative plasmid pFL35, bearing the *TRP1* marker gene. The distal *Nco*I site of *CDD1* was filled in and after re-ligation a *Nsi*I site was created. *Nsi*I digestion (1 unit/μg DNA for 5 min at 37 °C) of the pFL35 carrying the putative modified *CDD1* sequence was performed and the RJ202 yeast strain was transformed by electroporation to Trp$^+$ prototrophy. Trp$^+$ transformants were crossed with a wild-type strain to check the integration event of the *TRP1* gene sequence at the *CDD1* locus.

Enzyme assays. The following three assays were carried out:

(1) Cytidine deaminase (EC 3.5.4.5) assay. For a total volume of 8 μl, the standard mixture contained 4 μl of ^{14}C-2 cytidine (53.4 mCi/mmol) at a final concentration of 10 μCi/ml in 100 mM Tris-HCl pH 7.5 and 4 μl of crude extract adjusted to 5 mg/ml. After incubation at 37 °C, the cytidine deaminase assay was stopped by thermodenaturation in a water-bath for 2 min at 80 °C. After a short centrifugation, 4 μl of the reaction mixture, together with an appropriate amount of uridine, uracil and cytosine, were spotted on a PEI cellulose sheet (PEI cellulose F, Merck). Radioactive nucleosides and bases were separated by successive 1-Butanol:water (86:14) and water bi-dimensional thin-layer chromatography. After development of the chromatogram, it was dried and exposed for 12 h in an exposure cassette. Scanning the radioactive spots was achieved with a Molecular Dynamics 445 SI Phosphorimager.

(2) Uridine nucleosidase (EC 3.2.2.3) assay. A radioactive assay was developed with ^{14}C-2 uridine (62.4 mCi/mmol) and ^{14}C-2 cytidine (53.4 mCi/mmol) in Tris-HCl buffer (50 mM pH 7.5) at a final concentration of 2.5 μCi in 500 μl. Four microliters of ^{14}C-2 uridine or ^{14}C-2 cytidine were added to 4 μl of crude extract and the mixture was incubated at 37 °C. Separation of uridine and uracil was achieved on PEI cellulose (1-Butanol:water) and the radioactive spots quantified with a Molecular Dynamics 445 SI Phosphorimager.

Fig. 1 Deduced amino-acid sequence of Cdd1p showing the two consensus motifs (*in bold*) found in cytidine/deoxycytidine deaminases. The human cytidine deaminase sequence exhibits 32.8% identity with *S. cerevisiae*

S. cerevisiae	C I **C A E R S A M** I Q.......................Q C V **S P C A V C R Q F I N E F V V K** D F P
B. subtilis	C N **C A E R T A L** F K.......................G P V **S P C G A C R Q V I S E L C T K** D V I
Human	G I **C A E R T A I** Q K.......................D F I **S P C G A C R Q V M R E F G T N** W P V

(3) Uridine kinase (EC 2.7.1.48) assay. A radioactive assay was developed with ^{14}C-2 uridine (62.4 mCi/mmol) and ^{14}C-2 cytidine (53.4 mCi/mmol) as follows. For a total volume of 200 µl, the standard reaction mixture contained: 10 µl of Tris-HCl 1 M pH 7.5, 2.5 µl of NaF 1 M, and 20 µl of $MgCl_2$ 1 M. Each 10-µl assay was performed as follows: 2 µl of ATP (100 mM pH 7.0) were added to 4 µl of reaction mixture together with 4 µl of crude extract and 5 µCi of either ^{14}C-2 uridine or ^{14}C-2 cytidine. After incubation for 0, 3 and 6 min at 37 °C, the reaction was stopped by thermodenaturation (2 min in a 80 °C water bath). After a short centrifugation step, 6 µl of each supernatant were spotted onto a thin-layer PEI cellulose plate (Merck) and submitted to two-step chromatography (water over 18 cm followed by LiCl 1 M over 7 cm). After development of the chromatogram, it was dried and exposed for 12 h in an exposure cassette. Scanning the radioactive spots was achieved with a Molecular Dynamics 445 SI Phosphorimager.

Phenotypic identification of URK1. Strains lacking uridine kinase activity grow on media supplemented with uracil (5 µg/ml) and 5-fluorouridine (5-FUR) (5×10^{-3} M) as opposed to a wild-type strain for this gene. We used this test to check the presence or absence of the uridine kinase-encoding gene (*URK1*).

Radiolabelled nucleoside incorporation assay. Cells were grown for six generations in minimal medium supplemented with 400 mM of all the ^{14}C-labelled substrates. Incorporation into macromolecules was measured in the 5% trichloracetic acid (TCA) insoluble fraction after filtration through a 0.45-µm nitrocellulose membrane (Millipore). Counting was performed with a Kontron Betamatic.

Results

Selection of mutants impaired in cytidine metabolism

The 5-fluoropyrimidines 5-FU and 5-FC have been shown to be very efficient selective agents to obtain mutants specifically impaired in the pyrimidine-base salvage pathway (Grenson 1969; Jund and Lacroute 1970). Similarly, using 5-fluorocytidine (5-FCR) as a selective agent, we expected to obtain mutants in cytidine metabolism. Therefore, UV mutagenesis of a wild-type haploid strain was performed, followed by 5-fluorocytidine (10^{-3} M) selection. We obtained five classes of recessive mutants which were all allelic to either *fcy1*, *fcy2*, *fur1*, *urk1*, or *urh1* (Grenson 1969; Jund and Lacroute 1970; Kern 1990). Conversely, we found that all of these mutants which were initially selected as resistant to 5-FU (*fur1*), 5-FC (*fur1*, *fcy1*, *fcy2*) and 5-fluorouridine (*urk1*) showed a 5-fluorocytidine resistance profile. No cytidine deaminase mutant was found in any selection. These results indicated that in *S. cerevisiae* cytidine acts as a pyrimidine precursor (i.e. UMP) by multiple pathways. We proposed a model pathway (Fig. 2) in which cytidine may undergo three reactions (hatched arrows) to reach the already described salvage pathway (black arrows). The aim of this work was to validate and characterise these presumed steps.

In the model proposed, a first path may lead from cytidine to cytosine to uracil to UMP, which would require cytidine hydrolysis, an uncharacterised mechanism in *S. cerevisiae*, followed by cytosine deaminase (Fcy1p)- and uracil phosphoribosyltransferase (Fur1p)-catalysed steps. The finding that *urk1* and *urh1* mutants are resistant to 5-fluorocytidine and 5-fluorouridine led us to postulate the existence of a second pathway that necessarily involves uridine kinase (Urk1p), uridine nucleosidase (Urh1p) and presumably a cytidine deaminase (Cdd1p). Finally, as a third possibility, cytidine may be directly phosphorylated by Urk1p, leading to CMP.

Cytidine transport

Cytidine uptake was measured by classical initial velocity assays (Jund et al. 1977). In the wild-type strain, cytidine (10^{-4} M)-uptake kinetics is characterised by a low affinity constant (Km = 1.1×10^{-3} M). Cytidine

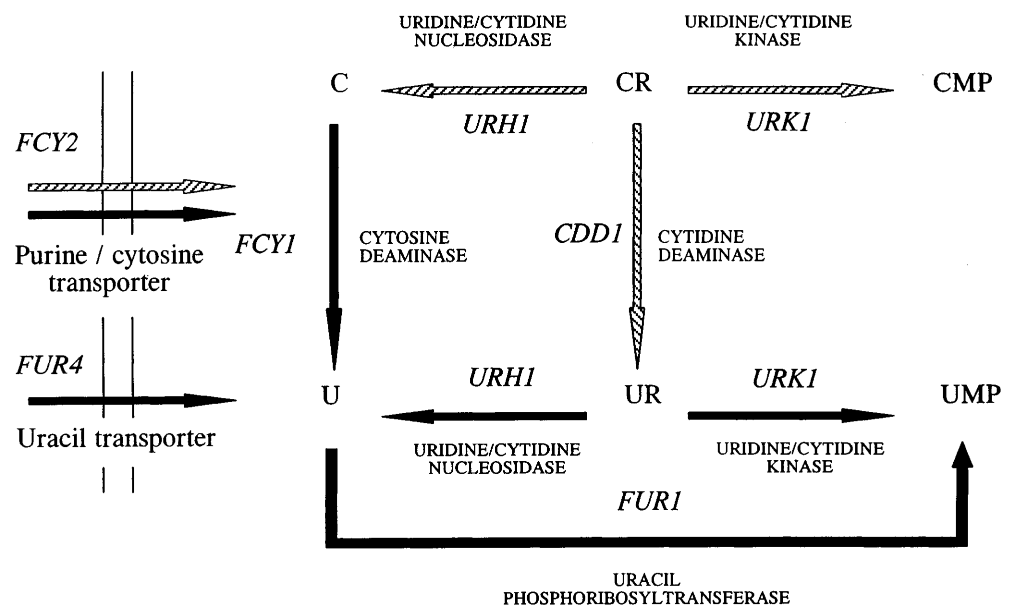

Fig. 2 Cytidine metabolism. *C* cytosine; *CR* cytidine; *CMP* cytidine 5′ monophosphate; *U* uracil; *UR* uridine; *UMP* uridine 5′ monophosphate. *Hatched arrows* presumed steps of cytidine pathway; *black arrows* previously described salvage pathway

transport is competitively inhibited by cytosine (Ki $\approx 10^{-6}$ M) and 5-fluorocytosine, but cytidine is unable to inhibit cytosine transport, even at a high concentration (10^{-3} M). Cytidine entry is undetectable in the wild-type in the presence of 2,4 dinitrophenol (3×10^{-4} M) and some mutants selected as resistant to 5-fluorocytidine are allelic to *fcy2*, demonstrating that cytidine enters the cell by active transport mediated by the Fcy2p purine/cytosine transporter.

Characterisation of the *CDD1* gene

A putative structural gene encoding cytidine deaminase (*CDD1*) in *S. cerevisiae* was cloned after PCR-amplification of ORF U20865 (Genbank) in the *S. cerevisiae* genome, which contains the signature sequences of the cytosine and cytidine deaminase families (Fig. 1) (Erbs et al. 1996). Disruption of this genomic ORF by integrative transformation with the pFL35 plasmid carrying an incomplete sequence of the putative cytidine deaminase gene led to a loss of enzyme activity (Fig. 3) and confirmed that this region constitutes the cytidine deaminase gene.

The expression plasmid *LEU2* p415 GPD, carrying the 429-bp *CDD1* coding fragment under the control of the yeast glyceraldehyde-3-phosphate dehydrogenase promoter (Mumberg et al. 1995), restores cytidine deaminase activity to the *cdd1*::*TRP1* strain (IRC100) and leads to overproduction of the enzyme (Fig. 4).

Subsequent DNA sequence analysis confirmed the identity of the 429-nucleotide open reading frame copied by PCR from the yeast genome (Genbank accession number AF080089). To further validate the different reactions that cytidine may undergo alongside deamination into uridine, mutants carrying a deficiency in different steps of the pyrimidine salvage pathway were crossed with the *cdd1*::*TRP1* strain (Table 1). The recombinants obtained after tetrad analysis were submitted to different assays.

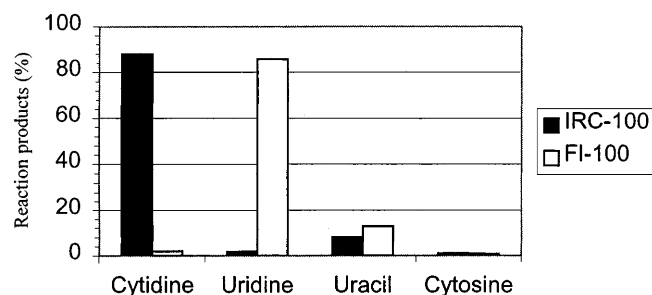

Fig. 3 Reaction products, resulting from the cytidine deaminase assay in the wild-type Fl100 and the disrupted strain IRC100, were quantified at time 8 min

Ability of uridine nucleosidase (Urh1p) to hydrolyse cytidine

Comparative plate analysis after cytidine deaminase assay with the wild-type (Fig. 5A) and with mutants which are only deficient in cytidine deaminase (*cdd1*::*TRP1*) (IRC100) (Fig. 5B) or which have lost both cytidine and cytosine deaminase activities (*cdd1*::*TRP1 fcy1-1*) (IRC102-1A) (Fig. 5C) was performed.

In the wild-type strain, cytidine was entirely converted into ^{14}C-2 uridine by the cytidine deaminase, and ^{14}C-2 uracil was detected as a product of the uridine nucleosidase activity (Fig. 5A).

In the disrupted strain under the same conditions, two spots were also detected; one of them was identified as ^{14}C-2 cytidine, the second one as ^{14}C-2 uracil (Fig. 5B). This result suggests that cytidine may also be converted into cytosine, by means of a putative nucleosidase, before deamination to uracil occurred by the cytosine deaminase. Finally, it appears that cytosine accumulates if cytosine deaminase is not functional (Fig. 5C), thereby confirming a cytidine nucleosidase activity. Further analysis of the chromatographic pattern obtained with extracts from *cdd1*::*TRP1 fcy1-1 urh1-2* (IRC103-9C) (Fig. 5D), in which uridine nucleosidase activity is abolished, shows a unique spot identified as cytidine. Considering that the same mutation in the *URH1* gene is responsible for the loss of both uridine and cytidine nucleosidase activities leads us to suggest that cytidine is also a substrate for uridine nucleosidase, despite previous results to the contrary obtained with the purified enzyme (Magni et al. 1975).

Ability of cytidine deaminase (Cdd1p) to deaminate deoxycytidine

The ability of Cdd1p to deaminate deoxycytidine was investigated in vitro, using labelled ^{14}C-deoxycytidine (48.7 mCi/mmol) as a substrate in the cytidine deaminase assay. After plate analysis, deoxyuridine resulting from deaminase activity was identified (data not shown), in agreement with data obtained with the purified enzyme (Magni et al. 1975).

Resistance to 5-fluorocytidine

In order to search for associated phenotypes, 5-fluorocytidine was added to the minimal culture medium and the growth of *URA2 cdd1*::*TRP1*, combined with (IRC101-1C) or without (IRC101-1B) *fcy1-1*, was compared to the wild-type (Fig. 6).

This test reveals that: (1) disruption of the *CDD1* gene confers the lowest level of resistance to 5-fluorocytidine among the 5-fluorocytidine-resistant mutants; (2) this level of resistance is increased if disruption is combined with *fcy1-1*. These data are consistent with the

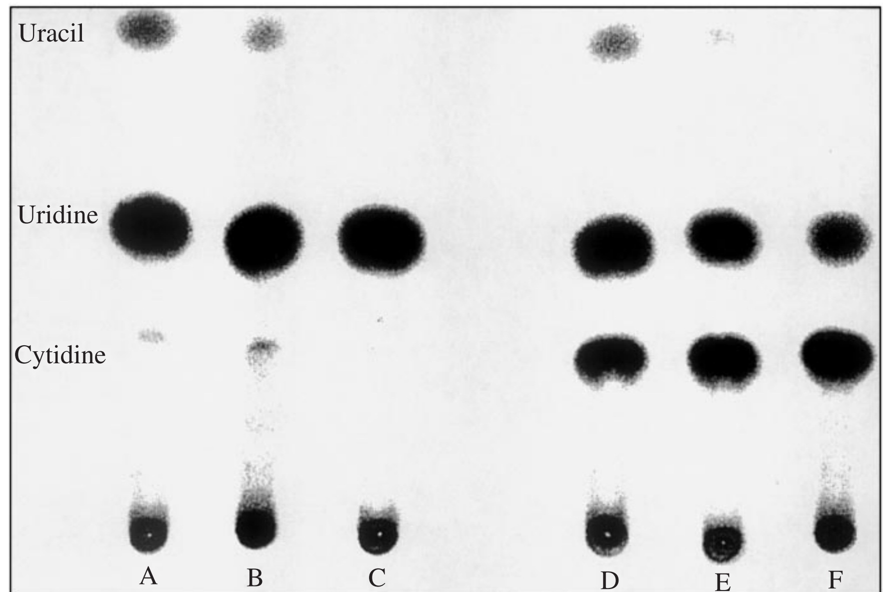

Fig. 4 Cytidine deaminase activity in *cdd1*::*TRP1* [pRS 415-CDD1] (*A*, *B*, *C*) and the wild-type Fl100 (*D*, *E*, *F*). Reactions were performed at time 4 min with a standard crude extract (*A*, *D*), a 1/3 dilution (*B*, *E*) and a 1/10 dilution (*C*, *F*). Cytidine was transformed into uridine with a specific activity of 5 nmol/min per mg of protein in the wild-type and was estimated to be 50 nmol/min per mg of protein in *cdd1*::*TRP1* [pRS 415-CDD1]

fact that exogenously supplied cytidine (200 μg/ml) as a pyrimidine source supports growth of a *ura2 cdd1*::*TRP1* strain, whereas growth under the same conditions is completely abolished in a *ura2 fcy1-1* strain.

Cytidine as a substrate for uridine kinase (Urk1p)

As *urk1* mutants show a resistance to 5-fluorocytidine, we investigated the ability of uridine kinase to phosphorylate cytidine. An in vivo approach was performed using steady state incorporation of ^{14}C-2 uridine (62.4 mCi/mmol) and ^{14}C-2 cytidine (53.4 mCi/mmol) into macromolecules as measured by growing cultures of *URK1*/*urk1 cdd1*::*TRP1 fcy1-1 urh1-2* strains (Table 2).

The results clearly demonstrated that not only uridine but also cytidine were substrates for the *URK1*-encoded uridine kinase (EC 2.7.1.48) as described in the rat myocard by Lortet et al. (1987), where uridine and

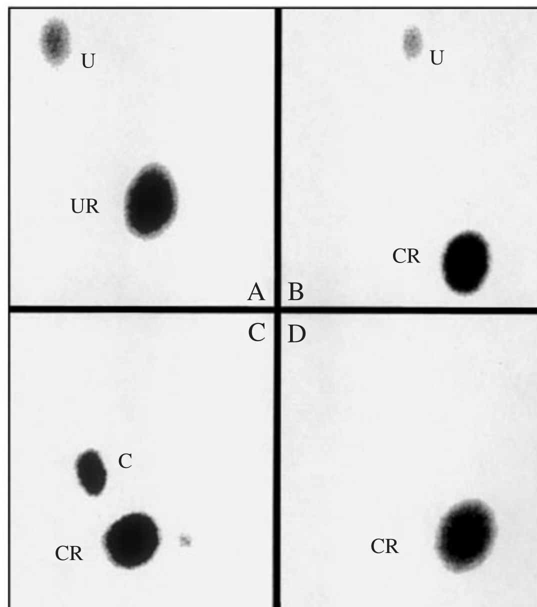

Fig. 5 A–D Bi-dimensional chromatographic pattern of cytidine metabolism in four different strains: Fl100 (**A**), *cdd1*::*TRP1* (**B**), *cdd1*::*TRP1 fcy1-1* (**C**) and *cdd1*::*TRP1 fcy1-1 urh1-2* (**D**). *UR* uridine, *CR* cytidine, *U* uracil, *C* cytosine

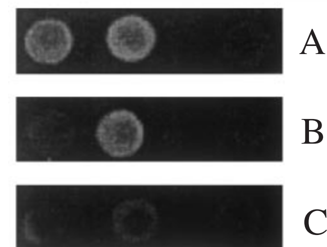

Fig. 6 Resistance pattern to 5-fluorocytidine, at concentrations of 2×10^{-3} M (**A**), 4×10^{-3} M (**B**), and 8×10^{-3} M (**C**). From left to right: strains *cdd1*::*TRP1*, *cdd1*::*TRP1 fcy1-1*, and wild-type Fl100

Table 2 Incorporation pattern of ^{14}C-2 uridine and ^{14}C-2 cytidine in the strains IRC103-8C and IRC103-9C. The steady state incorporation of uracil or adenine were internal controls demonstrating that the *urk1* mutation only affects cytidine and uridine fluxes, independently of overall nucleic acid synthesis

Strains	IRC103-8C cpm/DO	IRC103-9C cpm/DO	Ratio
Uracil	36.4	39.63	1.08
Uridine	1.42	12.24	8.6
Adenine	63.83	75.61	1.18
Cytidine	1.67	47.61	28.5

cytidine were substrates for the same kinase. Our uridine kinase assay confirmed these data (Fig. 7).

Discussion

In the present work the cytidine deaminase gene of *S. cerevisiae* has been identified as a unique sequence on chromosome XII sharing the consensus motifs of the deaminase families (Erbs et al. 1996). DNA sequence analysis revealed an open reading frame of 429 bp encoding a polypeptide of 143 amino acids consistent with prior protein-purification results (Magni et al. 1975). The disruption of *CDD1* was performed and a weak 5-fluorocytidine-resistant phenotype has been found for the null mutant. It is noteworthy that among the mutants in the six genes involved in cytidine metabolism, only the *CDD1* disrupted mutant exhibits strict resistance to 5-fluorocytidine. In spite of an in vitro cytidine deaminase activity with a similar level to that of other pyrimidine enzyme activities of the salvage pathway (5–10 nmol/min/mg), an unexpected result was that, in vivo, cytidine utilisation as an UMP source requires cytidine cleavage into cytosine. Our data demonstrate that this transformation is catalysed by the uridine nucleosidase encoded by *URH1*. Thus, a striking feature is the dual origin of uracil generated via cytosine deamination and uridine hydrolytic cleavage, which explains the resistance to high concentrations of 5-fluorocytidine (5×10^{-2} M) in *fur1* mutants impaired in uracil phosphoribosyltransferase activity. These results validate the presumed cytidine utilization pathway as shown in Fig. 1 (hatched arrows). In cancer therapy, phosphorylation by deoxycytidine kinase and deamination by cytidine deaminase are important processes in the activation and elimination of cytidine analogues, which represent an important class of chemotherapeutic agents (Bouffard et al. 1993). Forced expression of cytidine deaminase confers cellular resistance to analogues, including cytosine arabinoside (ara-C) and 2′,2′-difluorodeoxycytidine (gemcitabine), and therefore cytidine deaminase allows cells to survive the cytotox-

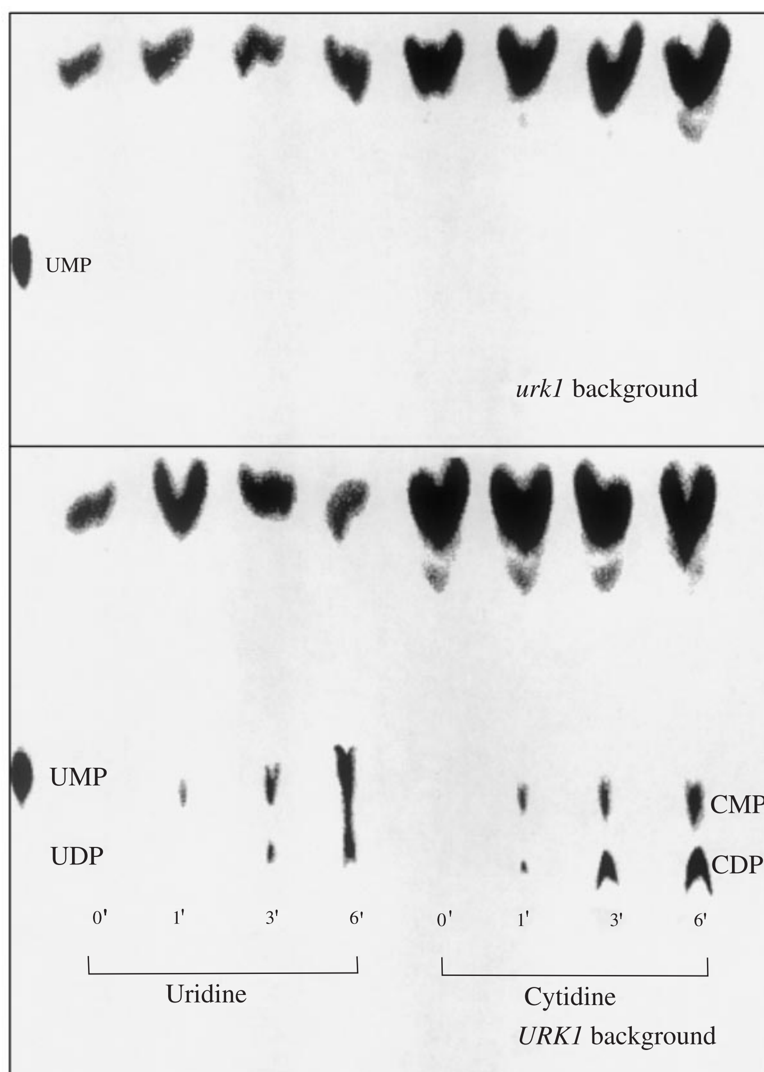

Fig. 7 Uridine/cytidine kinase activity in the IRC103-8 C (top) and the IRC103-9C (bottom) strains

icity exerted by these agents (Neff et al. 1996). The finding that cytidine can be phosphorylated into CMP via cytidine/uridine kinase may validate further utilisation of yeast as a model to check nucleoside analogues extensively used in cancer therapy and would be helpful in designing the most potent cell-growth inhibitors for their particular targets (Laliberte et al. 1992).

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