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Anna Andersson Rasmussen
Lund University

Dineshkumar Kandasamy
Lund University

Halfdan Beck
Lund University

Seth D. Crosby
Washington University School of Medicine in St. Louis

Olof Bjornberg
Lund University

See next page for additional authors

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Global Expression Analysis of the Yeast Lachancea (Saccharomyces) kluveri Reveals New URC Genes Involved in Pyrimidine Catabolism

Anna Andersson Rasmussen, Dineshkumar Kandasamy, Halfdan Beck, Seth D. Crosby, Olof Björnberg, Klaus D. Schnackerz and Jure Piskur

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Pyrimidines are important nucleic acid precursors which are constantly synthesized, degraded, and rebuilt in the cell. Four degradation pathways, two of which are found in eukaryotes, have been described. One of them, the URC pathway, has been initially discovered in our laboratory in the yeast Lachancea kluyveri. Here, we present the global changes in gene expression in L. kluyveri in response to different nitrogen sources, including uracil, uridine, dihydrouracil, and ammonia. The expression pattern of the known URC genes, URC1-6, helped to identify nine putative novel URC genes with a similar expression pattern. The microarray analysis provided evidence that both the URC and PYD genes are under nitrogen catabolite repression in L. kluyveri and are induced by uracil or dihydrouracil, respectively. We determined the function of URC8, which was found to catalyze the reduction of malonate semialdehyde to 3-hydroxypropionate, the final degradation product of the pathway. The other eight genes studied were all putative permeases. Our analysis of double deletion strains showed that the L. kluyveri Fullp protein transported uridine, just like its homolog in Saccharomyces cerevisiae, but we demonstrated that it was not the only uridine transporter in L. kluyveri. We also showed that the L. kluyveri homologs of DUR3 and FUR4 do not have the same function that they have in S. cerevisiae, where they transport urea and uracil, respectively. In L. kluyveri, both of these deletion strains grew normally on uracil and urea.
duced by the action of RutE or YdfG through the reduction of malonate semialdehyde (19).

In this report, we performed a global expression analysis in L. kluyveri and screened for additional URC genes. Microarrays were used to study changes in gene expression of all 5,321 predicted genes in response to different pyrimidines and other nitrogen-containing metabolites present as the sole nitrogen source. Here, we can show that the known URC genes are all upregulated by uracil, uridine, and, to some extent, dihydouracil but are downregulated by ammonia, which means they are under nitrogen catabolite repression (1). Nine putative URC genes were further studied through deletion analysis, and subsequently the function of a new URC gene, URC8, encoding a short-chain dehydrogenase, was determined through an enzyme assay.

**MATERIALS AND METHODS**

**Strains, media, and growth conditions.** The L. kluyveri strains used and developed in this study can be found in Table 1. The yeast strains were grown in rich yeast extract-peptone-dextrose (YPD) media unless stated differently. For selection, 100 μg/ml G418 was used.

For the microarray experiment and the growth test, N-minimal medium was used to inoculate flasks with N-minimal medium with uracil, dihydrouracil, uridine, urea, allantoin, proline, or ammonium sulfate, as described previously (13). For selection of transformants with the URA3 gene marker, SC URA dropout medium was used (1% succinic acid, 0.6% NaOH, 2% glucose, 0.19% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.1926% synthetic complete mixture [Kaiser], and dropout mixture lacking uracil [Formedium]).

*Escherichia coli*, which was used for protein production, was grown in LB medium (1% NaCl, 0.5% yeast extract, 1% peptone, pH 7).

**Cell cultivation for microarray experiment.** All cultivations of *L. kluyveri* were performed at 25°C, and *E. coli* was grown at 37°C. An overnight culture of the diploid sequenced strain Y057 (NRRL strain Y12651) in YPD was used to inoculate flasks with N-minimal medium with uracil, dihydrouracil, uridine, proline, or ammonia as the nitrogen source. Four parallel cultures (biological replicates) were made for each condition. The cells were grown to an optical density at 600 nm (OD600) of between 0.3 and 0.5 and then harvested by transferring the culture to small centrifugation tubes filled with crushed ice. The cells were collected by centrifugation at 4°C at 3,000 rpm for 10 min.

**RNA extraction and cDNA synthesis and labeling.** The yeast cells were immediately resuspended in 1 ml ice-cold TRizol reagent (Invitrogen) and then disrupted using a mini beadbeater (6 m/s, five times for 25 s each) using 0.5-μm glass beads. The samples were kept on ice, and total DNA was extracted according to the protocol, except that the DNA was precipitated at −20°C for 30 min. Cleanup was performed by running the RNA on RNasin spin columns (Qiagen) according to the manufacturer’s specifications. The RNA was treated on column with RNase-free DNase (Qiagen) according to the manufacturer’s protocol, and the integrity of extracted total RNA was analyzed by capillary electrophoresis (2100 Bioanalyzer; Agilent).

For cDNA synthesis, SuperScript III reverse transcriptase (RT; Invitrogen) was used, with 5 μg of total RNA as the template and the oligo(dT)12-18 primers (Invitrogen). The cDNA was labeled using a Pronto! plus labeling kit (Promega) according to the manufacturer’s protocol. Test samples were labeled with Cy3-dCTP (Amersham), and the reference was labeled with Cy5-dCTP. The protocols provided by the manufacturer were followed.

**Microarray hybridization and analysis.** Equimolar amounts of labeled test and reference cDNA were hybridized onto *L. kluyveri* microarray slides using the MAUI hybridization system. The microarrays, which were made on Corning epoxy slides, were provided by Mark Johnston, St. Louis, MO. Each spot contains a 65-base-long oligonucleotide representing one gene or open reading frame out of the total 5,353 included and is present in triplicates on each slide. In total, *L. kluyveri* has 5,321 predicted genes, but on the array some are represented by two different oligonucleotides; hence, 5,353 oligonucleotide-containing spots. The oligonucleotide sequences were based on the genome sequence determined by the Washington University School of Medicine (20), which was the only one available for *L. kluyveri* when the arrays were printed. Details of the platform can be found in the GEO database (platform no. GPL17318; http://www.ncbi.nlm.nih.gov/geo/). The slides were scanned and resulting images were analyzed in the GenePix Pro 4.1 software package (Molecular Devices, LLC).

Data analysis was conducted in R using the Bioconductor package. The raw data were normalized by within-print-tip-group local regression (LOESS) location normalization. An average of the signal from the three replicates on each array was then compared to the corresponding average of the control from the same array, and the log value of the sample/reference ratio was calculated. A standard t test was applied to assess the significance of the changes in expression, and the obtained P values were corrected for false discovery rates (FDR) due to the large sample size. The median of the log, ratios from the four...
biological replicates was used instead of the mean, since, in several cases, one out of the four replicates had large deviations from the other three. More details on the microarray experiment can be found in the GEO database (accession no. GSE48135).

Seven genes regulated in a manner similar to that of the known URCI-6 genes, i.e., upregulated by uracil and uridine and repressed by ammonia, were selected for further phenotypic studies. In addition, two other genes, both paralogs of genes selected from the microarray analysis, were included in the study. All nine genes can be found in Table 2.

**Cassettes for gene replacement.** Gene replacement cassettes were made for each of the nine genes and were used to create the knockout strains. The cassettes consisted of a marker gene flanked by regions of 470 to 520 bases homologous to the upstream and downstream sequence of the target gene (17). All sequences used, both coding and noncoding, were retrieved from the Genolevures database (http://www.genolevures.org/) (21). The constructs for the cassette were amplified separately by PCR, purified, and then assembled in a special primer-free assembly PCR, where about 48 ng marker gene and 20 ng flanking region were assembled with the help of overhangs on the flanking regions designed to be complementary to the marker gene. A second reaction with primers was run to amplify the correct product, using part of the assembly reaction as the complementary to the marker gene. A second reaction with primers was run to amplify the correct product, using part of the assembly reaction as the

**Creation of knockout strains.** Transformation of the gene replacement cassettes to create the knockout strains was performed by electroporation into competent *L. kluyveri* cells as described previously (13), with some minor adjustments. The cells were grown to an OD_{600} of 1 to 1.5, and the amount of cells used for each transformation corresponded to 2 to 3 ml of cells. For all transformations where minimal media was used for selection, an extra step of two washes with 2 ml of sterile water or minimal media was included after the incubation in YPD before spreading

### Table 1. *L. kluyveri* strains used and developed during this research project

| No. | Strain   | Genotype<sup>a</sup> | Originating strain        |
|-----|----------|-----------------------|---------------------------|
| 1   | Y057     | Diploid, prototroph   | NRRL Y-12651              |
| 2   | Y156     | MATα ura3            | GRY1175 (J. Strathern)    |
| 3   | Y1392    | MATα prototroph      | Y057                      |
| 4   | Y1161    | MATα ura3 urc2::KANMX3 | Y156                   |
| 5   | Y1616    | MATα urc3::KANMX3     | Y1392                     |
| 6   | Y1694    | MATα Lkfur3::KANMX3   | Y156                      |
| 7   | Y1700    | MATα ura3 Lkfur1::KANMX3 | Y156              |
| 8   | Y1701    | MATα ura3 Lkfur4::KANMX3 | Y156              |
| 9   | Y1702    | MATα ura3 Lkdal5::KANMX3 | Y156             |
| 10  | Y1703    | MATα ura3 Lkdal4::KANMX3 | Y156             |
| 11  | Y1705    | MATα ura3 Lkdur3::KANMX3 | Y156             |
| 12  | Y1704    | MATα ura3 SAKLA07480::KANMX3 | Y156            |
| 13  | Y1773    | MATα ura3 Lkfui1::KANMX3 Lkfui1 hom::URA3 | Y156          |
| 14  | Y1774    | MATα ura3 Lkfui1::KANMX3 Lkdal4::URA3 | Y156          |
| 15  | Y1775    | MATα ura3 Lkfui1::KANMX3 Lkdal5::URA3 | Y156          |
| 16  | Y1776    | MATα ura3 Lkfui1::KANMX3 Lkdal5 hom::URA3 | Y156          |
| 17  | Y1777    | MATα ura3 Lkfui1::KANMX3 Lkdal5 hom::KANMX3 | Y156         |
| 18  | Y1778    | MATα ura3 Lkfui1::KANMX3 SAKLA07480::URA3 | Y156          |
| 19  | Y1779    | MATα ura3 Lkfui1::KANMX3 Lkdur3::URA3 | Y156          |
| 20  | Y1780    | MATα ura3 Lkfui1 hom::KANMX3 | Y156          |
| 21  | Y1781    | MATα ura3 Lkdal5 hom::KANMX3 | Y156          |
| 22  | Y1782    | MATα ura3 Lkdur3::KANMX3 SAKLA07480::URA3 | Y156          |
| 23  | Y1783    | MATα ura3 Lkfui1 hom::KANMX3 Lkdal4::URA3 | Y156          |
| 24  | Y1784    | MATα ura3 Lkfui1 hom::KANMX3 Lkdur4::URA3 | Y156          |
| 25  | Y1785    | MATα ura3 Lkfui1 hom::KANMX3 Lkdal5::URA3 | Y156          |
| 26  | Y1786    | MATα ura3 Lkfui1 hom::KANMX3 Lkdal5 hom::URA3 | Y156          |
| 27  | Y1787    | MATα ura3 Lkfui1 hom::KANMX3 SAKLA07480::URA3 | Y156          |
| 28  | Y1788    | MATα ura3 Lkfui1 hom::KANMX3 Lkdur3::URA3 | Y156          |
| 29  | Y1789    | MATα ura3 Lkdal5::KANMX3 Lkdal5 hom::URA3 | Y156          |
| 30  | Y1790    | MATα ura3 Lkdal5::KANMX3 Lkdal4::URA3 | Y156          |
| 31  | Y1791    | MATα ura3 Lkdal5::KANMX3 Lkdur4::URA3 | Y156          |
| 32  | Y1792    | MATα ura3 Lkdal5::KANMX3 Lkdur3::URA3 | Y156          |
| 33  | Y1793    | MATα ura3 Lkdal5::KANMX3 SAKLA07480::URA3 | Y156          |
| 34  | Y1794    | MATα ura3 Lkdal5 hom::KANMX3 Lkdal4::URA3 | Y156          |
| 35  | Y1795    | MATα ura3 Lkdal5 hom::KANMX3 Lkdur4::URA3 | Y156          |
| 36  | Y1796    | MATα ura3 Lkdal5 hom::KANMX3 Lkdur3::URA3 | Y156          |
| 37  | Y1797    | MATα ura3 Lkdal5 hom::KANMX3 SAKLA07480::URA3 | Y156          |
| 38  | Y1798    | MATα ura3 Lkdal4::KANMX3 Lkdur4::URA3 | Y156          |
| 39  | Y1799    | MATα ura3 Lkdal4::KANMX3 Lkdur3::URA3 | Y156          |
| 40  | Y1800    | MATα ura3 Lkdal4::KANMX3 SAKLA07480::URA3 | Y156          |
| 41  | Y1801    | MATα ura3 Lkdur3::URA3 | Y156          |
| 42  | Y1802    | MATα ura3 Lkdur4::KANMX3 SAKLA07480::URA3 | Y156          |

<sup>a</sup> hom, homolog.
TABLE 2  L. kluyveri genes selected for knockout analysis and the name and function of their homologs in S. cerevisiae

| L. kluyveri name | Systematic | S. cerevisiae name | Systematic | Function in S. cerevisiae |
|-----------------|------------|--------------------|------------|--------------------------|
| LkFU1 hom       | SAKL0H30476g | FUI1              | YBL042C    | Uridine transporter      |
| LkDA5 hom       | SAKL0G16456g | DAL5              | YIR152W    | Ureidosuccinate/allantole transporter |
| LkDA5           | SAKL0C00352g | DAL5              | YIR028W    | Allantoin permease       |
| LkDA4           | SAKL0G04532g | DAL4              | YBR021W    | Uracil transporter       |
| LkFUR4          | SAKL0C11594g | FUR4              | YHL016C    | Urea/polyamine permease  |
| LkDUR3          | SAKL0A10010g | DUR3              | Noneb      | Transport of basic amino acids |
| URC8            | SAKL0H04730g |                  | YMR226C    | 3-Hydroxy acid reductase |

a All of these genes, except the LkFU1 homolog (hom) and LkDA5 homolog, were highly expressed on uracil but not on ammonia.

b Weakly similar to VBA2 (YBR293W).

the cells on the selective media to avoid transfer of the rich media to the selective minimal media.

Single deletions of all transporter-like genes were made by transformation of the cassettes with KANMX3 as the marker into the ura3 strain Y156. For the reductase gene, URC8, the same procedure was used but employing the prototrophic strain Y1392 instead of Y156, since no double deletion was planned for this gene and the URA3 marker was not needed. Transformants were selected on YPD with G418. Double knockouts were then created for all putative transporter genes by transforming the single knockout strains with deletion cassettes, now using URA3 as the marker gene, which allowed selection on SD media.

The correct insertion of the deletion cassette was confirmed by colony PCR in all deletion strains created. Cells were dissolved in 5 mM 0.02 M NaOH, incubated for 10 min at 95°C, and then spun down. Three μl of the supernatant was used as the template in a regular PCR using DreamTaq DNA polymerase (Fermentas).

All of the resulting strains, both single and double knockouts, were tested for growth on minimal media with various sources of nitrogen (described above). The sequenced strain Y057 and the parent strain were used as positive controls, and the Δura3 strain Y1616, which has a known URC phenotype, was used as the negative control. The strains were grown overnight in YPD and then harvested and washed in sterile water. They were diluted to ODs of 0.1 and 0.01 and spotted onto solid sole-nitrogen-source media. Analysis of the growth was done after 3 and 6 days.

The knockout strains created in this study are represented under the numbers 6 to 42 in Table 1.

UrCl8 overexpression and assay. The URCl8 gene from L. kluyveri was cloned and inserted into the pET151/D-TOPO vector (Invitrogen), which adds an N-terminal His tag to the protein product. The construct (P1027) was transformed into Escherichia coli strain BL21 (Invitrogen) for expression. A culture was grown at 37°C to an OD600 of 0.6, and then expression was induced with 0.1 mM IPTG (isopropyl β-D-thiogalactoside) followed by overnight incubation at 16°C. The cells were harvested and homogenized by French press two times at 16,000 and 18,000 lb/in3, and the protein was purified using nickel-nitriilotriacetic acid (NiNTA) chromatography. The storage buffer was subsequently changed to 10 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.1 M KF buffer, pH 7.4 using PD-10 columns (GE Healthcare).

The L. kluyveri β-alanine aminotransferase, PydH, was purified according to a previous description (15) and then used for production of malonate semialdehyde under the following conditions: 83 mM KF buffer, pH 7.4, 80.8 mM β-alanine, 2.6 mM α-ketoglutarate, 0.3 μg/μl PydH, 30°C, 5 min. NADPH and UrCl8 were added (0.13 μg/μl and 0.42 ng/μl, respectively) to assay the UrCl8 activity. Under these conditions, neither the PydH concentration nor the produced malonate semialdehyde should be limiting. The reaction was monitored by the decrease in absorbance at 340 nm, where NADPH has its absorption maximum.

Microarray data accession numbers. The microarray data determined in the course of this work have been deposited in the GEO database under accession no. GSE48135 and the microarray platform is found under accession no. GPL17318.

RESULTS

Global expression analysis. Global expression analysis was performed on L. kluyveri by specifically designed microarrays (GEO accession no. GSE48135; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48135), based on the first available genome sequence (20), in order to study the pyrimidine catabolism and its relationship to general nitrogen metabolism. To cover the different aspects of pyrimidine and nitrogen metabolism, we chose four growth conditions, where uracil, uridine, dihydroxyuracil, or ammonia was used are the only source of nitrogen. For each experiment, cells grown with proline as the nitrogen source were used as the control, since proline, like uracil, uridine, and dihydroxyuracil, is a poor nitrogen source and because its metabolism is not related to that of the other sources used. The top 20 upregulated genes under each condition are shown in Table 3. The highest number of upregulated genes was seen under the uridine condition, with 250 genes being at least 2-fold upregulated. On dihydroxyuracil at least 122 genes were 2-fold or more upregulated, on uracil 91 genes, and on ammonia 57 genes. The complete lists of genes which are at least 2-fold upregulated are found in Table S2 in the supplemental material.

Through this analysis, we show that all of the five already known URC genes (17) were upregulated in the presence of uracil and uridine, while no induction was observed on ammonia. For URC2, which encodes a transcription factor, the expression was comparatively constitutive, with only a 3-fold induction on uracil. An important observation was that URC1 is the most upregulated gene on both uracil and uridine, being 800 to 1,000 times induced, closely followed by URC4, which was increased 250 to 350 times. Surprisingly, we found that most of the URC genes were also induced by dihydroxyuracil, even though its metabolism is not related to that of uracil and uridine in yeast, in contrast to mammals and other organisms which possess the full reductive pathway. In addition, PYD2 and PYD3 of the reductive pathway also were upregulated on uracil, but the induction by dihydroxyuracil was stron-
| Condition and no. | ID          | Systematic name | Common name | S. cerevisiae homolog | Log2 fold induction | P value  |
|------------------|-------------|-----------------|-------------|-----------------------|---------------------|----------|
| **Uracil**       |             |                 |             |                       |                     |          |
| 1                | Contig145.1:A:821 | SAKL0H14498g    | URC1        | None                  | 9.703               | 0.00141  |
| 2                | Contig312.2:A:628 | SAKL0A09988g    | URC4        | None                  | 8.410               | 0.00104  |
| 3                | Skluy_39.4:2:2  | SAKL0C00352g    | DAL5        | None                  | 5.951               | 0.00107  |
| 4                | Skluy_32.2:16:16 | SAKL0H03479g    | FUI1        | None                  | 5.728               | 0.00069  |
| 5                | Skluy_1.3:12:12 | SAKL0E11792g    | URC6        | FUR1                  | 5.465               | 0.00061  |
| 6                | Skluy_36.1:5:5  | SAKL0H10560g    | URC3,5      | DUR1,2                | 5.264               | 0.00110  |
| 7                | Skluy_116.1:12:12 | SAKL0D01386g   | ATR1        | None                  | 4.976               | 0.00073  |
| 8                | Skluy_11.1:2:2  | SAKL0H23210g    | YGR015C     | None                  | 4.668               | 0.00175  |
| 9                | Skluy_104.1:4:4 | SAKL0C02178g    | None        | None                  | 4.626               | 0.000458 |
| 10               | Skluy_104.1:5:5 | SAKL0C02200g    | PRM1        | None                  | 4.550               | 0.00082  |
| 11               | Skluy_31.2:8:8  | SAKL0A10010g    | Dur3        | None                  | 4.483               | 0.00116  |
| 12               | Skluy_54.3:14:14 | SAKL0G04532g   | DAL4        | None                  | 4.280               | 0.000155 |
| 13               | Skluy_182.1:2:2 | SAKL0C00352g    | 5.951       | None                  | 5.951               | 0.00107  |
| 14               | Skluy_32.2:16:16 | SAKL0H03479g    | FUI1        | None                  | 5.728               | 0.00069  |
| 15               | Skluy_116.1:12:12 | SAKL0D01386g   | ATR1        | None                  | 4.976               | 0.00073  |
| 16               | Skluy_116.1:12:12 | SAKL0D01386g   | ATR1        | None                  | 4.976               | 0.00073  |
| **Dihydrouracil**|             |                 |             |                       |                     |          |
| 1                | Contig147.1:A:1027 | SAKL0C11748g   | PYD3        | None                  | 9.015               | 0.00129  |
| 2                | Skluy_13.2:3:3  | SAKL0B05588g    | PYD2        | None                  | 6.908               | 0.00326  |
| 3                | Skluy_177.1:3:3 | SAKL0B10120g    | DAL5        | None                  | 6.305               | 0.00135  |
| 4                | Skluy_30.1:33:33 | SAKL0B12562g    | UGA1        | None                  | 6.217               | 0.00097  |
| 5                | Skluy_182.1:2:2 | SAKL0C00352g    | 5.951       | None                  | 5.951               | 0.00061  |
| 6                | Skluy_76.1:28:28 | SAKL0H04730g    | URC8        | YMR226C               | 3.819               | 0.00206  |
| 7                | Contig29.2:E:116 | SAKL0H20526g    | PYD2        | None                  | 3.721               | 0.00337  |
| 8                | Skluy_13.2:3:3  | SAKL0B05588g    | PYD2        | None                  | 6.908               | 0.00326  |
| 9                | Contig312.2:A:628 | SAKL0A09988g   | HPA2        | None                  | 4.018               | 0.00103  |
| 10               | Skluy_30.1:35:35 | SAKL0B12606g    | GTP1        | None                  | 3.825               | 0.01224  |
| 11               | Skluy_23.5:2:2  | SAKL0G07480g    | HPA2        | None                  | 3.825               | 0.01224  |
| 12               | Contig312.2:A:628 | SAKL0A09988g   | HPA2        | None                  | 3.825               | 0.01224  |
| 13               | Skluy_104.1:4:4 | SAKL0C02178g    | FEN2        | None                  | 3.581               | 0.00405  |
| 14               | Skluy_45.3:31:31 | SAKL0E15290g    | PRM1        | None                  | 3.244               | 0.00460  |
| 15               | Skluy_104.1:5:5 | SAKL0C02200g    | FEN2        | None                  | 3.581               | 0.00405  |
| 16               | Skluy_20.1:9:9  | SAKL0F16632g    | ARG8        | None                  | 3.001               | 0.00127  |
| 17               | Skluy_3.3:12:12 | SAKL0E05016g    | ARG8        | None                  | 3.001               | 0.00127  |
| 18               | Contig6.3:B:1543 | SAKL0H11767g    | UGA4        | None                  | 3.475               | 0.00259  |
| 19               | Contig20.1:B:21 | SAKL0F16654g    | UGA4        | None                  | 3.475               | 0.00259  |
| 20               | Skluy_3.3:13:13 | SAKL0E04994g    | HER2        | None                  | 2.715               | 0.00242  |

(Continued on following page)
TABLE 3 (Continued)

| Condition and no. | ID          | Systematic name | Common name       | S. cerevisiae homolog | Log, fold induction | P value |
|-------------------|-------------|-----------------|-------------------|-----------------------|---------------------|---------|
| 17                | Skluy_36.1.5:5 | SAKLOH10560g     | URC3,5            | DUR1,2                | 4.326               | 0.00129 |
| 18                | Skluy_46.2.9:9 | SAKLOB03212g     |                  | ICL2                  | 4.315               | 0.00397 |
| 19                | Skluy_76.1.28:28 | SAKLOH04730g      | URC8              | YMR226C               | 4.161               | 0.00286 |
| 20                | Skluy_0.4.5:5  | SAKLOH08580g     |                  | YPL110C               | 4.123               | 0.00126 |

Ammonia

|     | ID          | Systematic name | Common name       | Log, fold induction | P value |
|-----|-------------|-----------------|-------------------|---------------------|---------|
| 1   | Skluy_88.1.2:2 | SAKLOD02948g     | AGPI              | 3.515               | 0.019478 |
| 2   | Skluy_140.1.3:3 | SAKLOD09152g      | CITI              | 2.660               | 0.009231 |
| 3   | Skluy_2.1.29:29 | SAKLOF04356g      | TIR3              | 2.417               | 0.026387 |
| 4   | Skluy_95.2.7:7  | SAKLOB02090g     | HSP12             | 2.190               | 0.05253 |
| 5   | Skluy_80.1.22:22 | SAKLOF13310g      | ITR2              | 2.123               | 0.03577 |
| 6   | Skluy_27.1.38:38 | Sequence on chromosome F, no matching genes in Génolevures |                  | 2.089               | 0.027403 |
| 7   | Skluy_22.1.1:1 | Sequence on chromosome G, no matching genes in Génolevures |                  | 2.054               | 0.042043 |
| 8   | Skluy_34.1.4:4 | SAKLOD04510g     | JEN1              | 2.024               | 0.013497 |
| 9   | Contig113.1A1140 | SAKLB01672g      | NOHBY648          | 1.961               | 0.007016 |
| 10  | Skluy_22.1.46:46 | SAKLGO1164g      | PDC1              | 1.924               | 0.021277 |
| 11  | Skluy_112.3.8:8 | SAKLOC11528g     | GAL7              | 1.906               | 0.040539 |
| 12  | Skluy_375.1.1:1 | Matches 10 retrotransposon Gag genes |                  | 1.882               | 0.205696 |
| 13  | Contig573.1A203 | SAKLOF13310g      | YHR214C-C         | 1.831               | 0.050309 |
| 14  | Skluy_105.1.10:10 | SAKLOA09394g    | OPT1              | 1.712               | 0.022063 |
| 15  | Skluy_40.1.1:1  | SAKLGD16038g     | CTR3              | 1.689               | 0.022914 |
| 16  | Skluy_12.2.10:10 | SAKLGD19140g    | MDHI              | 1.671               | 0.013244 |
| 17  | Skluy_2.1.30:30 | SAKLOF04334g     | PDC1              | 1.616               | 0.025753 |
| 18  | Skluy_25.3.2:2 | SAKLGO1414g      | HIP1              | 1.540               | 0.029876 |
| 19  | Skluy_15.3.12:12 | SAKLDO11946g   | SUC2              | 1.497               | 0.03344 |
| 20  | Skluy_161.1.5:5 | SAKLGO8052g      | YHR087W           | 1.490               | 0.036698 |

A similar but much weaker effect was seen for the last gene of the pathway, PYD4. The genes of the reductive pathway also were downregulated by ammonia. These results show that both the PYD and the URC genes in L. kluyveri are subjected to the nitrogen catabolite repression. The change in expression is represented in Table 4 and graphically visualized in Fig. 2.

DELETION STUDIES REVEALED NOVEL URC GENES. From the microarray results, seven potential new URC genes were selected for

| L. kluyveri name | S. cerevisiae homolog | Sequence ID | Log, fold regulation in response to: |
|-----------------|----------------------|-------------|--------------------------------------|
| URC1            | None                 | Contig145.1A821 | Uracil: 9.703, Dihydrouracil: 4.158, Uridine: 10.131, Ammonia: -2.016 |
| URC2            | YDR520C              | SAKLGD96.2:2  | Uracil: 1.715, Dihydrouracil: 0.726, Uridine: 0.939, Ammonia: -0.418 |
| URC3,5          | DUR1,2               | SAKLGD36.1:5:5 | Uracil: 5.264, Dihydrouracil: 5.544, Uridine: 4.326, Ammonia: -0.406 |
| URC4            | None                 | Contig31.2A628 | Uracil: 8.410, Dihydrouracil: 5.112, Uridine: 0.801, Ammonia: -0.301 |
| URC6            | FUR1                 | SAKLGD12.12:2 | Uracil: 5.465, Dihydrouracil: 4.622, Uridine: 4.502, Ammonia: -0.080 |
| PYD2            | None                 | Contig147.1A1027 | Uracil: 3.590, Dihydrouracil: 9.015, Uridine: 2.798, Ammonia: -0.106 |
| PYD4            | UGA1                 | SAKLGD29.2:17:17 | Uracil: 0.585, Dihydrouracil: 1.213, Uridine: -0.441, Ammonia: -0.941 |
| URC8            | YMR226C              | SAKLGD76.1:28:28 | Uracil: 3.819, Dihydrouracil: 4.984, Uridine: 4.161, Ammonia: -0.201 |
| DAL4            | DAL5                 | SAKLGD39.4:2:2 | Uracil: 5.951, Dihydrouracil: 1.801, Uridine: 5.786, Ammonia: -0.121 |
| DAL5 homolog    | DAL5                 | SAKLGD40.3:7:7 | Uracil: -0.513, Dihydrouracil: 1.105, Uridine: -1.614, Ammonia: -1.609 |
| DAL5 homolog    | DAL5                 | SAKLGD32.2:16:16 | Uracil: 5.728, Dihydrouracil: 2.406, Uridine: 6.070, Ammonia: -1.632 |
| DAL5 homolog    | DAL5                 | SAKLOH03498G   | Uracil: 3.890, Dihydrouracil: 5.903, Uridine: 2.406, Ammonia: -0.211 |
| SAKL0A07480     | None                 | SAKLGD104.1:4:4 | Uracil: 4.626, Dihydrouracil: 3.581, Uridine: 3.061, Ammonia: -1.632 |
| DUR3            | DUR3                 | SAKLGD31.2:8:8 | Uracil: 4.483, Dihydrouracil: 1.749, Uridine: 3.580, Ammonia: -0.351 |

- Weakly similar to VBA2 (YBR293W).
- The oligonucleotide matching this gene could not be identified; therefore, its systematic name found in the Génolevures database was used.
The selection was made on the basis (i) that their expression profile was similar to that of the known URC genes, i.e., upregulation on uracil and uridine and no change or downregulation on ammonia (Table 4), and (ii) that the function in *L. kluyveri* was unknown. Two additional genes (SAKL0H03498g and SAKL0G16456g), whose function in *L. kluyveri* were also unknown, were included as well. These two genes had different expression patterns but were included since they are paralogs of the FUI1 and DAL5 genes selected from the microarray study. An extensive collection of knockout strains, including 9 single and 26 double knockout strains (Table 1), was prepared through gene replacement for the selected genes. All of the created strains were found to be viable and were

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**FIG 2** Expression analysis of *L. kluyveri* grown on different nitrogen sources, as analyzed by microarrays. The oligonucleotides representing genes known to be involved in the URC and reductive pyrimidine catabolic pathways are marked in red. All of the genes selected for further analysis, which also are marked in the figure, were previously unknown but have expression patterns similar to those of the known URC genes or are homologous to a gene with a similar expression pattern. The exception is the FUI1 homolog SAKL0H03498g, which could not be identified among the oligonucleotides represented on the array. The y-axis shows the sample/reference signal log₂ ratio, and the x-axis is the average intensity of the total signal for each oligonucleotide (average of the technical and biological replicates).
TABLE 5 Growth phenotype analysis of single and double deletion strains

| Deleted gene(s) | Growth on: | Ammonia | Uracil | Uridine | DHU | Allantoin | Urea |
|----------------|------------|---------|--------|---------|-----|-----------|------|
| Single deletion |            |         |        |         |     |           |      |
| LkFU1         | +         | +       | +      | -       | +   | +         | +    |
| LkFU1 hom     | +         | +       | +      | +       | +   | +         | +    |
| LkDAL5        | +         | +       | +      | +       | +   | +         | +    |
| LkDAL5 hom    | +         | +       | +      | +       | +   | +         | +    |
| LkDAL4        | +         | +       | +      | +       | +   | +         | +    |
| LkFU4         | +         | +       | +      | +       | +   | +         | +    |
| LkDUR3        | +         | +       | +      | +       | +   | +         | +    |
| SAKL0A07480   | +         | +       | +      | +       | +   | +         | +    |
| URC8          | +         | /+       | +      | +/-     | +   | +         | +    |

| Double deletion |               |         |        |         |     |           |      |
| Δlkfur4 Δlkdal4 | +         | +       | +      | +       | +   | +         | +    |
| Δlkfur1 Δlkfur4 | +         | +       | +      | -       | +   | +         | +    |
| Δlkfur1 Δfui1 hom | +         | +       | +      | -       | +   | +         | +    |
| Δlkfur1 Δlkdar3 | +         | +       | +      | +       | +   | +         | +    |
| Δlkfur1 Δlkdal4 | +         | +       | +      | +       | +   | +         | +    |
| Δlkfur1 Δlkdal5 | +         | +       | +      | +       | +   | +         | +    |
| Δlkfur1 dal5 hom | +         | +       | +      | +       | +   | +         | +    |
| Δlkfur1 ΔSAKL0A07480 | +         | +       | +      | +       | +   | +         | +    |
| Δlkdar3 ΔSAKL0A07480 | +         | +       | +      | +       | +   | +         | +    |
| Δlkdar3 Δlkdal5 | +         | +       | +      | +       | +   | +         | +    |
| Δlkdar3 Δlkdal4 | +         | +       | +      | +       | +   | +         | +    |
| Δfui1 hom Δlkdar3 | +         | +       | +      | +       | +   | +         | +    |
| Δfui1 hom Δlkdal4 | +         | +       | +      | +       | +   | +         | +    |
| Δfui1 hom Δlkdal5 | +         | +       | +      | +       | +   | +         | +    |
| Δfui1 hom Δlkfur4 | +         | +       | +      | +       | +   | +         | +    |
| Δfui1 hom ΔSAKL0A07480 | +         | +       | +      | +       | +   | +         | +    |
| Δdal5 hom Δlkdal5 | +         | +       | +      | +       | +   | +         | +    |
| Δlkdal4 Δlkdal5 | +         | +       | +      | +       | +   | +         | +    |
| Δlkfur4 Δlkdal5 | +         | +       | +      | +       | +   | +         | +    |
| ΔSAKL0A07480 Δlkdal5 | +         | +       | +      | +       | +   | +         | +    |
| Δdal5 hom Δlkdal4 | +         | +       | +      | +       | +   | +         | +    |
| Δdal5 hom Δlkfur4 | +         | +       | +      | +       | +   | +         | +    |
| Δdal5 hom Δlkdar3 | +         | +       | +      | +       | +   | +         | +    |
| Δdal5 hom ΔSAKL0A07480 | +         | +       | +      | +       | +   | +         | +    |
| Δlkfur4 ΔSAKL0A07480 | +         | +       | +      | +       | +   | +         | +    |
| Δlkdal4 ΔSAKL0A07480 | +         | +       | +      | +       | +   | +         | +    |

a hom, homolog.
b Shading highlights the results which differed from those for the wild-type strain. + + +, wild-type-like growth; + +, growth, but less than that of the wild type; +, very limited and slow growth; −, no growth/equal to negative control; NA, not assayed. DHU, dihydrouracil. Growth was inspected after 3 and 6 days.

tested for growth on media with different nitrogen sources (Table 5) using the parental strain as a positive control and the Δurc2 strain as the negative control.

A majority of the genes selected for deletion analysis encode putative permeases. So far, no permeases have been characterized in *L. kluyveri*. Uptake of nutrients is crucial for the cell, so often there is more than one transporter for each nutrient, although their affinity for that nutrient may differ. Therefore, it is not very surprising that most of the permease gene deletions did not show any deviating phenotype; however, a few strains grew slower than their parental strain. The strain with the deletion of the *L. kluyveri* *FU1* (SAKL0H0476g) gene showed a decrease in growth on uridine. Several of the double-knockout strains where this gene was missing had a changed phenotype on uridine, and in a few cases a slight decrease in growth was seen on dihydrouracil as well. Two strains, in which LkFU1 was disrupted together with the LkFU1 paralog or LkFU4, only had a little background growth, similar to the negative control, thereby showing a stronger growth phenotype than the single deletion of LkFU1.

A slight but still clear decrease in growth on both uracil and uridine was observed for several double knockout combinations involving the LkDUR3 gene. No effect, however, was seen on urea, although this is the substrate of DUR3 in *S. cerevisiae* (22).

A newly characterized URC gene encodes a C3-modifying enzyme. The only non-permease-like gene selected for further analysis was SAKL0H04730g, which was later named URC8 (see Fig. S3 in the supplemental material). The deletion strain showed significantly reduced growth on uracil and uridine, indicating involvement of this gene in the URC pathway. In addition, it also showed less growth on dihydrouracil, which is degraded by the reductive pathway and would be expected to be unrelated to the URC pathway. The Urc8 homologs in *S. cerevisiae* (YMR226C) and *E. coli* (YdfG) have been characterized previously (23). They belong to the NADP+/NADPH-dependent short-chain dehydrogenase/reductase family.
observed during a 10-min period (Fig. 4), indicating that NADPH
is also the outcome of the URC pathway (17). We hypothesize that
the role of URC8 is similar to that of YdfG.

**Overexpression and purification of Urc8p.** We successfully
subcloned the URC8 gene of *L. kluyveri* and produced the protein
in *E. coli*, using a pET/TOPO vector in order to add a His tag to
facilitate purification. The purified protein was analyzed by SDS-
PAGE (Fig. 3), and a band just below 35 kDa was observed, which
should be compared to the predicted size of the monomer of the
native protein, 29.2 kDa, and the 4 kDa added by the vector. After
confirmation of the protein size, the concentration was deter-
mined by Bradford assay and the yield was estimated to be about
10 mg per liter of culture. The fractions containing the highest
protein concentration were pooled, desalted, and diluted for ac-

tivity assays.

**Urc8p catalyzes the reduction of malonate semialdehyde to
3-hydroxypropionate.** To test our hypothesis that Urc8p cata-
lyzes the same reaction as YdfG in the Rut pathway (19), i.e.,
converts malonate semialdehyde to 3-hydroxypropionate, we
used the purified protein in a coupled assay with the β-alanine
amino-transferase from *L. kluyveri*, Pyd4p, to make the proposed
substrate. β-Alanine amino-transferase catalyzes the fourth step
of the common reductive pyrimidine degradation pathway, where
β-alanine is converted to malonate semialdehyde (15). The puri-
ified Pyd4p enzyme was incubated with β-alanine and α-ketoglutarate (as an acceptor of the ammonia moiety) at 30°C for 5 min to
produce malonate semialdehyde before addition of NADPH and
the Urc8p protein. The conditions were optimized, so the concen-
trations of Pyd4p and its substrates should not be limiting.

The reaction was monitored by the absorbance change of
NADPH at 340 nm. A linear decrease from 0.825 to 0.494 was
observed during a 10-min period (Fig. 4), indicating that NADPH
is the reducing agent in the reaction. A negligible decrease in ab-
sorbance was seen without Urc8p added or when NADH was used
instead of NADPH. This confirms that Urc8p catalyzes the reduc-
tion of malonate semialdehyde to 3-hydroxypropionate, which is
a waste product of the URC pathway (Fig. 4).

**DISCUSSION**

We designed a microarray to deepen our knowledge on the URC
pathway. *L. kluyveri* had already been studied by microarray anal-
ysis (25, 26), but only with cells grown in rich medium and not
minimal media with different nitrogen sources. Our transcription
analysis of *L. kluyveri* clearly shows that all genes known to be
involved in the pyrimidine catabolism are also under nitrogen
catabolite repression (Table 4). The nitrogen catabolite repression
has been well studied in *S. cerevisiae* and in some filamentous fungi
(1, 27), and we confirmed here that this regulatory circuit also
exists in *L. kluyveri*, making the nitrogen metabolism more energy
efficient by using the best source available. All URC genes were
indeed induced when uracil or uridine, but not ammonia, was
used as the source of nitrogen (Table 4 and Fig. 2). However, the
fact that a quite substantial induction of most URC genes is also
observed in the dihydrooracil condition is more surprising, con-
sidering that the catabolism of uracil and dihydrooracil occurs by
two separate pathways in fungi, although they are both degraded
by the same reductive pathway in most other organisms. The fact
that the URC genes were strongly upregulated in response to uracil
and uridine implies that these compounds also can be utilized as a
source of nitrogen in nature. This would give *L. kluyveri* an advan-
tage in niches where preferred nitrogen sources, such as ammonia
glutamate, are absent but uracil or uridine is present.

In contrast to mammals, insects, plants, and many bacteria,
which have a complete reductive pathway in which degradation of
uracil proceeds through reduction to dihydrooracil, yeasts that are
able to degrade pyrimidines possess two separate pathways, one
for uracil and one for dihydrooracil catabolism (17). The dihy-
droacil is degraded by the same reductive pathway as that in
other organisms, but the enzyme performing the first step,

![FIG 3 Purified His-tagged Urc8 protein from *L. kluyveri* run on SDS-PAGE.](image1)

![FIG 4 Assay for the new URC enzyme, Urc8p. For the Urc8p assay, the absorbance was monitored at 340 nm for 10 min, corresponding to the decrease of NADPH. In the inset in the lower left corner, the Urc8p reaction, where malonic semialdehyde is converted into 3-hydroxypropionate with the use of NADPH as the reductant, is presented. No change in absorbance was observed when NADH was used instead of NADPH in a parallel sample.](image2)
genes involved in the reductive pathway, namely, PYD2 (13) and PYD3 (14), were significantly upregulated by uracil, although the induction by dihydrouracil was higher. Similarly, the URC genes were found to be upregulated not only by uracil but also by dihydrouracil. In short, all URC and PYD genes were strongly regulated by their expected inducers. The observed induction by uracil and dihydrouracil could be a leftover process from the complete reductive pathway in early unicellular eukaryotes. It could also be explained by the fact that the two molecules only differ by one double bond in the pyrimidine ring and might be similar enough to activate the specific regulator proteins for each other’s pathways. A similar overlap could be seen in the transport, where the same transporter carries both uracil and dihydrouracil (28). Lastly, one could also speculate that the two molecules get slowly interconverted in the yeast cell.

The high induction by uracil observed for URC1 and URC4 (about 830 and 350 times, respectively) can be compared to a previous study of uracil induction in Schizosaccharomyces pombe; however, that study was performed in the presence of ammonia. Here, the URC1 homolog urg1 was upregulated 100-fold. As in L. kluyveri, the S. pombe homologs of URC1, URC4, and URC6 were the three most strongly regulated genes in response to uracil, although the URC6 homolog urg2 was stronger than the URC4 homolog urg3 (29).

After our microarray analysis, we developed a large collection of strains with single and double gene disruptions for the eight putative transporters included in this study (Table 1). The single deletion strains were all viable, meaning that none of the genes was essential. Likewise, all of the double deletions created were found to be viable.

All deletion strains of FUI1 showed restricted growth on the minimal plate containing uridine as a sole nitrogen source (Table 5). Together, these results suggest that FUI1, which is a high-affinity uridine permease in S. cerevisiae (30), also is responsible for uridine transport in L. kluyveri. However, in L. kluyveri, there are additional permeases whose functions overlap this function. The double deletions where FUI1 was disrupted together with FUR4 or the LkFUI1 paralog had a null phenotype, indicating that these two genes function as weak uridine transporters. In S. cerevisiae, FUR4 is known to be the only uracil transporter (31), which is clearly not the case with the L. kluyveri homolog, since no effect at all was seen on uracil for any of its deletions.

The existence of several transporters for uridine explains why strains with the single deletion of FUI1 still grow slightly on uridine. The function of the LkFUI1 paralog (SAKL0H03498g) has not been characterized before, but it is 70% identical to LkFUI1p and 69% identical to S. cerevisiae FUI1p. We speculate that the function of the three proteins is similar. The main FUR4 function in S. cerevisiae is transport of uracil. Interestingly, no deviating phenotype was observed for any other double deletion strain where FUR4 was disrupted; instead, they all grew like the parental strain, even on uracil (Table 5). However, we cannot rule out uracil transport as a function of FUR4 in L. kluyveri, since other transporters might have overlapping functions, but it is clear that the uracil transporter in L. kluyveri is different from that in S. cerevisiae, where FUR4 is the only uracil transporter (31, 32).

DUR3 also seems to have some role in the transport of both uracil and uridine, since several of its double knockout combinations showed some deviation in phenotype on these media, although no change was seen for the single knockout strain (Table 5). The other genes deleted in these double combinations apart from FUI1, namely, SAKL0A07480, DUR4, and DUR5, might also play a small role in uracil and uridine transport. The slight phenotype observed for the dur3 double knockouts, with fui1 and the Lkfui1 paralog, indicates that it also is involved in the transport of dihydrouracil. In S. cerevisiae, DUR3 is known to be a permease for polyamines and urea (22, 33), but no deviating phenotype was observed on urea for any dur3 strain in our growth tests, so it seems that the function of DUR3 in L. kluyveri is different.

The new URC gene presented in this paper, URC8, had a similar expression level under dihydrouracil, uridine, and uracil conditions (Table 4), and the corresponding knockout grew weakly on uracil, uridine, and dihydrouracil. We successfully overproduced and purified the encoded protein, Urc8p, and showed that it can catalyze the reduction of a 3-carbon molecule just like its homologs in S. cerevisiae and E. coli, YMR226c and YdflG. The confirmed reaction, i.e., reduction of malonate semialdehyde to 3-hydroxypropionate, is the same reaction catalyzed by RutE and YdfG in E. coli (19). However, the Urc8p homologs YMR226c and YdfG are known multisubstrate enzymes, meaning that Urc8p is likely to be one too. We speculate that the now confirmed reaction is the one which is required for the URC pathway, since 3-hydroxypropionate has already been confirmed as a waste product of this pathway (17).

Now that the function of URC8 is clear, its regulation by uracil and dihydrouracil also makes sense, as this gene apparently provides a link between the reductive and URC pathways (Fig. 5). Malonate semialdehyde, the substrate of Urc8p, is formed in both pathways and then processed by Urc8p. In the URC pathway, it is likely formed directly or spontaneously from the three-carbon part when the pyrimidine ring is opened and in the reductive pathway from beta-alanine by the action of Pyd4 (10). The reactions needed for extracting the two nitrogen sources from the pyrimidine ring are still unrelated in the two pathways, as Urc8p is only involved in taking care of the carbon waste product. The Urc8p homolog in E. coli, YdfG, degrades the malonate semialdehyde formed in the Rut pathway, producing 3-hydroxypropionate, thereby overlapping in function with RutE, which is a malonate semialdehyde reductase not homologous to YdfG or Urc8p. The known catalytic amino acids (34) are conserved in the E. coli and S. cerevisiae homologs of Urc8p. They are present also in the L. kluyveri protein (see Fig. S2 in the supplemental material), suggesting a similar function. In addition, the E. coli homologs produce the same product as that formed in the URC pathway. These facts together led us to hypothesize that Urc8p performed the same reaction as YdflG. In E. coli, the reaction is proposed to have a detoxifying role, helping to speed up the removal of toxic intermediates formed during the degradation (19).

The fact that the Δurc8 strain is still able to grow on both uracil and dihydrouracil, but much slower than the parent strain, also points to a detoxifying role of URC8. This is the first report on functional homology among the four pyrimidine degradation pathways.

In conclusion, we have now provided proof of nitrogen catabolite repression in L. kluyveri and provided a deeper knowledge of the regulation of genes involved in general nitrogen metabolism and pyrimidine degradation from the microarrays. A new URC gene, URC8, was found necessary for full growth of L. kluyveri on
Overview of pyrimidine degradation in *L. kluyveri*, showing a link between the reductive and the URC pathways. At the top is the reductive pathway, which degrades dihydrouracil to β-alanine via ureidopropionate using the PYD2 and PYD3 gene products. In yeast, the gene encoding the dihydropyrimidine dehydrogenase, which catalyzes the reduction of uracil to dihydrouracil, does not exist, but other species, like mammals, plants, and insects, possess the complete reductive pathway. In yeast, uracil is degraded by the URC pathway (bottom). Malonate semialdehyde is produced both in the reductive pathway when Pyd4p dehydrogenase, which catalyzes the reduction of uracil to dihydrouracil, does not exist, but other species, like mammals, plants, and insects, possess the complete reductive pathway. In yeast, uracil is degraded by the URC pathway, and its function was determined. Our growth test analysis of the large single and double knockout collection for putative transporters brought new insight into the complex transport of pyrimidines and other metabolically related compounds. LkFu1p was found to be involved in the uptake of uridine, but unlike its homolog in *S. cerevisiae*, it is not the only uridine transporter in *L. kluyveri*.

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