5.1. Příprava nástrojů genového inženýrství pro *Z. rouxii*

Poznání procesů probíhajících v kvasince *Z. rouxii* na molekulární úrovni je dosud velmi limitované, a to hlavně z důvodu chybějícího souboru metod pro genové manipulace v této kvasince. Pro identifikaci a charakterizaci genů *Z. rouxii* se doposud využívala převážně jejich heterologní exprese v *S. cerevisiae*. *Z. rouxii* je nicméně zajímavá právě z hlediska svých specifických vlastností, které jsou pravděpodobně zajištěny specifickými geny. Vzhledem k tomu, že izolace a studium specifických genů nemusí být prostřednictvím jejich exprese v heterologním hostiteli umožněno jednoduše z toho důvodu, že v heterologním hostiteli buď nefungují vůbec nebo je jejich funkce odlišná, je pro identifikaci a charakterizaci specifických genů *Z. rouxii* esenciální možnost genových manipulací přímo v této kvasince.

Soubor nástrojů umožňující manipulovat s genovou výbavou *Z. rouxii* dosud neexistoval. Zatím byly k dispozici pouze dva auxotrofní mutantní kmeny: *Z. rouxii* UL4 (*ura3*), derivát divokého kmene CBS 732^T připravený autorkou této dizertační práce v rámci její diplomové práce (Přibylová, 2002), a *Z. rouxii* MA11 (*leu2*), derivát divokého kmene ATCC 42981 (Ushio *et al.*, 1988). Jediným dominantním selekčním genem využitým dosud v *Z. rouxii* byl *kanMX*. Možnosti selekce transformantů tak byly omezeny. Neexistovaly specifické plasmidy pro *Z. rouxii* s polylinkerem, ani jednoduchá a účinná technika transformace. Jediná publikovaná metoda pro transformaci *Z. rouxii* byla pracná technika využívající protoplastů (Ushio *et al.*, 1988). Nebyl k dispozici ani nástroj umožňující vícenásobné delece, jakkoli již bylo známo, že pro cílené delece v *Z. rouxii* lze využít homologní rekombinaci (Iwaki *et al.*, 1999; Tang *et al.*, 2005; Watanabe *et al.*, 1995).

V této dizertační práci se nám podařilo připravit soubor nástrojů pro genové manipulace v *Z. rouxii*, zahrnující účinnou a jednoduchou metodu transformace, možnost exprese genů z centromerových nebo episomálních plasmidů a selekce transformantů prostřednictvím různých selekčních genů, systém pro vícenásobné delece, a též možnost značení proteinů v buňkách *Z. rouxii* pro určení jejich vnitrobuněčné lokalizace. Výsledky jsme zveřejnili (publikace č. 1 - 3) nebo odeslali k recenznímu řízení (publikace č. 4).

5.1.1. Publikace č. 1: Efficient transformation of the osmotolerant yeast *Zygosaccharomyces rouxii* by electroporation

Přibylová, L., Sychrová, H. (2003) *J Microbiol Methods* **55**(2): 481 - 484

Prvním krokem pro vytvoření souboru nástrojů pro genové manipulace v *Z. rouxii* bylo optimalizovat metodu transformace. V současné době je jednou z nejjednodušších a nejvíce používaných metod transformace kvasinek elektroporace. V této práci jsme navázali na výsledky získané v autorčině diplomové práci, ve které byl připraven kmen *Z. rouxii* UL4 s mutací *ura3* (Přibylová, 2002).

Vytvořili jsme jednoduchý protokol pro transformaci dvou dostupných mutantních kmenů *Z. rouxii* - UL4 (*ura3*) a MA11 (*leu2*; Ushio *et al.*, 1988) elektroporací. Zjistili jsme, že kmeny pro účinnou transformaci vyžadují odlišné podmínky inkubace s látkami rozvolňujícími buněčný povrch. Na buňky kmene UL4 bylo třeba před aplikací elektrického pulzu působit kromě DTT ještě ionty Li^+, účinnost transformace kmene MA11 se působením Li^+ naopak snižovala. Kmen UL4 též vyžadoval delší dobu působení látek rozvolňujících buněčný povrch než kmen MA11.

Vzhledem k tomu, že typovým kmenem *Z. rouxii* je CBS 732^T, jehož celková sekvence genomu bude v brzké době veřejně dostupná, bylo výhodné připravit nástroje genového inženýrství pro manipulaci DNA ve kmenech s genetickým pozadím CBS 732^T. Pro přípravu nástrojů pro manipulaci DNA *Z. rouxii* byl proto v následujících pracích využit hlavně kmen *Z. rouxii* UL4.

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Note

Efficient transformation of the osmotolerant yeast *Zygosaccharomyces rouxii* by electroporation

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Abstract

A rapid and simple electroporation method to transform osmotolerant yeast *Zygosaccharomyces rouxii* has been developed and conditions for efficient transformation of mutants derived from different *Z. rouxii* wild-type strains optimized.

Keywords: Electroporation; Osmotolerance; Transformation; *Zygosaccharomyces rouxii*

Zygosaccharomyces rouxii belongs to the group of hemiascomycetous yeasts with high tolerance to osmotic stress. This typical feature is supposed to be due to the fact that this yeast possesses some sets of specific genes conferring the ability to grow in media with high concentrations of salts and/or sugars, i.e. under conditions restrictive to *Saccharomyces cerevisiae*. Thus, cloning of *Z. rouxii* genes involved in osmoresistance and their expression in the industrial strains of *S. cerevisiae* could improve the growth and fermentation capacities of industrial strains in adverse conditions.

Though the physiological and biochemical processes of *Z. rouxii* have been studied extensively (Kurtzman and Fell, 1998; van Zyl et al., 1990), our knowledge of cell properties at the molecular level lags far behind those of *S. cerevisiae* and other model yeast species. One of the reasons for this situation is the high resistance of *Z. rouxii* cells to routine and quick transformation procedures. The method mainly used for its transformation so far is the time consuming and laborious transformation of spheroplasts in the presence of polyethyleneglycol (PEG) and Ca^{2+} cations (Ushio et al., 1988).

To develop an efficient and quick transformation of *Z. rouxii*, we used auxotrophic mutant strains derived from two different *Z. rouxii* wild types. UL4 is a *ura3* strain derived from *Z. rouxii* ATCC 56077 (CBS 732) and MA11 is a *leu2* mutant of ATCC 42981. MA11 has been already transformed with a *Z. rouxii* pSR1-based vector containing the *ScLEU2* (Ushio et al., 1988). Plasmid pLU1 used in this work contains the *ZrLEU2* gene (Sychrova, 2001) cloned into YCplac33 (*S. cerevisiae CEN/ARS* and *URA3*) and complements the auxotrophies of both mutant strains. Cells were grown overnight in the YPD standard yeast rich medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose) at 30 °C with shaking (200 rpm) and harvested when the OD_{600} reached 0.6–0.8 (2–2.7 × 10^7 cells/ml). All experiments were carried out

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at least in triplicate and results summarized in tables correspond to mean values. Mean deviations were in all cases less than 10%.

First, we tried to transform the MA11 strain using several techniques widely used for yeast cells. Neither the classical transformation of intact yeast cells in the presence of Li^+ and PEG (Ito et al., 1983; Ogawa et al., 1990) nor the transformation of frozen cell stocks used for *Pichia* species (Passoth and Hahn-Hagerdahl, 2000) were efficient for *Z. rouxii*. Using these two methods, we obtained less than 10 transformants/μg of pDNA and also the electroporation techniques used for *S. cerevisiae* (Bloch et al., 1992; Meilhoc et al., 1990) or *C. maltosa* (Kasuske et al., 1992) gave only few transformants. To increase the efficiency of electroporation, we first tested different conditions of cell treatment with dithiothreitol (15–50 mM DTT in H_2O, room temperature vs. 30 °C, incubation 5–30 min and standing on bench vs. shaking). The best results were obtained if harvested cells were shaken at 30 °C with 25 mM DTT for 30 min. After this treatment, the electroporation gave tens to hundred transformants per microgram pDNA.

Another factor influencing the transformation efficiency could be the composition of the growth medium. One of the major components of *Z. rouxii* cell wall is chitin, and it was shown that *Z. rouxii* cells grown in a mild salt stress contained less chitin and probably thinner cell wall (Tomita et al., 1996). To check whether the cell-wall thickness could influence the transformation efficiency, we performed the transformation of cells grown in the presence of NaCl (150, 300 or 600 mM, respectively) and we found that addition of 300 mM NaCl to YPD increased the number of transformants about five times.

Table 1
Yield of *Z. rouxii* MA11 transformants/μg pDNA as a function of pDNA concentration

pDNA added to competent cells (μg)	Transformants/μg pDNA
0.05	2820
0.1	3490
0.25	2492
0.5	1696
1.0	787

Experiments were carried out at least in triplicate; results summarized in the table correspond to mean values. Mean deviations were in all cases less than 10%.

Table 2
Effect of Li^+ concentration on transformation efficiency of *Z. rouxii* UL4

LiAc concentration in DTT-preincubation mixture (mM)	Transformants/μg pDNA
0	10
5	650
20	1010
60	1100
100	830

Experiments were carried out at least in triplicate; results summarized in the table correspond to mean values. Mean deviations were in all cases less than 10%.

Electroporation was carried out using standard electroporation cuvettes (0.2 cm) and GHT 1287-B electropulsator (JOUAN, France) delivering square-wave pulses where voltage (up to 1 500 V) and pulse duration (up to 24 ms) were independently adjustable. We tested the voltage ranging from 470 to 1500 V and pulse duration from 6 to 24 ms. Most transformants were obtained when 625 V was applied for 24 ms. Table 1 summarizes the yields of *Z. rouxii* MA11 transformants depending on pDNA amount added to competent cells. The quantity of pDNA was not the limiting step for the transformation efficiency; the highest numbers of transformants were obtained with less than 0.5 μg pDNA in the transformation mixture. The same efficiency of transformation was observed when the competent MA11 cells were transformed with the multicopy plasmid pKU24 (not shown).

If the optimum conditions (30-min preincubation with DTT, 0.1 μg pDNA, electroporation 625 V/24 ms) giving approx. 3500 transformants/μg pDNA for the MA11 strain were used to transform the

Table 3
Effect of pulse (625 V) duration on transformation efficiency of *Z. rouxii* UL4

Duration of pulse (ms)	Transformants/μg pDNA
6	100
12	390
18	580
24	1000

Experiments were carried out at least in triplicate; results summarized in the table correspond to mean values. Mean deviations were in all cases less than 10%.

Panel 1
Protocols for *Z. rouxii* transformation by electroporation

(A) Strains derived from ATCC 42981	(B) Strains derived from ATCC 56077 (CBS 732)
• Grow cells in 80 ml of YPD-300 mM NaCl to $OD_{600} \sim 0.7-0.8$	
• Wash cells with ddH_2O (3500×*g*, 3.5 min)	
• Resuspend in 8 ml of 25 mM dithiothreitol in ddH_2O. Incubate at 30 °C for 30 min with shaking	• Resuspend in 16 ml of 25 mM dithiothreitol+20 mM LiAc in TE, pH 8. Incubate at 30 °C for 60 min with shaking
• For next steps chill on ice	
• Add 4 ml of ice-cold H_2O. Pellet (3500×*g*, 4 °C, 3.5 min)	
• Resuspend in 3 ml of ice-cold EB (10 mM Tris–HCl, 0.1 mM $MgCl_2$, 270 mM sucrose, pH 7.5)	
• Pellet (3500×*g*, 4 °C, 3.5 min). Resuspend in 800 μl of ice-cold EB	
• Transfer 100 μl of the cell suspension into a chilled electroporation cuvette. Add 0.1–0.25 μg pDNA	
• Place the cuvette in the electroporation chamber and apply an electric pulse of 3.13 kV/cm, 24 ms (GHT 1287-B Jouan, 625 V, 0.2 cm cuvettes)	
• Add immediately 100 μl of ice-cold ddH_2O and incubate for 10 min, RT	
• Plate on selective medium	

UL4 mutant strain (derived from ATCC 56077), surprisingly, only about 10 transformants were obtained.

Changes in the strength and duration of the electric pulse did not lead to a significantly higher efficiency of UL4 transformation (not shown). To increase the transformants' number, the addition of Li^+ cations and buffering the DTT-preincubation mixture to pH 8.0 (25 mM DTT in a TE buffer, pH 8.0) was necessary. As shown in Table 2, the lowest concentration of LiAc increasing significantly the number of transformants was 5 mM, and the maximum was obtained with cells preincubated in the presence of 20 mM LiAc. UL4 cells treated with Li^+ and DTT were electroporated under different conditions (voltage and pulse duration, amount of pDNA), and similarly as for the MA11 strain, maximum number of transformants was obtained with 0.1 μg pDNA and 625 V/24 ms (cf. Table 3). The same efficiency was observed if a multicopy pSR1-based vector with *ScURA3* was used instead of the monocopy pLU1 (not shown).

Table 4
Transformation efficiency of *Z. rouxii* strains according to protocols A and B

Strains derived from	Transformants/μg pDNA	
	Protocol A	Protocol B
ATCC 42981	3490	550
ATCC 56077 (CBS 732)	10	1000

Experiments were carried out at least in triplicate; results summarized in the table correspond to mean values. Mean deviations were in all cases less than 10%.

The two protocols for efficient transformation of UL4 and MA11 strains are summarized in Panel 1. If the same conditions as for UL4 were tested for MA11, the presence of 20 mM LiAc decreased the transformation efficiency for cells derived from *Z. rouxii* ATCC 42981 (Table 4).

We have optimized the electroporation procedure to yield the maximum number of *Z. rouxii* transformants per microgram of input DNA. The optimal conditions for transformation of *Z. rouxii* by electroporation differ not only from those used for other yeast species, e.g. *S. cerevisiae* (Bloch et al., 1992), *Schizosaccharomyces pombe* (Suga and Hatakeyama, 2001), *Candida* spp. (Kasuske et al., 1992; Thompson et al., 1998), but they are not the same for different *Z. rouxii* strains.

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5.1.2. Publikace č. 2: Expression of the *Saccharomyces cerevisiae MPR1* gene encoding N-acetyltransferase in *Zygosaccharomyces rouxii* confers resistance to L-azetidine-2-carboxylate

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Pro selekci transformantů a především delečních mutantů se při genových manipulacích kvasinek využívají dominantní selekční geny. Mezi nejrozšířenější patří *kanMX*. Jeho využití jako selekčního genu v *Z. rouxii* již bylo publikováno, nicméně ve kmenech s jiným genetickým pozadím než CBS 732^T (Tang *et al.*, 2005; Ushio *et al.*, 1988). Využití *kanMX* pro selekci transformantů a delečních mutantů odvozených od kmene CBS 732^T jsme potvrdili v publikaci č. 4. Při genových manipulacích je však výhodné mít více možností selekce, a tedy je žádoucí i větší výběr dominantních selekčních genů.

Za účelem rozšíření možností dominantní selekce v *Z. rouxii* jsme zkoumali využití genu *MPR1* kvasinky *S. cerevisiae* jako selekčního genu. *ScMPR1*, kódující enzym detoxifikující analog prolinu (AZC), byl nalezen ve kmeni *S. cerevisiae* Σ1278b (Shichiri *et al.*, 2001). Tento kmen je proto rezistentní k přítomnosti AZC v médiu. Většina laboratorních kmenů *S. cerevisiae* funkční kopii genu *ScMPR1* neobsahuje, a je k přítomnosti AZC citlivá. Pokud exprimují gen *ScMPR1*, stanou se rezistentními. V databázi Génolevures 1, která představuje soubor sekvencí získaných při částečné sekvenaci genomu *Z. rouxii* CBS 732^T, jsme nenalezli žádný gen homologní k *ScMPR1*, dalo se tedy předpokládat, že i buňky *Z. rouxii* budou k AZC citlivé.

V této práci jsme potvrdili, že buňky *Z. rouxii* jsou citlivé k přítomnosti AZC v růstovém médiu, přičemž exprese genu z plasmidu je učinila rezistentními. Nicméně, při selekci transformantů na základě jejich rezistence k AZC (tj. selekčním médiem bylo médium obsahující AZC) docházelo k výskytu spontánně rezistentních kolonií. Gen *ScMPR1* lze tedy v *Z. rouxii* použít pouze jako pomocný selekční gen, nikoli dominantní. Totéž jsme potvrdili pro *S. cerevisiae*.

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Expression of the *Saccharomyces cerevisiae MPR1* Gene Encoding *N*-Acetyltransferase in *Zygosaccharomyces rouxii* Confers Resistance to L-Azetidine-2-carboxylate

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ABSTRACT. The osmotolerant yeast *Zygosaccharomyces rouxii* is sensitive to the toxic L-proline analogue, L-azetidine-2-carboxylate (AZC). The possibility of use of the *Saccharomyces cerevisiae MPR1* gene (*ScMPR1*) encoding the AZC-detoxifying enzyme as a dominant selection marker in *Z. rouxii* was examined. The heterologous expression of *ScMPR1* in two *Z. rouxii* strains resulted in AZC-resistant colonies, but that of *ScMPR1* as a dominant marker gene in vectors was affected by a high frequency of spontaneously resistant colonies. The same was found for an AZC-sensitive *S. cerevisiae* strain in which the *ScMPR1* was expressed. In both yeasts, *ScMPR1* can be used only as an auxiliary marker gene.

Zygosaccharomyces rouxii is a hemiascomycetous yeast with a high tolerance to osmotic stress. This species apparently possesses some sets of specific genes conferring the ability to grow on media with high concentrations of salts and/or sugars, *i.e.* under conditions restrictive to *Saccharomyces cerevisiae*. Thus the cloning of *Z. rouxii* genes involved in osmoresistance and their expression in industrial strains of *S. cerevisiae* could improve the growth and fermentation capacities of industrial strains in adverse conditions. Although the physiological and biochemical processes of *Z. rouxii* have been studied extensively (van Zyl *et al.* 1990; Kurtzman and Fell 1998), our knowledge of cell properties at the molecular level lags far behind that of *S. cerevisiae*. One of the reasons is the lack of tools for genetic engineering with this yeast. In our previous study, we optimized the transformation procedure to efficiently introduce plasmid DNA (Přibylová and Sychrová 2003), but the possibilities to select *Z. rouxii* transformants remain few. As far as auxotrophic mutants are concerned, only the *leu2* (Ushio *et al.* 1998) or *ura3* (Přibylová and Sychrová 2003) mutants are available, and the only one dominant selection marker successfully used in *Z. rouxii* so far is the *kanMX* gene.

L-Azetidine-2-carboxylate (AZC) is a proline analogue that is incorporated into proteins competitively with L-proline and causes the synthesis of abnormal misfolded proteins. It inhibits cell growth due to its alteration of the protein structure (Fowden *et al.* 1963), thus providing a possible mechanism for use as selection reagent for transformation. Two AZC-resistance genes (*MPR1* and *MPR2*) have been isolated from the genome of *S. cerevisiae* Σ1278b (Takagi *et al.* 2000; Shichiri *et al.* 2001) and proven to have a role in oxidative stress (Nomura and Takagi 2004). Mpr1p is a 229 amino-acid-long acetyltransferase that has been shown to detoxify AZC by acetylating it in the cytoplasm (Shichiri *et al.* 2001). The resulting *N*-acetyl-AZC is not recognized by L-prolyl-tRNA synthetase, so that only L-proline is incorporated into proteins and the yeast can grow in the presence of AZC.

Compared to Σ1278b, other *S. cerevisiae* strains, *e.g.* S288C, are AZC-sensitive and do not contain a functional copy of the *MPR1/2* genes (Kimura *et al.* 2002). On the other hand, *MPR1* genes or highly homologous DNA fragments were found in the genomes of *Schizosaccharomyces pombe* (ppr1$^+$), *S. paradoxus* (*SpaMPR1*), *S. bayanus*, *S. pastorianus*, *S. mikatae*, *S. kudriavzei* and *S. kluyveri* (Kimura *et al.* 2002; Nomura *et al.* 2003; Nomura and Takagi 2004). Among *S. paradoxus*, *S. bayanus* and *S. pastorianus*, only *S. paradoxus* was resistant to AZC; the *S. bayanus* and *S. pastorianus* gene products probably lost acetyltransferase activity due to gene mutation(s) at the essential region for catalysis, or due to a functional defect of the promoter or terminator region (Kimura *et al.* 2002). Expression of the *ScMPR1* and *ScMPR2* genes from the *S. cerevisiae* Σ1278b strain conferred AZC resistance to sensitive *S. cerevisiae* strains (Takagi *et al.* 2000). Hence, the detoxication of AZC could serve as a dominant selection tool after transformation with

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vectors containing *ScMPR1* as a marker gene. In this work, we tested the sensitivity and/or tolerance of two *Z. rouxii* strains to AZC, and the use of *ScMPR1* as a dominant selection marker for *Z. rouxii* or *S. cerevisiae* transformation.

MATERIALS AND METHODS

Strains and plasmids. All yeast strains used were haploid. The *Z. rouxii* wild-type strains were ATCC 2623 (CBS 732) and ATCC 42981. *Z. rouxii* UL4 was an *ura3* derivative of CBS 732 (Přibylová and Sychrová 2003). *Z. rouxii* MA11 (*leu2*) was provided by Prof. Y. Watanabe (*Ehime University*, Ehime, Japan). The *S. cerevisiae* wild-type strains were Σ1278b (*MATa*), S288C (*MATa*) and FL100 (*MATa*). FRJ1 (*MATa ura2 trp1-4*) was the FL100 derivative (Schacherer *et al.* 2004). The plasmids used were: YCplac33 (*ScURA3 CEN4 ARS1* Amp^r; Gietz and Sugino 1988), pLU1 (*ScURA3 ZrLEU2 CEN4 ARS1* Amp^r; Přibylová and Sychrová 2003), pMH1 (*ScMPR1 ScURA3* 2μ Amp^r; Takagi *et al.* 2000) and YCpAZC. YCpAZC was constructed by inserting the 2.4 kb *Bgl*II–*Sac*I fragment of the pMH1 plasmid containing the *ScMPR1* gene and its promoter region into the *Bam*HI–*Sac*I restriction sites of YCplac33.

Media, growth experiments and transformation. Yeast cells were grown in standard YPD (in %: *Bacto* peptone 2, glucose 2, yeast extract 1) or YNB-NH_4 (0.67 % Yeast Nitrogen Base without amino acids, 2 % glucose) media at 30 °C. YNB-Pro and YNB-urea were the YNB-NH_4 media where diammonium sulfate was substituted for 0.1 % proline or 0.1 % urea. To test the effect of AZC, media were supplemented with 0–1 g/L AZC (*Sigma*) after autoclaving. AZC was stocked at 4 °C as a 20 g/L water solution sterilized by filtration (0.22 μm; *Millipore*). To estimate the AZC sensitivity of stationary cells (drop tests), YNB-NH_4 plates with increasing concentration of AZC were inoculated with 5 μL drops of serial 15-fold dilutions of saturated yeast cultures previously stored on solid YPD at 4 °C. To explore the AZC sensitivity of cells growing exponentially in liquid YPD + 0.3 mol/L NaCl, the 100 μL aliquots (≈3 × 10^6 cells) of the *Z. rouxii* culture ($A_{600} \approx 0.5$) were spread on solid YNB-NH_4 or YPD media supplemented with 0.3–0.9 g/L AZC. *S. cerevisiae* and *Z. rouxii* cells were transformed by electroporation (Přibylová and Sychrová 2003). For starvation experiments, immediately after application of the electric pulse and before plating, transformed *Z. rouxii* MA11 cells were transferred to 500 μL of distilled H_2O and incubated with shaking (12.5 Hz, 2 h, 30 °C).

RESULTS AND DISCUSSION

The presence of *ScMPR1* homologous sequences has been already found in the genomes of several yeast species (Kimura *et al.* 2002; Nomura *et al.* 2003; Nomura and Takagi 2004). A blast search performed in the *Génolevures2* protein database (Dujon *et al.* 2004) detected homologous sequences also in *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Debaryomyces hansenii* and *Candida glabrata*. Exploring the *NCBI* protein database, we also found homologous proteins in *Candida albicans*. On the other hand, a blast search in the partially sequenced *Z. rouxii* genome in the *Génolevures1* database (de Montigny *et al.* 2000) did not reveal any *ScMPR1* homologues.

To test the sensitivity or resistance of *Z. rouxii* wild-type strains, (CBS 732, *i.e.* ATCC 2623 and ATCC 42981) to AZC, a drop test was performed (Fig. 1). Resistant (Σ1278b) and sensitive (S288C) *S. cerevisiae* strains were used as controls. After inoculation of stationary cells, only *S. cerevisiae* Σ1278b cells harboring the *MPR1* gene started to grow on plates with AZC. This test showed that *Z. rouxii* wild-type cells were sensitive to AZC, and similar sensitivity to AZC as for wild types was observed for *Z. rouxii* auxotrophic mutants (UL4 and MA11; *not shown*).

To study the function of *ScMPR1* gene in *Z. rouxii*, and its possible use as a dominant selection marker, *Z. rouxii* UL4 cells were transformed with YCpAZC or "empty" YCplac33 (as the control). The YCpAZC plasmid was constructed because 2 μ-based vectors are not replicable in *Z. rouxii*, and thus the original multicopy pMH1 could not be used. The use of centromeric plasmid (YCpAZC) harboring the *ScMPR1* gene with its own promoter and the *ScURA3* marker made it possible to select transformants for uracil prototrophy and/or for AZC resistance. First, cells were selected for uracil prototrophy on minimal YNB-NH_4 media, and Ura$^+$ colonies were then tested for AZC resistance (Fig. 2). Only *Z. rouxii* cells harboring the YCpAZC plasmid were able to grow on media with 0.5 g/L AZC, thus the *ScMPR1* gene proved to be transcribed from its own promoter in *Z. rouxii* cells, and its product was functional in AZC-detoxication.

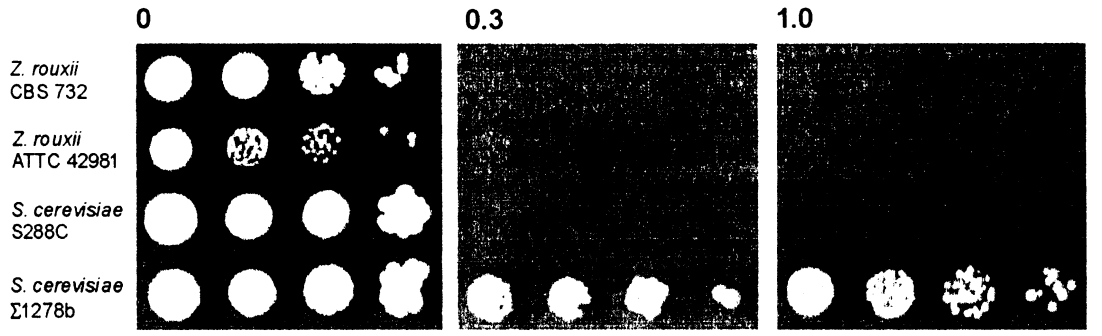

Fig. 1. Growth of *Z. rouxii* on YNB-NH_4 medium supplemented with 0, 0.3 and 1.0 g/L AZC; strains *S. cerevisiae* S288C (AZC-sensitive) and Σ1278b (AZC-resistant) were used as controls.

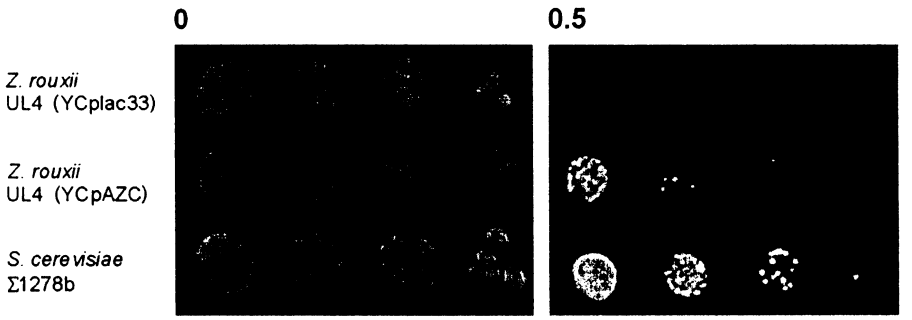

Fig. 2. Growth of *Z. rouxii* UL4 transformants on YNB-NH_4 medium with 0 and 0.5 g/L AZC; *S. cerevisiae* Σ1278b was used as positive control.

To test the possible use of *ScMPR1* as a dominant selection marker in *Z. rouxii*, ATCC 42981 wild-type cells were transformed with the YCpAZC plasmid and transformants were selected directly on YNB-NH_4 media supplemented with 0.5 g/L AZC. A very high number of colonies appeared on the negative control (without plasmid DNA added), which made it impossible to distinguish between transformants and non-transformed cells. This experiment suggested that exponentially growing cells used for the electroporation procedure were more resistant to AZC compared to stationary cells used in drop tests.

The AZC sensitivity of two wild-type *Z. rouxii* strains exponentially growing in liquid YPD + 300 mmol/L NaCl, *i.e.* under conditions used for *Z. rouxii* transformation was tested. Exponentially growing cells ($\approx 3 \times 10^6$) were directly spread on plates supplemented with AZC. On rich medium (YPD), a very high number of colonies of both strains appeared even in the presence of the highest concentration of AZC (0.9 g/L) after 3–4 d. In the case of YNB-NH_4 medium supplemented with AZC, the ATCC 42981 strain formed thousands of colonies, the CBS 732 strain only about 20 colonies per plate (the number of colonies did not vary at different AZC concentrations tested). To verify the stability of the AZC-resistant phenotype, ten ATCC 42981 colonies were transferred on a new YNB-NH_4 plate with AZC. The cells also grew there, the phenotype was stable, and cells had gained resistance. This result confirmed that exponentially growing cells cultured for efficient electroporation were able to better resist the toxic effect of AZC than stationary cells. Thus the selection of transformants directly on media with AZC was not possible for the ATCC 42981 strain. For the CBS 732 strain (and thus also for its derivative UL4), the YNB-NH_4 medium with AZC could be considered a selective one. This strain always formed several colonies on this medium, both at low (0.3 g/L) and high (0.9 g/L) AZC concentrations. The concentration of 0.5 g/L AZC was chosen for further experiments as the background growth could represent only a minority of colonies that appear after electroporation.

Z. rouxii UL4 cells were transformed with the YCpAZC vector (0.2 μg) and transformants were selected on YNB-NH_4 medium with 0.5 g/L AZC and 15 mg/L uracil (uracil was added so that the only selective pressure was the presence of toxic AZC). Several tens of colonies appeared on the selection medium when the cells were transformed with YCpAZC and also on the negative control (cells subjected to the transformation procedure without plasmid DNA; Table I). Because the numbers of AZC-resistant colonies were

very similar for cells without YCpAZC and for cells harboring the *ScMPR1* gene on plasmid (60 *vs.* 100, *cf.* Table I), our next approach was to lower the undesirable background growth.

Table I. Transformation of *Z. rouxii* UL4 by the YCpAZC plasmid

Plasmid DNA[a]	*n*[b]
None	0
	60[c]
pLU1[d]	200
YCpAZC	180
	100[c]

[a]0.2 μg.
[b]Number of colonies growing on the YNB-NH_4 medium after electroporation.
[c]Supplemented with 0.5 g/L AZC + 15 mg/L uracil.
[d]Positive control.

As shown above *Z. rouxii* stationary cells were more sensitive to AZC than exponentionally growing cells; the possibility of rendering the transformed *Z. rouxii* cells more sensitive to the AZC effect by starvation was therefore tested. *Z. rouxii* MA11 cells were transformed with the pLU1 vector, and starved for 2 h prior to plating. The yield of transformants after this procedure was 5× lower than in cells plated within 20 min after electroporation. Starvation after the electroporation greatly reduced transformation efficiency, and therefore could not be an effective approach to improve the selection of transformed cells on AZC-containing plates.

Another possible way to lower the background growth after transformation was to change the nitrogen source (ammonium) in the selection media for a nonrepressing nitrogen source (*i.e.* proline, urea). It was shown that AZC as a proline analogue is efficiently imported into *S. cerevisiae* cells by four amino acid transporters, including two nitrogen-regulated transporters (*Sc*Put4 and *Sc*Gap1) and two transporters that are regulated by the SPS sensor of extracellular amino acids (*Sc*Agp1 and *Sc*Gnp1) (Andreasson *et al.* 2004). *Sc*Gap1 ("general amino acid permease") and *Sc*Put4 (proline-specific transporter) are inactive in the medium with ammonium as nitrogen source. In the presence of a nonrepressing, poor nitrogen source, these transporters are active and facilitate the uptake of amino acids and their analogues, including proline and AZC, into cells (ter Schure *et al.* 2000). Hence, more AZC molecules might be transported into the cell and the toxic effect enhanced during growth with a poor nitrogen source. On the other hand, AZC is a toxic competitor of L-proline (Takagi *et al.* 1997), and less AZC might enter the cells in the presence of L-proline. To test the influence of the nitrogen source on the *Z. rouxii* (CBS 732 and ATCC 42981) resistance to AZC, cells were grown on YNB-NH_4, YNB-Pro and YNB-urea plates containing different concentrations of AZC (0–1 g/L) with *S. cerevisiae* Σ1278b used as a resistant control. Plates were inoculated with cells cultured to the mid-exponential growth phase in the liquid YPD medium. In the case of urea, only the growth of *Z. rouxii* CBS 732 and *S. cerevisiae* Σ1278b was tested, because the ATCC 42981 strain was not able to use urea as a nitrogen source. A better growth of both *Z. rouxii* wild types with AZC when L-proline was used as a nitrogen source was found. This suggested a competition between L-proline and AZC (probably on the level of transport) as had been described for *S. cerevisiae* and tobacco cells (Takagi *et al.* 1997; Zhang *et al.* 2004). This enhanced AZC resistance of *Z. rouxii* cells was not observed when urea was used as a nitrogen source, and the cell growth on YNB-urea plates in the presence of AZC was inhibited similarly as on YNB-NH_4 + AZC.

To test the use of YNB-urea + AZC for selection of AZC-resistant transformants, UL4 was transformed with the YCpAZC vector (0.2 μg) and transformants were selected on YNB-urea medium containing 0.5 g/L AZC and 15 mg/L uracil. Several tens of colonies appeared on the selective medium when the cells were transformed with YCpAZC and also on the negative control. Spontaneously resistant cells appeared at a frequency of 7.5×10^{-6}. Thus, similar results with urea and ammonium as nitrogen sources were obtained. Cells after the transformation procedure showed a higher resistance to AZC toxicity; we were not able to distinguish between transformants expressing the *ScMPR1* gene and colonies originated from spontaneously resistant cells.

The use of *ScMPR1* as a dominant selection marker in its natural host *S. cerevisiae* was examined. AZC-sensitive *S. cerevisiae* FRJ1 was transformed with YCpAZC and transformants were selected on YNB-NH_4 supplemented with 0.5 g/L AZC and 15 mg/L tryptophan and uracil. As for *Z. rouxii* transformation, several tens of spontaneously resistant colonies appeared on negative-control plates (no plasmid DNA added). The same result was obtained when the pMH1 plasmid (episomal, *i.e.* with more copies of *ScMPR1* per cell) was used for transformation. There was no significant difference in the number of colonies on negative-control plates and on plates with pMH1 transformed cells. Both *Z. rouxii* UL4 and *S. cerevisiae* FRJ1 expressing *ScMPR1* could grow on YNB-NH_4 medium with AZC, but the positive selection for transformants on AZC-containing medium was not possible due to the presence of spontaneously resistant colonies, and a first selection based on complementation to prototrophy was necessary. Similar results have also been obtained for *ScMPR1* expression in tobacco plants (Zhang *et al.* 2004). The use of a strong promoter for the *MPR1* gene expression in *Z. rouxii* cells would probably not help, as many spontaneously resistant colonies appear after transformation. Whether these colonies result from a sudden switch of the *Z. rouxii MPR1* homo-

logue expression in some cells remains to be established when the complete genome sequence of *Z. rouxii* genome is available.

Although the *ScMPR1* gene is functional in AZC detoxication in *Z. rouxii*, it cannot be used as a dominant selection marker for this yeast species.

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5.1.3. Publikace č. 3: Characterization of *Zygosaccharomyces rouxii* centromeres and construction of first *Z. rouxii* centromeric vectors

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Pro expresi genů v kvasinkách jsou často používány centromerové plasmidy, které zajistí stabilní přenášení genu do dalších generací hostitele a jeho přítomnost v buňkách v max. dvou kopiích. Takové plasmidy pro *Z. rouxii* dosud neexistovaly. Pro expresi genů sice bylo možno použít centromerové plasmidy *S. cerevisiae*, protože ARS *S. cerevisiae* je rozeznávána i replikačním aparátem *Z. rouxii*, ale nebyla zajištěna regulace počtu kopií plasmidu v buňce ani jeho stabilní pomnožování (Ushio *et al.*, 1988). Sekvence centromery *Z. rouxii* dosud nebyla známa.

Za účelem konstrukce centromerových plasmidů pro *Z. rouxii* jsme v této práci identifikovali a funkčně charakterizovali centromery *Z. rouxii*. V databázi Génolevures 1 jsme na základě homologie s oblastmi DNA blízce příbuzné kvasinky *S. cerevisiae* obsahujícími centromery identifikovali čtyři fragmenty představující patrně centromery *Z. rouxii*. Dva fragmenty jsme vybrali pro funkční analýzu. Úseky představující pravděpodobné centromery jsme vložili do plasmidu obsahujícího pouze ARS *S. cerevisiae*, a funkčnost plasmidů jsme sledovali jak v buňkách *Z. rouxii*, tak *S. cerevisiae*. Prokázali jsme, že v *Z. rouxii* plasmidy fungují jako centromerové, a fragmenty tak skutečně představují centromery. Hybridizační analýzou DNA-DNA (Southern blot) jsme zjistili, že se jedná o centromery chromosomů II. resp. VII. Centromery jsme potvrdili jako druhově specifické - plasmid nesoucí centromeru *Z. rouxii* nebyl rozpoznán jako centromerový v *S. cerevisiae* a naopak.

Zjistili jsme, že centromery *Z. rouxii* mají obdobnou strukturu jako centromery blízce příbuzných kvasinek *S. cerevisiae* nebo *K. lactis* - též jsou uspořádané do oblastí CDE I, II a III, přičemž úsek CDE II *Z. rouxii* je delší než příslušný úsek *S. cerevisiae* a kratší než CDE II *K. lactis*. Právě rozdílná délka tohoto úseku patrně z velké části přispívá k druhové specificitě kvasinkových centromer.

Vzhledem k sekvenční homologii pravděpodobně představují všechny čtyři fragmenty identifikované v této práci centromery *Z. rouxii*. Jejich sekvence byla odeslána do databáze GenBank. Připravené plasmidy představují první centromerové plasmidy *Z. rouxii*.

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Characterisation of *Zygosaccharomyces rouxii* centromeres and construction of first *Z. rouxii* centromeric vectors

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Abstract

Zygosaccharomyces rouxii is a hemiascomycetous yeast known for its high osmotolerance, the basis of which still remains unknown. By exploring the Génolevures I database, four *Z. rouxii* fragments homologous to *Saccharomyces cerevisiae* centromeres were identified. Two of them were subjected to further analysis. Their function as centromeres in *Z. rouxii* was proved, and they were localized to *Z. rouxii* chromosomes II and VII, respectively. The species-specificity of centromeres was observed; plasmids with a *Z. rouxii* centromere were not recognized as centromeric in *S. cerevisiae*, and a *S. cerevisiae* centromere did not function as a centromere in *Z. rouxii*. Constructed plasmids bearing *Z. rouxii* centromeres serve as the first specific centromeric plasmids, and thus contribute to the so-far limited set of genetic tools needed to study the *Z. rouxii* specific features.

Introduction

Zygosaccharomyces rouxii is a non-conventional hemiascomycetous yeast closely related to *Saccharomyces cerevisiae*. Unlike this model species and most other yeasts, *Z. rouxii* is capable of growing in highly concentrated environments (3.4 M NaCl, 60% sugar; Barnett *et al.* 1990, Hosono 1992), which makes it one of the most osmotolerant yeast species. However, the basis of its unique osmotolerance still remains unknown. The close relation between *Z. rouxii* and *S. cerevisiae* could result in interesting biotechnological applications for *S. cerevisiae*. *Z. rouxii*'s high osmotolerance is supposedly due to specific genes; the identification and cloning of these genes and their expression in industrial strains of *S. cerevisiae* could then improve the growth and fermentation capacities of industrial strains under adverse conditions. The identification of *Z. rouxii* specific genes remains difficult, as the system of genetic tools for manipulating *Z. rouxii* DNA is not well established—so far, only two auxotrophic mutants (*leu2* and *ura3*) and few multicopy vectors are available (Ushio *et al.* 1988, Pribylova & Sychrova 2003).

To develop new tools, e.g. stable monocopy plasmids, the identification and functional characterization of centromeric sequences is necessary. The identification of *Z. rouxii* centromeres can also assist in studying yeast evolution: the organization of a centromeric sequence and its adjacent genes is related to speciation and represents relevant information to adjust the position of the species in the phylogenetic tree.

Z. rouxii together with *Kluyveromyces lactis* belong to the *Kluyveromyces* clade and are closely related to the *Saccharomyces* clade (Souciet *et al.* 2000). *S. cerevisiae* and *K. lactis* share similar organization of their centromeric sequences. The *S. cerevisiae* centromere is a short DNA sequence 125 bp in size, a so-called point centromere, and is composed of three CDE elements: an 8 bp sequence PuTCACPuTG (CDE I), followed by a 78–86 bp AT rich (> 90%) region (CDE II) and a 25 bp sequence TGTTT(T/A)TGNTTTCCGAAANNNAAAAA (CDE III; Clarke 1998). In *K. lactis* the centromere is also organized into CDE blocks, as in *S. cerevisiae*, but the CDE II region is about twice as long (161–164 bp; Heus *et al.* 1993). The organization of a centromere into three CDE regions is also found in *Candida glabrata*, but not in other budding yeasts such as *Debaryomyces hansenii*, *Yarrowia lipolytica* or *C. albicans* (Dujon *et al.* 2004, Sanyal *et al.* 2004).

During the Génolevures I project a low-coverage genomic exploration of *Z. rouxii* DNA was performed (de Montigny *et al.* 2000). A set of 3000 random sequence tags (RST) was obtained and further analysed. This set of sequences provided a tool for searching for potential *Z. rouxii* centromeres.

In this study four *Z. rouxii* putative centromeric sequences were identified. Two of them were further analysed by a plasmid-based assay and chromoblot analysis. They were proved to function as centromeres in *Z. rouxii*, but not in *S. cerevisiae*. The newly identified *Z. rouxii* centromeres made it possible to construct the first centromeric, i.e. low-copy and stably propagated plasmids for this yeast species.

Materials and methods

Strains and growth conditions

Z. rouxii CBS 732 (wt) was used for the isolation of the putative *Z. rouxii* centromeres and chromoblot analysis. *Z. rouxii* UL4 (*ura3*; Pribylova & Sychrova 2003) and *S. cerevisiae* W3031A (*MAT*a *ade 2-1 can1-100 his3-11/15 leu2-3/112 mal10 trp1-1 ura3-1*; Wallis *et al.* 1989) were used as hosts for the plasmids. Non-transformed cells were grown in YPD media. Transformants were grown on standard YNB-NH_4 plates with auxotrophic supplements (20 μg/ml) when required. For growth curves an overnight preinoculum was used: 20 ml of the medium was then inoculated with the preinoculum to a density of A_{600} 0.06 and cultures were cultivated at 30°C, 200 rpm.

Plasmids

Plasmids were yeast/*E. coli* shuttle vectors (*ScARS1*, *ori*, Amp^R) based on pUC19 (Yanisch-Perron *et al.* 1985) (Table 1). pLU1 contained a *S. cerevisiae* centromere. pZCA containing a 816 bp fragment of the putative *Z. rouxii* centromere A and pZCC with a 588 bp fragment of the putative *Z. rouxii* centromere C were constructed by PCR-based cloning: Genomic DNA of *Z. rouxii* CBS 732 was used as a template, prepared as previously described (Hoffman & Winston 1987). The primers are listed in Table 2. The *Nhe*I (forward primers) or *Spe*I (reverse primers) restriction sites, respectively were introduced into the 5′ ends of primers. The amplified fragments were digested by *Nhe*I and *Spe*I and cloned into YCplac33 (Gietz & Sugino 1988) instead of the *Spe*I-*Nhe*I fragment (1251 bp) containing *ScCEN4*. YRp33 without a centromeric sequence was constructed from YCplac33 by removing the *Spe*I-*Nhe*I fragment (1251 bp) containing *ScCEN4* and circularization of the resulting 4.4 kb *Spe*I-*Nhe*I fragment by ligation.

Yeast transformation

Cells of both yeast species were transformed by electroporation in 0.2 cm cuvettes. *Z. rouxii* UL4 cells were transformed according to Pribylova &

Table 1. Plasmids used for *Z. rouxii* and *S. cerevisiae* transformation

Plasmid	Size (kb)	Centromere	Marker gene	Reference
pZCA	5.2	*Z. rouxii* A	*ScURA3*	This work
pZCC	5.0	*Z. rouxii* C	*ScURA3*	This work
YRp33	4.4	none	*ScURA3*	This work
pLU1	8.2	*ScCEN4*	*ScURA3*, *ZrLEU2*	Pribylova & Sychrova 2003
YCplac33	5.6	*ScCEN4*	*ScURA3*	Gietz & Sugino 1988

Table 2. Primers used for cloning of *Z. rouxii* fragments containing putative centromeres A and C

Primer	Sequence (5′–3′)
Af	CTCTACTAGTAGGCATTTGTTTATTACCAG
Ar	CAGTGCTAGCATCGATCGGGCACTAACTTAGTGTCA
Cf	CTCTACTAGTCAGTATACTCTTGGTTAGAT
Cr	CAGTGCTAGCATCGATATAGCTTAAATATTTAGTT

f. Forward primer; r. reverse primer. *Spe*I and *Nhe*I restriction sites are underlined.

Sychrova (2003) with slight modifications: cells were washed with 1 M sorbitol and electroporation was performed using an Eppendorf Electroporator (9 kV/cm; 6 ms). *S. cerevisiae* cells were treated before electroporation according to Meilhoc *et al.* (1990), and an electric pulse (3.13 kV/cm, 24 ms) was applied in the GHT 1287-B Jouan electroporator.

DNA manipulations

DNA manipulations were performed according to standard protocols (Sambrook *et al.* 1989). To avoid mismatch base pairing during the synthesis of PCR products, *Pfx* DNA polymerase (Invitrogene) with intrinsic proofreading activity was used. DNA sequencing was performed in the ABI PRISM 3100 DNA sequencer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Plasmid loss test

Transformants carrying the plasmids were grown with shaking in 20 ml YPD at 30°C for 24 h. The cultures were then diluted and spread on YPD media to obtain single colonies (approximately 200 colonies per plate). Colonies were replica plated on YNB-NH_4 media with or without uracil and the percentage of colonies that were unable to grow without uracil (i.e. lost the plasmid) was estimated.

Pulse-field gel electrophoresis (PFGE)

Entire chromosomal DNA was prepared according to a protocol designed for *S. cerevisiae* (Vezinhet *et al.* 1990) with slight modifications: lyticase from *Arthrobacter luteus* (Sigma) was used for cell treatment (100 μg/1.5 × 10^9 cells/ml). The 1% agarose gels were prepared from pulse-field certified agarose (Bio-Rad Laboratories) and run on a Bio-Rad CHEF (contour-clamped homogeneous electric field) apparatus in 0.5 × TBE at 14°C. The operating conditions were: switch time 300 s, run time 100 h, angle 106°, voltage 3 V/cm.

Southern blot and chromoblot analyses

For Southern blot analysis the total DNA was isolated (Hoffman & Winston 1987) and digested with *EcoR*I and *BamH*I. DIG-labelled DNA probes were prepared using a DNA labelling and detection kit (Roche). Oligonucleotides used for probe amplification were the same as for plasmid construction (cf. Table 2). After hybridization the membranes were photographed with a digital camera (Nikon Coolpix 4500) and densitometry analyses of the Southern blot digital images were performed using the computer program Aida 3.28 (Advanced Image Data Analyzer, Germany). Chromoblot analysis was performed using the same probes and membranes containing the chromosomes previously separated by PFGE.

Results and discussion

In-silico identification of putative centromeres

As described previously (Sychrova *et al.* 2000), the *Z. rouxii* CBS 732 genome consists of seven chromosomes. To identify the *Z. rouxii* centromeres, the set of sequences obtained by partial sequencing of *Z. rouxii* CBS 732 genomic DNA during the Génolevures I project was used.

As the specific regions of centromeres (CDE blocks) are short and not conserved enough (especially CDE II) among yeasts, a blast(n) analysis could not be applied to identify the centromeric sequences in the large set of Génolevures I data. For this purpose we selected all the genomic DNA inserts with the first RST including a centromeric

orthologous gene located on the right arm of a defined chromosome and the second RST including a centromeric orthologous gene on the left arm of the same chromosome (for example YLL001 and YLR001). With chromosomal map conservation between *Z. rouxii* and *S. cerevisiae*, the non-sequenced DNA region between the two RST could contain a *Z. rouxii* centromere. The sequencing of these regions revealed four putative *Z. rouxii* centromeric sequences (A, B, C and D).

Z. rouxii putative centromeres (fragments A, B, C and D) were organized into CDE I, II and III blocks, similarly to the centromeres of *S. cerevisiae* or *K. lactis* (Figure 1). The CDE I and III regions were well conserved and flanked the CDE II AT-rich area. In the CDE I region the TCA motif was conserved among all three yeasts, as was also the case for the TTCCGAA motif in the CDE III region. However, the *Z. rouxii* CDE II region proved to be longer (134–136 bp) than *S. cerevisiae* CDE II (78–86 bp) but shorter than *K. lactis* CDE II (161–164 bp). ORF flanking the *Z. rouxii* A, B and C putative centromeres were orthologues of *S. cerevisiae* ORF flanking *CEN12*, *CEN15* and *CEN7*, respectively. Putative centromere D was surrounded by orthologues of *YNL001w* (neighbour of *S. cerevisiae CEN14*) and *YCR002c* (neighbour of *S. cerevisiae CEN3*). As *S. cerevisiae* has 16 chromosomes (Goffeau *et al.* 1996) and *Z. rouxii* only seven (Sychrova *et al.* 2000), it is evident that there were different rearrangements of genomes during the evolution of these two relatives, e.g. whole genome duplication in a *S. cerevisiae* ancestor. The differences in the surroundings of the *Z. rouxii* and *S. cerevisiae* centromeres could result from those rearrangements.

Functional analyses of putative centromeres

For further functional analyses of the putative *Z. rouxii* centromeres, A and C DNA fragments were chosen. In order to prove that they act as centromeres in *Z. rouxii*, we used a functional approach (Murphy & Fitzgerald-Hayes 1990). The putative *Z. rouxii* centromeres were inserted into a plasmid containing a selectable marker gene and a sequence that enables plasmid replication (autonomously replicating sequence, ARS). We wanted to demonstrate that such a plasmid (a) would be stably propagated in *Z. rouxii* and (b) would be present in only one or two copies per cell. These properties are attributed exclusively to a centromeric plasmid by the presence of a functional centromere. The centromere in the plasmid uses the same partitioning machinery as chromosomes, which explains why a plasmid will be propagated in generations almost as stably as an entire chromosome; an excess of centromeres in a yeast cell causes chromosome missegregation and thus is toxic for the cell.

For *Z. rouxii* the ARS still remains unknown, although it has already been shown that a plasmid carrying *S. cerevisiae ARS1* was replicable in *Z. rouxii* (Ushio *et al.* 1988) and the *ScURA3* gene complemented the *Z. rouxii ura3* mutation (Pribylova & Sychrova 2003). To prepare plasmids with putative *Z. rouxii* centromeres the YCplac33 plasmid was used. Its *ScCEN4* fragment was replaced with

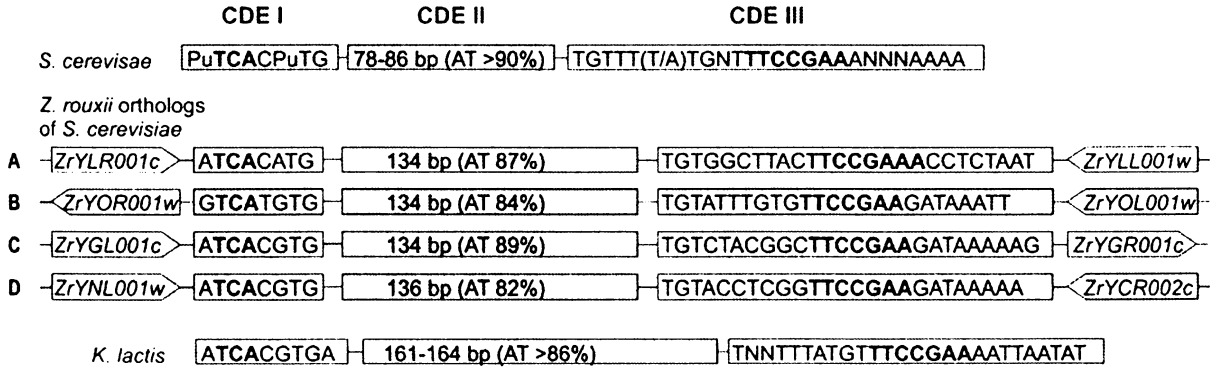

Figure 1. *Z. rouxii* putative centromeres and their comparison with *S. cerevisiae* and *K. lactis* centromere consensus. Rectangles represent CDE regions, arrows symbolize *Z. rouxii* orthologues of *S. cerevisiae* genes flanking the CDE regions, bars represent non-coding regions. Conserved sequence motifs are in bold. The GeneBank Accession Nos. for A, B, C and D are AJ298246, EF512461, AJ303361 and AJ303360.

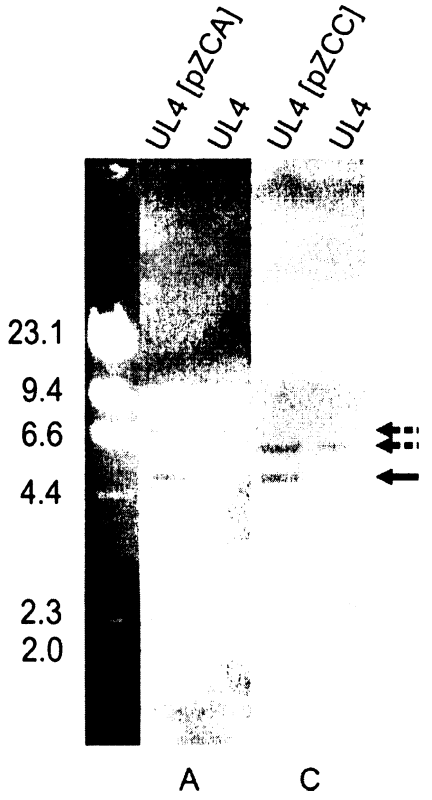

Figure 2. Southern blot analysis of *Z. rouxii* UL4 transformants. DNA isolated from UL4 [pZCA] was digested with *EcoRI* (a unique site in the pZCA backbone); DNA from UL4 [pZCC] with *BamHI* (a unique site in the pZCC backbone). Thus, a probe against the A fragment detected a linearized pZCA plasmid and a single larger genomic *EcoRI* fragment. Similarly, the C-fragment probe visualized the linearized pZCC and a genomic fragment containing the C sequence. As control, the labelling of a non-transformed *Z. rouxii* UL4 genomic DNA digested with *EcoRI* or *BamHI* is shown. Broken arrows indicate bands corresponding to chromosomal centromeric sequences, full arrows point to bands corresponding to plasmid DNA. A: DNA labelled with the probe against the *Z. rouxii* putative centromere A; C: DNA labelled with the probe against the putative *Z. rouxii* centromere C.

fragments carrying *Z. rouxii* putative centromeres A or C, respectively, which resulted in the pZCA (carrying the putative centromere A) and pZCC (carrying the putative centromere C) construction (Table 1). *Z. rouxii* UL4 cells were transformed with pZCA, pZCC and YRp33 (as a control) and Ura^+ transformants were selected. The cells transformed with a plasmid without a centromere (YRp33) did not show a stable Ura^+ phenotype. To verify the pZCA and pZCC stability in cells, the transformants were grown under non-selective conditions (in YPD) for 24 h. After this cultivation the loss of the Ura^+ phenotype (i.e. loss of the plasmid carrying the selectable *ScURA3* gene) was estimated (Table 3). Both plasmids were stably maintained in *Z. rouxii* cells, even under non-selective conditions. The difference in plasmid losses (0.2% for pZCA versus 3.1% for pZCC) could be due to different lengths of the centromeric fragments A and C present on the plasmids (816 bp and 588 bp, respectively) and/or to different lengths of entire plasmids, or to the different effect of circularization of the centromeres on their function. The efficient propagation of the plasmids in *Z. rouxii* was also confirmed by growth curves in selective YNB-NH_4 medium: the growth of *Z. rouxii* UL4 [pZCA] or [pZCC] transformants was similar to the growth of the non-transformed strain in YNB-NH_4 + uracil or to the growth of wild-type (CBS 732) cells in YNB-NH_4 (Figure 3).

To confirm that pZCA and pZCC really represent centromeric, i.e. low-copy plasmids for *Z. rouxii*, their copy number in *Z. rouxii* transformants was estimated by Southern blot analysis (Figure 2). In both cases two bands were obtained–one corresponding to the chromosomal copy of A or C, the other to the A or C fragments present in pZCA and pZCC plasmids, respectively. The intensity of bands revealed, when assuming that a haploid cell contains a single chromosomal copy of the centromere per cell,

Table 3. Transformation efficiency and plasmid loss of pZCA, pZCC and pLU1 in yeast hosts

Plasmid	Number of transformants/μg DNA		Plasmid loss (%)	
	Z. rouxii	*S. cerevisiae*	*Z. rouxii*	*S. cerevisiae*
pZCA	1.4×10^4	1.1×10^4	0.2	63.2
pZCC	1.6×10^4	1.5×10^4	3.1	64.8
pLU1	1.3×10^4	1.0×10^4	64.7	7.7

Values are averages from three independent analyses.

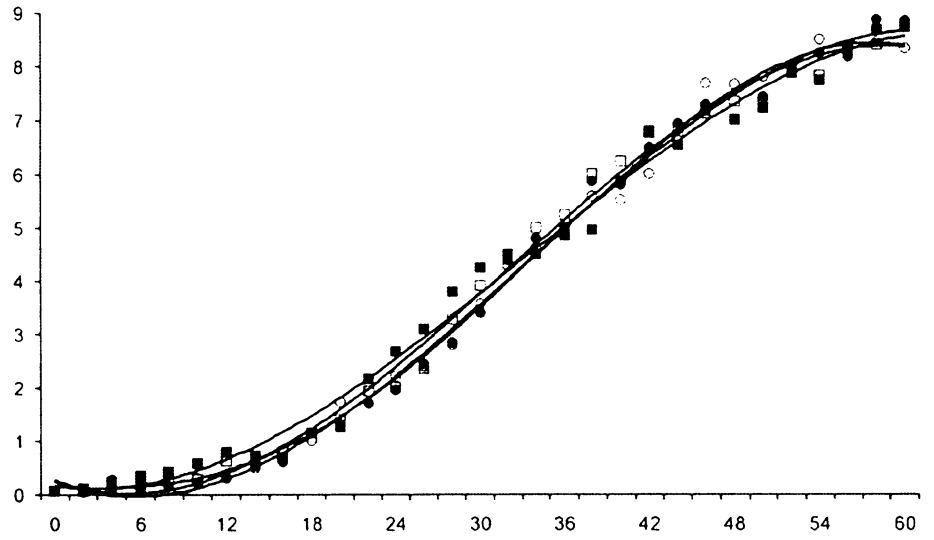

Figure 3. Growth curves of *Z. rouxii* UL4 [pZCA] and [pZCC] transformants in the minimal YNB-NH_4 medium. As a control, the growth of *Z. rouxii* UL4 in YNB-NH_4 medium supplemented with uracil and of *Z. rouxii* CBS 732 in YNB-NH_4 is shown. Symbols: ■, *Z. rouxii* UL4 [pZCA]; ●, *Z. rouxii* UL4 [pZCC]; □, *Z. rouxii* UL4; ○, *Z. rouxii* CBS 732.

that the copy numbers of pZCA and pZCC corresponded to 1.1 and 1.0 per cell, respectively. These results confirmed that pZCA and pZCC behaved as stable and monocopy, i.e. centromeric plasmids in *Z. rouxii*.

To reveal which *Z. rouxii* chromosomes the A and C sequences belong to, a chromoblot analysis was performed with the same probes as used for the Southern blot analysis. The probe against putative centromere A hybridized to chromosome II, the other one to chromosome VII of *Z. rouxii* CBS 732 (not shown).

Species-specificity of centromeres

Our data showed that all plasmids efficiently transformed both *Z. rouxii* or *S. cerevisiae* hosts ($1–2 \times 10^4$ transformants/μg DNA), i.e. *ScARS1* was replicable in both yeast species (Table 3). However, only the plasmids carrying the putative *Z. rouxii* A or C centromeres were stably maintained in *Z. rouxii* although, on the other hand, they were unstable in *S. cerevisiae* transformants. Similarly, pLU1 carrying the *S. cerevisiae* centromere was stable in *S. cerevisiae*, but not in *Z. rouxii*. These results showed that the A and C inserts were recognized as centromeres only in *Z. rouxii*, and *ScCEN4* was recognized as a centromere only in *S. cerevisiae*. This species-specificity of centromeres might be due to the length of the CDE II region, which was previously shown as crucial in *S. cerevisiae* (Murphy & Fitzgerald-Hayes 1990). The *Z. rouxii* CDE II region may be too long to enable the *Z. rouxii* centromere to adopt a functional conformation in *S. cerevisiae*, and the *S. cerevisiae* centromere may be equally too short to function properly in *Z. rouxii*. The species-specificity of centromeres was also observed for *K. lactis* (Heus *et al.* 1993). Our results thus correspond to a general assumption that yeast centromeres do not function in other species, even in closely related ones. From our results and those obtained for *K. lactis* (Heus *et al.* 1993), it can be concluded that it is mainly the length and sequence of the CDE II region that underlies this centromere specificity.

Conclusions

Our functional analysis of A and C DNA fragments proved that they represent the *Z. rouxii* centromeres of chromosomes II and VII, and that these *Z. rouxii* centromeres are species-specific. Based on the sequence similarity and synteny with the *S. cerevisiae* genes flanking the centromeres, it can be assumed that the other two identified DNA fragments (B and D) may also represent *Z. rouxii* centromeres. The remaining three centromeres can be identified

using the same approach (searching for neighbour orthologues of genes flanking *S. cerevisiae* centromeres) when the whole *Z. rouxii* genome sequence is available (the Génolevures III project). The pZCA and pZCC plasmids constructed in this work represent the first low-copy, stably propagated centromeric plasmids for *Z. rouxii* and can serve for genetic manipulations of this osmotolerant yeast species, which is by its phylogenetic position an interesting preduplication model.

Acknowledgements

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5.1.4. Publikace č. 4: Tools for the genetic manipulation of *Zygosaccharomyces rouxii*

Přibylová, L., de Montigny, J., Sychrová, H.

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Vytvoření transformačního protokolu, možnost využití *ScMPR1* jako pomocného selekčního genu a příprava centromerových plasmidů představovaly rozšíření do té doby limitovaných možností pro genové manipulace v *Z. rouxii*. V kvasinkách, a zvláště v modelové kvasince *S. cerevisiae*, však nástrojů pro genové manipulace existuje celá řada. Velmi často se například používají pro expresi genů episomální plasmidy (s replikonem 2μ), které zajistí, že vnášený gen bude v buňkách stabilně pomnožován v několika kopiích (viz kap. 2.3.2.1.). Tyto plasmidy však v *Z. rouxii* není možné použít, protože replikační aparát *Z. rouxii* nerozeznává 2μ. *Z. rouxii* nicméně obsahuje přirozený plasmid pSR1, který je velmi podobný 2μ, a který již byl využit pro konstrukci plasmidů (Ushio *et al.*, 1988); jednalo se však o plasmidy neobsahující polylinker, který by umožnil vnášení genů.

Další velmi užitečnou technikou pro genové manipulace v kvasinkách je možnost cílené delece genů prostřednictvím delečních kazet a homologní rekombinace (viz kap. 2.3.3.). V kvasince *Z. rouxii* již byla potvrzena možnost cílených disrupcí/delecí genů využívajících homologní rekombinaci (Iwaki *et al.*, 1999; Tang *et al.*, 2005), nebyl však k dispozici nástroj umožňující vícenásobné delece. Ani jedna z disrupcí/delecí navíc nebyla provedena ve kmeni s genetickým pozadím CBS 732^T.

Pro expresi genů v kvasinkách je též výhodné využít regulovatelných promotorů (jedním z nejčastěji používaných regulovatelných promotorů je *ScGAL1*) a značení jejich proteinových produktů pro určení jejich lokalizace v buňkách (GFP; viz kap. 2.3.2.). Takové možnosti pro *Z. rouxii* zatím nebyly.

V této práci jsme proto metody pro genové manipulace *Z. rouxii* rozšířili do té míry, abychom získali soubor nástrojů umožňující jak expresi genů z různých plasmidů, tak vícenásobné delece genů. Jelikož jediným dosud dostupným auxotrofním mutantem odvozeným od typového kmene *Z. rouxii* CBS 732^T byl kmen *Z. rouxii* UL4, rozšířili jsme možnosti selekce o další auxotrofní mutanty a plasmidy obsahující příslušné selekční geny. Byly připraveny kmeny nesoucí různé kombinace mutací *ura3*, *leu2* a *ade2* a zkonstruovány specifické episomální a centromerové plasmidy s různými auxotrofními selekčními geny

(*ScURA3*, *ZrLEU2* a *ZrADE2*) a polylinkerem. Pro konstrukci episomálních plasmidů byla na základě dostupných informací o plasmidu pSR1 využita část jeho sekvence zajišťující replikaci a stabilitu plasmidů v buňkách. Sada centromerových plasmidů byla připravena odvozením od centromerového plasmidu zkonstruovaného v rámci publikace č. 3. Sekvence připravených plasmidů byly odeslány do databáze GenBank. Byl vytvořen systém pro vícenásobnou deleci genů v *Z. rouxii* využívající opakovatelně použitelné deleční kazety *loxP-kanMX-loxP* připravené prostřednictvím PCR a plasmidu nesoucího rekombinasu *cre*. Pro umožnění lokalizace proteinů v buňkách *Z. rouxii* byl zkonstruován plasmid pZGFP. Byla testována funkce promotoru *ScGAL1* v *Z. rouxii* – ukázalo se, že k expresi genů pod kontrolou tohoto promotoru dochází v *Z. rouxii* i při růstu buněk na glukose, a tento promotor tak není možno použít pro konstrukci plasmidů pro regulovatelnou expresi genů.

Vytvořené metody představují soubor nástrojů genového inženýrství využitelný pro genové manipulace kvasinky *Z. rouxii*, a tak významně rozšiřují možnosti studia specifických vlastností této kvasinky.

Tools for the genetic manipulation of *Zygosaccharomyces rouxii*

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Running title: *Zygosaccharomyces rouxii* genetic tools

Keywords

Zygosaccharomyces rouxii; auxotrophic mutants; centromeric plasmid; episomal plasmid; targeted gene deletion; GFP-tagging

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Abstract

A set of tools for the genetic manipulation of the osmotolerant yeast *Zygosaccharomyces rouxii* was developed. Auxotrophic mutants (*ura3 leu2*, *ura3 ade2*, *ura3 leu2 ade2*) derived from the CBS 732 type strain were prepared. Centromeric and episomal *Z. rouxii/E. coli* shuttle plasmids with different marker genes (*ScURA3*, *ZrLEU2*, *ZrADE2*) and with multiple cloning sites were constructed, together with a plasmid enabling GFP-tagging. A system for repeatable targeted gene deletion in *Z. rouxii* was established, involving first the integration of a PCR-generated *loxP-kanMX-loxP* cassette, and second the removal of *kanMX* from the genome using a *Z. rouxii* plasmid harbouring *cre* recombinase.

Abbreviations

GFP, green fluorescent protein

Introduction

Zygosaccharomyces rouxii is a hemiascomycetous yeast closely related to the model yeast *Saccharomyces cerevisiae*. It is known for its ability to grow in environments with high concentrations of salts and/or sugars, which restrict the growth of most other yeast species (incl. *S. cerevisiae*). As one of the most osmotolerant yeast species, it is one of the main spoilage microorganisms in the food industry. The reason for its high osmotolerance has not yet been discovered, and is probably due to some sets of specific genes. Considering the close phylogenetic relationship between *Z. rouxii* and *S. cerevisiae* (de Montigny *et al.*, 2000), identifying the genes that are responsible for *Z. rouxii*'s osmotolerance would be of great interest to the food industry - their heterologous expression in industrial strains of *S. cerevisiae* could improve the growth capacities of these strains under adverse conditions. *Z. rouxii* is also interesting as a species that phylogenetically precedes the event of whole genome duplication, which took place in an ancestor of *S. cerevisiae* (Wolfe & Shields, 1997; de Montigny *et al.*, 2000). The DNA of the CBS 732 type strain has already been sequenced and is currently being annotated (the Génolevures 3 project, http://cbi.labri.fr/Genolevures/).

Though the physiological and biochemical processes of *Z. rouxii* have been studied extensively (van Zyl *et al.*,1990; Kurtzman & Fell, 1998; Jansen *et al.*, 2003; Martorell *et al.*, 2007), our knowledge of its cell properties at the molecular level lags far behind that of *S. cerevisiae*, mainly due to a lack of tools for *Z. rouxii* genetic manipulation. For this reason, most of the *Z. rouxii* genes and their products that have been studied so far were identified and/or characterized by heterologous expression in *S. cerevisiae* mutants (Iwaki *et al.*, 1999; Sychrova *et al.*, 1999; Sychrova, 2001; Wang *et al.*, 2002; Kwon *et al.*, 2003; Watanabe *et al.*, 2004). To characterize *Z. rouxii* specific genes, a set of tools for manipulationg its genome, *i.e.* an efficient transformation procedure, various auxotrophic mutants, centromeric and episomal plasmids with different auxotrophic markers, a system for repeated gene deletions and protein-tagging would be helpful. Such a set of genetic tools has so far been missing for *Z. rouxii*.

In our previous studies, we optimized a transformation procedure to efficiently introduce plasmid DNA into *Z. rouxii* by electroporation (Pribylova & Sychrova, 2003; Pribylova *et al.*, 2007b), but the available methods for selecting *Z. rouxii* transformants remained few. So far, the only auxotrophic mutant derived from the *Z. rouxii* CBS 732 type strain was a *ura3* strain (UL4; Pribylova & Sychrova, 2003). A *leu2* mutant was also available, *Z. rouxii* MA11 (Ushio *et al.*, 1988), but unfortunately in a different genetic background. Both mutants were prepared by UV mutagenesis. Of the dominant markers, the use of *kanMX* to select transformants (Ushio *et al.*, 1988) or prepare mutants (Tang *et al.*, 2005) has been described. Attempts to adopt another gene (*ScMPR1*) as a dominant selection marker failed (Pribylova & Sychrova, 2006).

As far as the gene disruptions or deletions in *Z. rouxii* are concerned, there have been a few studies employing a *ScLEU2* disruption cassette with several hundred bp of target sequence homology (Watanabe *et al.*, 1995, 1999; Iwaki *et al.*, 1998, 1999) and one study employing a *kanMX* deletion cassette with 40 bp target sequence homology (Tang *et al.*, 2005). Although these papers proved that the frequency of homologous recombination in *Z. rouxii* is high enough to be employed in targeted gene deletions, they did not provide a suitable general tool, as there was no way to delete more genes within the same strain. Moreover, none of the gene deletions were performed in a strain with the *Z. rouxii* CBS 732 genetic background, *i.e.* in the strain where the complete genome sequence will soon be available.

As far as plasmids for *Z. rouxii* are concerned, some of the *S. cerevisiae* centromeric plasmids can be used for transformation, but they are not stably maintained in *Z. rouxii*

cells, *i.e.* do not behave as centromeric (Pribylova *et al.*, 2007b). Our study of *Z. rouxii* centromeres resulted in the first *Z. rouxii*-specific plasmid with a multiple cloning site, *Z. rouxii* centromere and the *ScURA3* marker, pZCA (Pribylova *et al.*, 2007b). The 2μ replicon of *S. cerevisiae* does not function in *Z. rouxii* (Araki *et al.*, 1985; Ushio *et al.*, 1988), and thus *S. cerevisiae* episomal plasmids cannot be used. The replicon of pSR1, a cryptic plasmid of *Z. rouxii* similar to the 2μ plasmid of *S. cerevisiae*, was already being used for plasmid constructions (Ushio *et al.*, 1988), but no *Z. rouxii* episomal plasmid with a multiple cloning site was available.

In this paper, we report a set of tools developed for genetic manipulations in *Z. rouxii*, *i.e.* (i) various auxotrophic mutants derived from CBS 732, (ii) specific centromeric and episomal plasmids with different markers and a multiple cloning site, (iii) a plasmid for GFP (Green Fluorescent Protein)-tagging and (iv) a system for repeated targeted gene deletion employing a PCR-generated *loxP–kanMX–loxP* cassette and *cre* recombinase expression.

Materials and methods

Strains and growth conditions

The *Z. rouxii* strains used were UL4 (*ura3*; Pribylova & Sychrova, 2003), MA11 (*leu2;* Ushio *et al.*, 1988) and the CBS 732 wild type. *S. cerevisiae* W3031A (*MATa ade 2-1 can1-100 his3-11/15 leu2-3/112 mal10 trp1-1 ura3-1*; Wallis *et al.*, 1989) was used for the construction of plasmids by homologous recombination and for testing plasmid functionality in a *S. cerevisiae* host. *E. coli* XL1-Blue (Stratagene) was routinely used as the host strain for plasmid amplification. Yeasts were grown in standard YPD or YNB-NH_4 media with 2% glucose. Growth curves were measured as described previously (Pribylova *et al.*, 2007b). When required, auxotrophic supplements (20 μg ml^{-1}) or G418 (100 μg ml^{-1}, MP Biochemicals, Germany) were added. For growth tests on galactose, galactose was substituted for glucose in the media. *E. coli* transformants were grown in standard LB medium supplemented with ampiciline (100 μg ml^{-1}, Sigma).

DNA manipulations

DNA manipulations were performed according to standard protocols (Sambrook *et al.*, 1989). Genomic DNA and plasmid DNA from yeast cells were isolated according to Hoffman & Winston (1987), plasmid DNA from *E. coli* was isolated using the GenElute™

Plasmid Miniprep Kit (Sigma). GENECLEAN® Turbo was used for the isolation of DNA fragments from agarose gels. To avoid mismatch base pairing during the synthesis of PCR products, a mixture of Taq polymerase (PPP Master Mix, Top-Bio) and ISIS™ DNA Polymerase (QBIOgene) with intrinsic proofreading activity was used (2.5 U Taq and 0.1 U ISIS per 20 µl reaction). DNA sequencing was performed in an ABI PRISM 3100 DNA sequencer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Plasmids

The plasmids used are listed in Table 1. New plasmids were constructed either by standard restriction and ligation, or by homologous recombination in *S. cerevisiae* (Zaragoza, 2003). The primers used for plasmid constructions are listed in Table 2. pZEU with the *ScURA3* marker gene was prepared by restriction and ligation: a 1207 bp long fragment of pSR1 (positions 2509 – 3716; Araki *et al.*, 1985) was amplified by PCR using pSRT5 (Ushio *et al.*, 1988) as a template. *Cla*I restriction sites were introduced into the 5' and 3' ends of the primers. The amplified fragment was digested with *Cla*I and cloned into the *Cla*I site of pFL34 (Bonneaud *et al.*, 1991). Plasmids pZEA and pZCAA with the *ZrADE2* marker gene (Sychrova *et al.*, 1999) and pZEL and pZCAL with the *ZrLEU2* marker gene (Sychrova, 2001) were constructed by homologous recombination: Fragments containing *ZrADE2* or *ZrLEU2* were amplified by PCR using the genomic DNA of *Z. rouxii* CBS 732 as a template. The amplified fragments were cloned into pZEU or pZCA (Pribylova *et al.*, 2007b) plasmids by replacing the *ScURA3* gene. pCUG6 was constructed by insertion of the *ScARS1/ScCEN6* fragment from pFL38 (Bonneaud *et al.*, 1991) into pUG6 (Guldener *et al.*, 1996): pFL38 was digested by *Cla*I, and the recessed 3' termini of the fragment harbouring *ScARS1/ScCEN4* were filled in with the Klenow fragment (DNA Blunting and Ligation Kit, Fermentas). The resulting 811 bp fragment was cloned into the *Pvu*II site of pUG6. pZCRE was constructed by digesting the pSH47 plasmid (Guldener *et al.*, 1996) with *Sac*I and *Sal*I endonucleases and inserting the resulting 1919 bp fragment containing *cre* recombinase behind the *ScGAL1* promoter into the *Sac*I-*Sal*I restriction sites of pZEU. The pGLG plasmid harbouring the GFP-tagged *ZrLEU2* gene behind the *ScGAL1* promoter was prepared by homologous recombination: A fragment containing the *ZrLEU2* gene was amplified by PCR using pLU1 as a template. The amplified fragment was cloned into YCpCJ025 (De Craene *et al.*, 2001) behind the *ScGAL1* promoter and in frame with the GFP sequence. pZGFP for GFP-tagging in *Z. rouxii* was constructed by restriction and ligation: pGRU1 was digested with *Eco*RI and *Stu*I and the 1352 bp fragment containing

the GFP coding sequence was cloned into pZEU by replacing the 669 bp *Eco*RI/*Stu*I fragment.

Yeast transformation and plasmid loss

Z. rouxii and *S. cerevisiae* cells were transformed and plasmid losses were measured as described previously (Pribylova *et al.*, 2007b).

Construction of the *loxP–kanMX–loxP* deletion cassettes

Cassettes containing 40 or 80 bp long target sequence homology were amplified by PCR with pUG6 as a template. The primers used for amplification are listed in Table 3. The PCR products were purified in an agarose gel. A cassette containing the long (268 and 1354 bp) target sequence homology for the deletion of *ZrLEU2* was constructed via several steps: The cassette with 40 bp-long regions homologous to the *ZrLEU2* locus was amplified by PCR with pUG6 as a template and cloned by homologous recombination (in *S. cerevisiae*) into the pLU1 plasmid by replacing a 1646 bp fragment of the *ZrLEU2* coding sequence. The resulting plasmid was digested with *BamH*I and *Sph*I endonucleases and the 3243 bp fragment representing the *loxP–kanMX–loxP* cassette with the long (268 and 1354 bp) regions homologous to the *ZrLEU2* locus was purified in an agarose gel.

Gene deletions and removal of the integrated *kanMX* marker

Z. rouxii cells were transformed with 1 μg of the deletion cassette. Before plating on selective YPD + 100 μg ml^{-1} G418, the transformation mixtures were grown for 2 h in YPD at 30 °C. Targeted integration of the cassette was verified by phenotypic (drop test) and genotypic (PCR) analyses. For removal of the G418 resistance marker (*kanMX*) from the genome, cells were transformed with pZCRE and transformants were grown selectively on YNB-NH_4 plates for two days and then non-selectively in liquid YPD for 24 hours. Cultures were diluted and plated on YPD to obtain single colonies. Three days later, colonies were replica-plated on YPD, YPD + 100 μg ml^{-1} G418 and YNB-NH_4 and clones with both Ura^- and G418-sensitive phenotypes were selected. The removal of *kanMX* from the genome was confirmed by PCR analysis.

Fluorescence microscopy

Cultures were grown exponentionally in YNB-NH_4. When the OD_{600} reached 0.3, cells were harvested by centrifugation and immediately observed with an Olympus AX70

fluorescent microscope. For GFP visualizing, an U-MWB fluorescent cube was used with excitation filter 450–480 nm and barrier filter 515 nm.

Results and Discussion

Construction of *Z. rouxii* episomal plasmids with different markers

So far, there has been no *Z. rouxii* episomal plasmid available with a multiple cloning site. To construct the first *Z. rouxii* episomal plasmids, a 1.2 kb pSR1 fragment was selected as a replicon, as this fragment was previously shown to contain ARS and elements for stable partitioning into subsequent generations (Jearnpipatkul *et al.*, 1987a, 1987b). Three *Z. rouxii*-specific episomal plasmids with multiple cloning sites were constructed, pZEU, pZEA and pZEL, harbouring *ScURA3*, *ZrADE2* or *ZrLEU2* as marker genes, respectively, and pSR1 as a replicon (Fig. 1A).

To confirm the function of the pSR1-derived plasmids in yeasts, the function of pZEU was tested in both *Z. rouxii* and *S. cerevisiae*. *Z. rouxi*i UL4 and *S. cerevisiae* W3031A were transformed with pZEU and YEp352 (a *S. cerevisiae* plasmid containing the 2μ replicon), and Ura^+ transformants were selected. pZEU transformed both species with high efficiency, which proved that the pSR1 fragment contained an ARS functional in both *Z. rouxii* and *S. cerevisiae* (Table 4A). The YEp352 plasmid transformed only *S. cerevisiae* cells, which was due to the fact that 2μ-derived plasmids do not replicate in a *Z. rouxii* host (Araki *et al.*, 1985; Ushio *et al.*, 1988). To test the stability of pZEU in *Z. rouxii* and *S. cerevisiae* transformants, plasmid loss was measured (Table 4B). pZEU loss from *Z. rouxii* cells (in absence of selection pressure) was much lower than from *S. cerevisiae* cells (56.1 % compared to 98.8 %), which confirmed the function of the pSR1 fragment present in the plasmid in the maintenance of pZEU in *Z. rouxii*, but not in *S. cerevisiae* cells. The loss of pSR1 from *Z. rouxii* was higher compared to the loss of YEp352 from *S. cerevisiae* (56.1 % versus 34.5 %). To test the function of pZEU in complementing the *Z. rouxii ura3* mutation and its efficient propagation in *Z. rouxii*, growth curves of the *Z. rouxii* UL4 [pZEU] transformant in selective minimal YNB-NH_4 medium and of a non-transformed *Z. rouxii* UL4 strain in YNB-NH_4 medium supplemented with uracil were measured. Both strains grew similarly, which confirmed that the pZEU plasmid efficiently complemented the *ura3* mutation and was efficiently propagated (not shown).

The multicopy status of pZEU in *Z. rouxii* was confirmed, when the *ZrSOD2-22* gene encoding a plasma membrane antiporter (Kinclova *et al.*, 2001) was inserted into pZEU

and the resulting plasmid pZEU_*ZrSOD2-22* expressed in a *sod2-22* mutant of *Z. rouxii* UL4. The pZEU_*ZrSOD2-22* harbouring transformants grew better with high NaCl in the media than wild-type *Z. rouxii* UL4 harbouring empty pZEU, proving that the pZEU_*ZrSOD2-22* transformants contained more copies of the antiporter, *i.e.* several copies of the plasmid (not shown).

The pZEA (*ZrADE2* marker) and pZEL (*ZrLEU2* marker) plasmids were constructed as derivatives of pZEU. They complemented the *ade2* and *leu2* mutations respectively in both *Z. rouxii* and *S. cerevisiae*, and the efficiency of transformation of both species was similar to that of pZEU.

The pZEU, pZEA and pZEL plasmids represent the first *Z. rouxii/E. coli* shuttle plasmids that are episomal in *Z. rouxii* and contain different marker genes and a multiple cloning site. These constructed plasmids' features are summarized in Table 5.

Construction of centromeric plasmids with different markers

In our previous work, we constructed a *Z. rouxii* centromeric plasmid with the *ScURA3* marker gene, pZCA, which was stably maintained in *Z. rouxii* but not *S. cerevisiae* cells and was monocopy in *Z. rouxii* (Pribylova *et al.*, 2007b). To enrich the set of *Z. rouxii*-specific plasmids, two *Z. rouxii* centromeric plasmids with different markers were constructed, pZCAA (containing *ZrADE2*) and pZCAL (containing *ZrLEU2*). Both plasmids are derivatives of pZCA (Fig. 1B). They complemented the *ade2* and *leu2* mutations respectively in both *Z. rouxii* and *S. cerevisiae*, were stably maintained only in *Z. rouxii* and the efficiency of transformation was similar to that of pZCA, *i.e.* approx. 10^4 transformants μg^{-1} DNA.

The pZCAA and pZCAL plasmids represent new *Z. rouxii/E. coli* shuttle plasmids, are centromeric (*i.e.* stable and monocopy) in *Z. rouxii* and contain different marker genes and a multiple cloning site. These plasmids' features are summarized in Table 5.

Function of the *ScGAL1* promoter

In *S. cerevisiae*, a galactose promoter (for example *ScGAL1*) is used for regulable gene expression, *i.e.* repression in media with glucose and activation in media with galactose (Mumberg *et al.*, 1994). In order to explore the possible use of the *ScGAL1* promoter for regulated gene expression in *Z. rouxii*, the pGLG plasmid harbouring the *ZrLEU2* gene under the *ScGAL1* promoter was constructed (Table 5). *Z. rouxii* MA11 was transformed with pGLG and pLU1 (containing the *ZrLEU2* gene expressed from its own promoter) as a

control and transformants were selected on YNB-2% galactose. The efficiency of transformation was similar for both plasmids (approx. 10^4 transformants μg^{-1} DNA). To reveal the function of the *ScGAL1* promoter in *Z. rouxii*, growth curves of MA11 [pGLG] and MA11 [pLU1] transformants in YNB-NH_4 with 2% glucose or 2% galactose were measured. Both pGLG and pLU1 harbouring transformants grew in the medium with glucose, although the growth of MA11 [pGLG] was reduced to about 63 % of MA11 [pLU1] (not shown). This indicated that in medium with glucose, the *ScGAL1* promoter was either only slightly repressed or simply not as active as the intrinsic *ZrLEU2* promoter. The growth of both transformants on galactose was weak, which suggested that galactose is not an efficient source of carbon for *Z. rouxii* CBS 732 derivatives. The variable assimilation of galactose by *Z. rouxii* CBS 732 has been reported previously (Kurtzman & Fell, 1998).

The results obtained showed that the *ScGAL1* promoter, as it was not repressed significantly by glucose, cannot be used in *Z. rouxii* for regulable gene expression, and that in *Z. rouxii*, genes under the control of the *ScGAL1* promoter are expressed in media with glucose.

Function of GFP and construction of a new plasmid for GFP-tagging

In *S. cerevisiae* and other organisms, GFP is used for the determination of protein localization in cells (Niedenthal *et al.*, 1996). To explore the use of GFP in *Z. rouxii*, a pGLG plasmid containing the *ZrLEU2* gene fused to the GFP sequence was introduced to MA11 cells. In *S. cerevisiae*, the beta-isopropylmalate dehydrogenase encoded by the *LEU2* gene is soluble and localized in the cell cytosol. The MA11 [pGLG] transformants were observed in a fluorescent microscope together with the control, MA11 [pLU1] transformants. The fluorescent signal was detected only in the cytosol of MA11 [pGLG] cells. This experiment proved that the GFP is functional in *Z. rouxii* and can thus be used for localization of GFP-tagged proteins. The pZGFP plasmid was constructed for such purpose, which is multicopy in *Z. rouxii* and has a multiple cloning site upstream of the GFP sequence (Table 5). As the plasmid is replicable in *S. cerevisiae* and can be maintained there under selection pressure, the construction of a plasmid harbouring the gene of interest fused to the GFP sequence can be performed easily by homologous recombination of overlapping 40 bp regions in *S. cerevisiae*.

Development of a system for targeted gene deletion

In *S. cerevisiae*, the targeted gene deletion requires only 40 bp of target sequence homology and is very efficient (20 – nearly 100 % depending on the targeted locus; Baudin *et al.*, 1993). However, for some other yeasts, such as *Kluyveromyces lactis, Candida albicans* or *Yarrowia lipolytica*, the necessity of using longer target sequence homology, sometimes extrapolated even to several hundred bp, has been reported (Sanglard *et al.*, 1997; Fickers *et al.*, 2003; Kooistra *et al.*, 2004). From previous studies in *Z. rouxii*, it was not clear how long a target sequence homology is required for an efficient targeted gene disruption/deletion (Watanabe *et al.*, 1995, 1999; Iwaki *et al.*, 1998, 1999; Tang *et al.*, 2005). Besides, no disruptions/deletions have been performed in the CBS 732 genetic background.

To reveal how long a target sequence homology is necessary for gene deletions in the *Z. rouxii* CBS 732 genetic background, we attempted to delete the *ZrLEU2* gene in the UL4 strain using a system employing the *loxP-kanMX-loxP* deletion cassette, originally designed for *S. cerevisiae* (Guldener *et al.*, 1996).

First, the sensitivity of *Z. rouxii* UL4 to G418 was tested. A concentration of 100 μg ml^{-1} G418 in YPD plates showed to be sufficient to suppress growth. To test whether *kanMX* is efficient in G418 detoxification in *Z. rouxii* UL4, the pCUG6 plasmid harbouring the *loxP–kanMX–loxP* module and a *S. cerevisiae* centromere was constructed (Table 5) and used for *Z. rouxii* UL4 transformation. Transformants were selected on YPD + 100 μg ml^{-1} G418 with an efficiency of ~ 10^4 transformants μg^{-1} DNA, which proved the correct functioning of the *kanMX* encoded enzyme in G418 detoxification.

To analyse the effect of the length of the sequence homology on gene deletion in *Z. rouxii*, three types of a *loxP–kanMX–loxP* deletion cassette differing in the length of their regions homologous to *ZrLEU2* locus were constructed: A) 40 bp, B) 80 bp and C) 268 bp upstream and 1354 bp downstream, respectively (Fig. 2). *Z. rouxii* UL4 was transformed with the purified cassettes. Table 6 shows the frequencies of $G418^R$ transformants and deletion mutants obtained and the targeting efficiency. Only cassettes containing longer, *i.e.* 80 bp or 268/1354 bp target sequence homology, were effective in replacing the *ZrLEU2* gene with the *loxP–kanMX–loxP* module. This result suggests that in the *Z. rouxii* CBS 732 background, 40 bp target sequence homology is not long enough to be efficiently used for gene deletion. To determine whether this observation also applied for other loci, a deletion of *ZrSOD2-22* was attempted using deletion cassettes with 40 bp or 80 bp target sequence homology, respectively. No $G418^R$ clones were obtained with 40 bp homology,

whereas 80 bp was enough to target the cassette to the *ZrSOD2-22* locus. This suggested that the impossibility of using short target sequence homology for gene deletion in *Z. rouxii* UL4 is probably general, not dependent on the locus. As different *Z. rouxii* strains have already been shown to have different properties and to respond differently to the treatments used in molecular biology techniques (Pribylova & Sychrova, 2003; Pribylova *et al.*, 2007a), the fact that Tang *et al.* (2005) succeeded in deleting a gene in the *Z. rouxii* NRRL Y2547 strain using only 40 bp target gene homology, could be due to the different genetic background of the strains used.

Both deletion cassettes with longer homologous regions were effective in the *ZrLEU2* deletion, and had similar targeting efficiency (approximately 1 %). Although increasing the length of the homologous regions from 80 to several hundred bp had no influence on targeting efficiency, it resulted in a higher frequency of $G418^R$ transformants, and thus also in a higher number of deletion mutants obtained (Table 6). A similar observation has already been made for *K. lactis* (Kooistra *et al.*, 2004).

Though the cassette with the longest homologous regions was the most effective in targeted gene deletion in *Z. rouxii* UL4, such a large deletion construct has to be prepared by several laborious cloning steps. For this reason, a PCR-generated deletion cassette containing an 80 bp-long region homologous to the target sequence represents an optimal tool for targeted gene deletions in the *Z. rouxii* CBS 732 genetic background.

Construction of various auxotrophic mutants and repeated use of the *loxP–kanMX–loxP* cassette

While testing the use of a *loxP–kanMX–loxP* deletion cassette in *Z. rouxii*, *Z. rouxii* DL1 (*ura3 leu2Δ::kanMX*), a derivative of CBS 732 type strain, was obtained. To enrich the collection of *Z. rouxii* auxotrophic mutants derived form CBS 732, the deletion of *ZrADE2* in *Z. rouxii* UL4 was performed. *Z. rouxii* UL4 was transformed with the PCR-generated *loxP–kanMX–loxP* cassette containing 80 bp long regions homologous to the *ZrADE2* locus, and the *Z. rouxii* DA1 (*ura3 ade2Δ::kanMX*) mutant strain was selected from among the $G418^R$ transformants. The Ade^- and Leu^- phenotypes of the two mutants were verified by PCR, drop tests and by complementation with the corresponding genes on plasmids.

In *S. cerevisiae*, the *loxP–kanMX–loxP* module can be removed from the locus by recombination between the two *loxP* sites mediated by *cre* recombinase expressed under the control of the *ScGAL1* promoter from the pSH47 plasmid (Guldener *et al.*, 1996). To test the *cre/loxP* system in *Z. rouxii*, *Z. rouxii* DL1 was transformed with pSH47 (*URA3*

marker). However, the plasmid was poorly replicable in *Z. rouxii*, as the growth of DL1 [pSH47] was very slow in selective YNB-NH_4 medium without uracil and supplemented with leucine. This was possibly due to the *ScARS1/ScCEN6* replicon of pSH47, which did not function well in *Z. rouxii*. For this reason, pZCRE bearing the *Z. rouxii* pSR1 replicon and *cre* recombinase under the *ScGAL1* promoter was constructed (Table 5) and used for removing *kanMX* from the *ZrLEU2* locus of the *Z. rouxii* DL1 strain. As the *ScGAL1* promoter is not repressed on media with glucose in *Z. rouxii* (*cf.* above), the procedure of removing *kanMX* did not involve the activation of *cre* recombinase expression by growing of transformants with galactose, but growth on standard selective YNB-NH_4 with glucose was sufficient for *cre* expression. PCR analysis and a G418-sensitivity test of the resulting strain confirmed that the *kanMX* marker was removed from the genome leaving a single *loxP* site in its place, thus producing the *ura3 leu2Δ::loxP* strain, DL2. Similarly, the *kanMX* marker was removed from *Z. rouxii* DA1, producing the DA2 strain (*ura3 ade2Δ::loxP*).

To prove that the *loxP–kanMX–loxP* module can be used repeatedly, a deletion of *ZrADE2* in *Z. rouxii* DL2 was performed using the same cassette as for the *ZrADE2* deletion in *Z. rouxii* UL4, and the *Z. rouxii* DLA1 (*ura3 leu2Δ::loxP ade2Δ::kanMX*) strain was obtained. The *kanMX* marker was then removed from the genome by pZCRE, producing the DLA2 (*ura3 leu2Δ::loxP ade2Δ::loxP*) strain.

In summary, the first double *ura3 leu2* or *ura3 ade2* and triple *ura3 leu2 ade2* auxotrophic *Z. rouxii* mutants were constructed. Their genotypes are summarized in Table 7. All these CBS 732 derived mutants can be used for further gene deletions.

Conclusions

Three new *Z. rouxii* auxotrophic mutants (with different combinations of the *ura3*, *leu2* and *ade2* mutations), and six new *Z. rouxii* plasmids with multiple cloning sites were constructed: three episomal (pZEU with *ScURA3*, pZEL with *ZrLEU2* and pZEA with the *ZrADE2* marker genes), two centromeric (pZCAL with *ZrLEU2* and pZCAA with *ZrADE2* marker genes) and one for GFP-tagging (pZGFP). Tables 5 and 7 summarize the features of all the plasmids and strains constructed in this work. Together with the *Z. rouxii ura3* strain, UL4 (Pribylova & Sychrova, 2003), and the pZCA plasmid which is centromeric in *Z. rouxii* and contains the *ScURA3* gene as a marker gene (Pribylova *et al.*, 2007b), this series of auxotrophic mutants and plasmids represents an efficient set of tools for selecting

Z. rouxii transformants. For repeatable gene deletions in *Z. rouxii*, a system employing the *loxP–kanMX–loxP* deletion cassette and the *cre/loxP* system was established, employing the newly constructed plasmid harbouring *cre* recombinase, pZCRE.

This set of tools for genetic manipulations with *Z. rouxii* will serve to enable the study of *Z. rouxii*-specific features, mainly the basis of its high osmotolerance. Together with the fact that the complete *Z. rouxii* CBS 732 genome sequence will soon be available, the existence of such genetic tools makes this species an interesting model to study yeast osmotolerance. Moreover, as a species that phylogenetically precedes the event of whole genome duplication, *Z. rouxii* also represents an interesting "preduplication" model.

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Tables

Table 1. Plasmids used in this work

Plasmid	Reference
pFL34	(Bonneaud *et al.*, 1991)
pFL38	(Bonneaud *et al.*, 1991)
pGRU1	NCBI, Acc. No AJ249649
YCpCJ025	(De Craene *et al.*, 2001)
pLU1	(Sychrova, 2001)
pSH47	(Guldener *et al.*, 1996)
pSRT5	(Ushio *et al.*, 1988)
pUG6	(Guldener *et al.*, 1996)
pZCA	(Pribylova *et al.*, 2007b)
YCplac33	(Gietz & Sugino, 1988)
YEp352	(Hill *et al.*, 1986)

Table 2. Primers used for plasmids' construction

Primer	Sequence (5'-3')	For plasmid	Description
pZEUf	CGTGATCGATAGATCCTACA AAATATGTAA	pZEU	*Cla*I restriction site underlined
pZEUr	CTCTATCGATTTAGTTTAGCT TAATATAAT	pZEU	*Cla*I restriction site underlined
pZELf	tcactgcccgctttccagtcgggaaacctgtcgt gccagcGATCATTGCTCATTGTC CCA	pZEL	Lower case, region homologous to pZEU upstream *ScURA3*; upper case, region homologous to *ZrLEU2* locus
pZELr	accgcctctccccgcgcgttggccgattcatta atgcagcGTTTATGTGTACACAA TAAT	pZEL	Lower case, region homologous to pZEU downstream *ScURA3*; upper case, region homologous to *ZrLEU2* locus
pZCAAf	gtgagtttagtatacatgcatttacttataatacag ttttACGTGACCATGGTATCAC AT	pZCAA, pZEA	Lower case, region homologous to pZCA or pZEU upstream *ScURA3*; upper case, region homologous to *ZrADE2* locus
pZCAAr	gattcggtaatctccgaacagaaggaagaacg aaggaaggCAATATAAAAGACA CAGAAA	pZCAA, pZEA	Lower case, region homologous to pZCA or pZEU downstream *ScURA3*; upper case, region homologous to *ZrADE2* locus
pZCALf	gtgagtttagtatacatgcatttacttataatacag ttttGATCATTGCTCATTGTCCC A	pZCAL	Lower case, region homologous to pZCA upstream *ScURA3*; upper case, region homologous to *ZrLEU2* locus
pZCALr	gattcggtaatctccgaacagaaggaagaacg aaggaaggGTTTATGTGTACACA ATAAT	pZCAL	Lower case, region homologous to pZCA downstream *ScURA3*; upper case, region homologous to *ZrLEU2* locus
pGLGf	tacctctatactttaacgtcaaggagaaaaaact ataATGTCAAAAAACATCGTT GTCCT	pGLG	Lower case, region homologous to *pScGAL1* in YCpCJ025; upper case, region homologous to *ZrLEU2*
pGLGr	tactgttaattgctccagcaccagcaccagcac ctgctccTGCCAAGATTTCCTTG ACTG	pGLG	Lower case, region homologous to GFP sequence in YCpCJ025; upper case, region homologous to *ZrLEU2*

f, forward primer; r, reverse primer; *pScGAL1*, *ScGAL1* promoter.

Table 3. Primers used for amplification of deletion cassettes

Primer	Sequence (5'-3')	Description
*ZrLEU2*_40f	atgtcaaaaaacatcgttgtcctaccaggtgaccatgcc gTTCGTACGCTGCAGGTCG	Lower case, region homologous to *ZrLEU2* locus; upper case, region homologous to pUG6 upstream *loxP-kanMX-loxP*
*ZrLEU2*_40r	tcatgccaagatttccttgactgccttggcgatggcgtca GCATAGGCCACTAGTGGA	Lower case, region homologous to *ZrLEU2* locus; upper case, region homologous to pUG6 downstream *loxP-kanMX-loxP*
*ZrLEU2*_80f	gacctagtatcatgtcaaaaaacatcgttgtcctaccagg tgaccatgccggtcaagaaatcgctcaagaagccatca aaTTCGTACGCTGCAGGTCG	Lower case, region homologous to *ZrLEU2* locus; upper case, region homologous to pUG6 upstream *loxP-kanMX-loxP*
*ZrLEU2*_80r	taatttattaatcatgccaagatttccttgactgccttggcg atggcgtcaccgacttcagaagtgctgttagaaccacc GCATAGGCCACTAGTGGA	Lower case, region homologous to *ZrLEU2* locus; upper case, region homologous to pUG6 downstream *loxP-kanMX-loxP*
*ZrADE2*_80f	agggataataatgaattcgcaaactgttggtatacttggt ggtggccaattgggtcgtatggttgttgaagctgctaata TTCGTACGCTGCAGGTCG	Lower case, region homologous to *ZrADE2* locus; upper case, region homologous to pUG6 upstream *loxP-kanMX-loxP*
*ZrADE2*_80r	tactgaattatttactcaaggcccatgtaattcttgtaacca gaattttccaatttttttagccttttctagcacttctacGCA TAGGCCACTAGTGGA	Lower case, region homologous to *ZrADE2* locus; upper case, region homologous to pUG6 downstream *loxP-kanMX-loxP*

Names of primers are composed of the name of the targeted locus followed by "f" for forward primers and by "r" for reverse primers. Numbers (40, resp. 80) in the names signify the use for amplification of cassettes containing 40, resp. 80 bp target sequence homology.

Table 4. Transformation efficiency and plasmid loss of pZEU and YEp352 in yeast hosts

	A Number of transformants/μg DNA		**B** Plasmid loss (%)	
Plasmid	*Z. rouxii*	*S. cerevisiae*	*Z. rouxii*	*S. cerevisiae*
pZEU	2.5×10^4	1.1×10^4	56.1	98.0
YEp352	0	1.2×10^4	-	34.5

Table 5. Plasmids constructed in this work

Plasmid	Size (kb)	Yeast replicon	Marker gene	LacZ	Construction	Other features	Acc. No.
A *Z. rouxii* centromeric plasmids							
pZCAL	5.7	*ScARS1/ZrCENA*	*ZrLEU2*	yes	HR		**AM697671**
pZCAA	6.4	*ScARS1/ZrCENA*	*ZrADE2*	yes	HR		**AM697672**
B *Z. rouxii* episomal plasmids							
pZEL	5.1	pSR1	*ZrLEU2*	yes	HR		**AM696690**
pZEA	6.1	pSR1	*ZrADE2*	yes	HR		**AM696691**
pZEU	4.8	pSR1	*ScURA3*	yes	R+L		**AM696689**
C Other plasmids							
pCUG6	4.8	*ScARS1/ScCEN4*	*kanMX*	no	R+L	*loxP-kanMX-loxP*	**AM701829**
pGLG	7.8	*ScARS1/ScCEN6*	*ZrLEU2*, *ScURA3*	no	HR	*pScGAL1*, *GFP*	
pZCRE	6.7	pSR1	*ScURA3*	no	R+L	*pScGAL1*, *cre*	**AM697668**
pZGFP	5.5	pSR1	*ScURA3*	yes	R+L	*GFP*	**AM697669**

All plasmids are *Z. rouxii/E. coli* shuttle vectors (*ori*, Amp^R). *Zr*, *Z. rouxii*; *Sc*, *S. cerevisiae*; HR, construction by homologous recombination in *S. cerevisiae*; R+L, construction by restriction and ligation; *pScGAL1*, *ScGAL1* promoter. Plasmid sequences have been submitted to the EMBL Nucleotide Sequence Database.

Table 6. The effect of different length of target sequence homology on efficiency of gene deletions

Length of target sequence homology (bp)	Frequency of $G418^R$ transformants	Frequency of deletion mutants	Targeting efficiency (%)
40	0	0	0
80	2.0×10^{-8}	2.1×10^{-10}	1.1
268/1354	2.3×10^{-7}	2.3×10^{-9}	1.0

Frequency of $G418^R$ transformants was calculated by dividing the number of $G418^R$ transformants by the number of cells subjected to transformation. Frequency of deletion mutants was calculated by dividing the number of deletion mutants obtained by the number of $G418^R$ transformants. Targeting efficiency was calculated by dividing the Frequency of deletion mutants by the Frequency of $G418^R$ transformants, and multiplying the resulting number by 100.

Table 7. *Z. rouxii* CBS 732 derived mutants constructed in this work

Strain	Genotype
DL1	*ura3 leu2Δ::kanMX*
DL2	*ura3 leu2Δ::loxP*
DA1	*ura3 ade2Δ::kanMX*
DA2	*ura3 ade2Δ::loxP*
DLA1	*ura3 leu2Δ::loxP ade2Δ::kanMX*
DLA2	*ura3 leu2Δ::loxP ade2Δ::loxP*

Figure legends

Fig. 1. Structure of episomal (A) and centromeric (B) *Z. rouxii* plasmids with different markers and multiple cloning sites (MCS). Each schematic map corresponds to three plasmids harbouring *ScURA3*, *ZrLEU2* or *ZrADE2* marker genes, respectively. A: The pZEx schematic map represents the pZEU (*ScURA3* marker), pZEL (*ZrLEU2* marker) and pZEA (*ZrADE2* marker) plasmids. B: The pZCAx schematic map represents the pZCA (*ScURA3* marker), pZCAL (*ZrLEU2* marker) and pZCAA (*ZrADE2* marker) plasmids. Unique restriction sites are shown. MCS box is for pZEU: *Eco*RI, *Sac*I, *Kpn*I, *Xma*I, *Sma*I, *Xba*I, *Sal*I, *Pst*I, *Sph*I, *Hind*III; for pZEL: *Eco*RI, *Sac*I, *Xma*I, *Sma*I, *Pst*I, *Hind*III; for pZEA: *Sac*I, *Kpn*I, *Xma*I, *Sma*I, *Sal*I, *Pst*I, *Sph*I, *Hind*III; for pZCA: *Sph*I, *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Xma*I, *Sma*I, *Kpn*I, *Sac*I, *Eco*RI; for pZCAL: *Pst*I, *Bam*HI, *Xma*I, *Sma*I, *Sac*I, *Eco*RI; for pZCAA: *Sph*I, *Pst*I, *Sal*I, *Bam*HI, *Xma*I, *Sma*I, *Kpn*I, *Sac*I.

Fig. 2. Schematic map of deletion cassettes with different lengths of target sequence homology constructed to delete *ZrLEU2*. A: Deletion cassette with 40 bp target sequence homology. B: Deletion cassette with 80 bp target sequence homology. C: Deletion cassette with 268/1354 bp target sequence homology. Lines represent sequences homologous to *ZrLEU2* gene flanking regions, full boxes correspond to sequences homologous to *ZrLEU2* coding sequence, open squares represent the *loxP-kanMX-loxP* module.

Fig. 1.

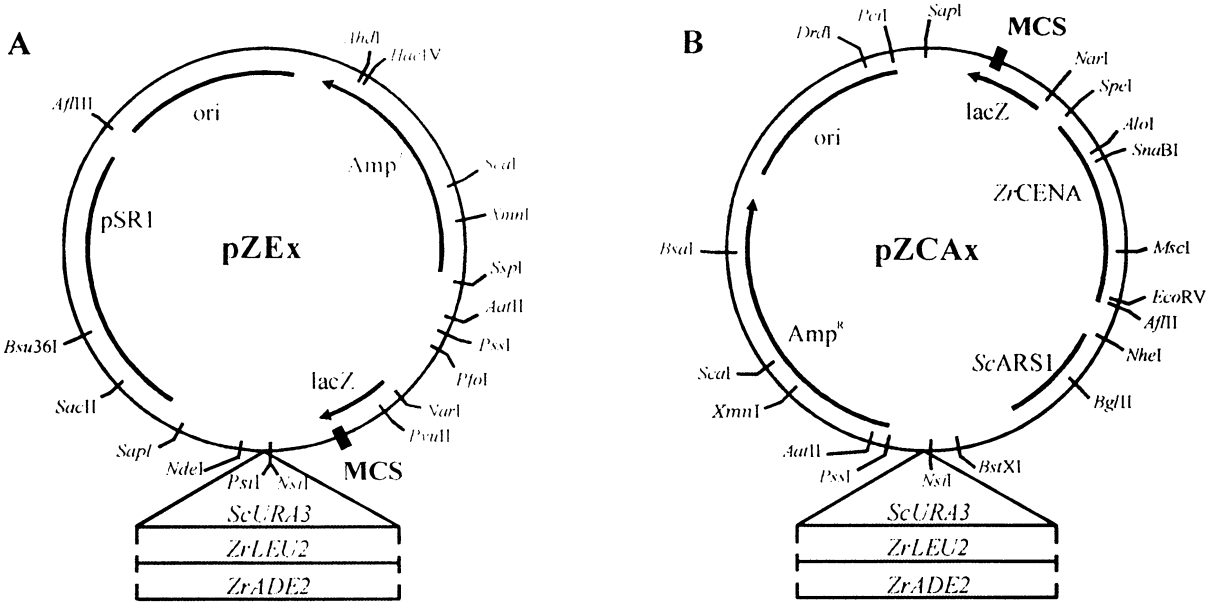
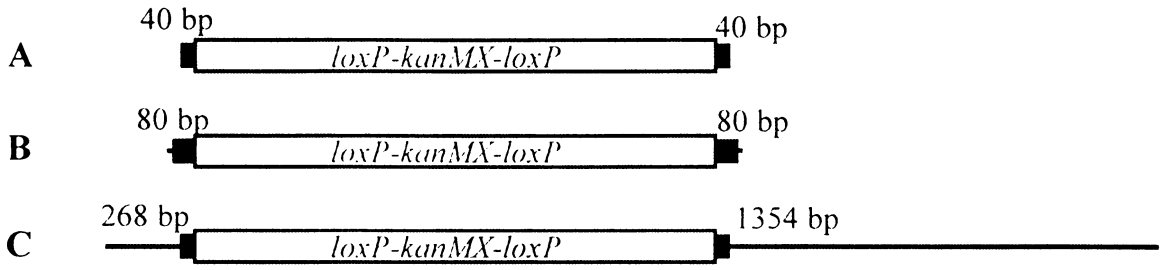

A
DraI
HaeIV
AflIII
ori
Amp
ScaI
XmnI
pSR1
pZEx
SspI
AatII
PssI
PfoI
Bsu36I
lacZ
SacII
VarI
PvuII
SapI
NdeI
PstI
NsiI
MCS
ScURA3
ZrLEU2
ZrADE2
B
DraI
PciI
SapI
MCS
NarI
lacZ
SpeI
AloI
ori
SnaBI
ZrCENA
BsaI
pZCAx
MscI
EcoRV
AflII
Amp
ScARS1
NheI
ScaI
BglII
XmnI
AatII
PssI
NsiI
BstXI
ScURA3
ZrLEU2
ZrADE2

Fig. 2.


A
40 bp
40 bp
loxP-kanMX-loxP
B
80 bp
80 bp
loxP-kanMX-loxP
C
268 bp
1354 bp
loxP-kanMX-loxP

5.2. Osmotolerantní vlastnosti *Z. rouxii*

Nejčastěji studovanými divokými kmeny *Z. rouxii* jsou CBS 732^T a ATCC 42981. Již z výsledků získaných v publikaci č. 1 bylo patrné, že se tyto kmeny ve svých vlastnostech liší. Buňky jejich derivátů vyžadovaly jiné podmínky pro rozvolnění svých buněčných povrchů před elektroporací, což naznačovalo odlišné složení či strukturu buněčných stěn. U několika druhů kvasinek již bylo prokázáno, že vlastnosti buněčných stěn jsou provázány s vlastnostmi osmotolerantními (viz kap. 2.1.2.). Že se budou vlastnosti osmotolerantní a vlastnosti buněčné stěny obou kmenů *Z. rouxii* navzájem ovlivňovat, naznačoval již fakt, že k jejich úspěšné elektroporaci bylo zapotřebí buňky kultivovat za mírného solného stresu (v přítomnosti 300 mM NaCl; viz publikace č. 1).

Rozhodli jsme se proto tyto dva divoké kmeny porovnat, a to hlavně z hlediska jejich vlastností týkajících se osmotolerance a buněčných stěn. Zjistili jsme významné rozdíly v toleranci k solím, produkci a asimilaci glycerolu, složení buněčných stěn, karyotypu. Získané výsledky jsme zveřejnili ve vědeckých časopisech (publikace č. 5 a 6).

5.2.1. Publikace č. 5: Osmoresistant yeast *Zygosaccharomyces rouxii*: the two most studied wild-type strains (ATCC 2623 and ATCC 42981) differ in osmotolerance and glycerol metabolism

Přibylová, L., de Montigny, J., Sychrová, H. (2007) *Yeast* **24**(3): 171-180

Buňky kmenů *Z. rouxii* ATCC 2623 (tj. CBS 732^T) a ATCC 42981 byly porovnány z hlediska svých morfologických, fyziologických a genomových vlastností. Kmen ATCC 42981 vykazoval vyšší toleranci k solím, vyšší produkci glycerolu a na rozdíl od kmene CBS 732^T byl schopen asimilovat glycerol. Za podmínek osmotického stresu produkovaly oba kmeny *Z. rouxii* výrazně méně glycerolu než *S. cerevisiae*, což by naznačovalo přítomnost přenašeče aktivně transportujícího uniknuvší glycerol zpět do buňky nebo schopnost efektivně zadržet produkovaný glycerol v buňce, jak tomu již bylo navrženo v práci van Zyl *et al.* (1990). Rozdíly byly nalezeny též v v karyotypu - kmen ATCC 42981 obsahuje o jeden chromosom více a má celkově větší genom než CBS 732^T, a dále v morfologii buněk - buňky kmene CBS 732^T jsou elipsoidní až protáhlé a menší než ATCC 42981, zatímco buňky ATCC 42981 jsou kulaté a větší, což naznačuje odlišné vlastnosti buněčných stěn těchto kmenů.

Analýza mezigenových sekvencí prostřednictvím PCR potvrdila, že se i přes zjištěné rozdíly jedná o kmeny *Z. rouxii*. Nedávno byla zveřejněna hypotéza, že kmen ATCC 42981 je ve skutečnosti zřejmě kmenem hybridním - tedy vznikl pravděpodobně sloučením genomu kmene *Z. rouxii* a jiného kmene *Zygosaccharomyces* (James *et al.*, 2005). Tomu by odpovídaly i námi zjištěné rozdíly kmenů v karyotypu a fakt, že ve kmeni ATCC 42981 jsou často nalézány geny ve dvou kopiích. Zároveň je možné, že schopnost kmene ATCC 42981 asimilovat glycerol je důsledkem právě hybridního původu. Přesný původ kmene ATCC 42981 zbývá ještě stanovit; námi provedená analýza PCR dokládá, že genom ATCC 42981 minimálně zčásti odpovídá druhu *Z. rouxii*.

Schopnosti kmene ATCC 42981 růst v přítomnosti vyšší koncentrace solí než CBS 732 nebo produkovat více glycerolu mohou být dány právě tím, že kmen ATCC 42981 obsahuje geny podílející se na osmotoleranci (např. *SOD*, *HOG*, *GPD*; viz kap. 2.2.) ve dvou kopiích. Jeho vysoká tolerance k solím v médiu pramení patrně z toho, že se jedná o izolát z tradiční japonské slané kořenící směsi zvané miso; kmen CBS 732^T byl naopak izolován z vinného moštu, tedy z prostředí, kde vysokou osmolaritu způsobují cukry, nikoli sole, a nemá tedy natolik účinné systémy umožňující růst v přítomnosti solí.

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Research Article

Osmoresistant yeast *Zygosaccharomyces rouxii*: the two most studied wild-type strains (ATCC 2623 and ATCC 42981) differ in osmotolerance and glycerol metabolism

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Abstract

The yeast *Zygosaccharomyces rouxii* is known for its high tolerance to osmotic stress, which is thought to be caused by sets of specific genes. Relatively few *Z. rouxii* genes have been identified so far, all of them having homologues in *Saccharomyces cerevisiae*; none of them was *Z. rouxii*-specific. Most of the known *Z. rouxii* genes were isolated from two wild-type strains, ATCC 2623 and ATCC 42981. In this study, we compared these two strains with regard to some of their morphological, physiological and genomic properties. Important differences were found in their salt tolerance and assimilation of glycerol and karyotype; slight differences were also present in their cell morphology. The ATCC 42981 strain showed a higher resistance to salts, higher glycerol production and, unlike ATCC 2623, was able to assimilate glycerol. Under conditions of osmotic stress, the glycerol production in both *Z. rouxii* strains was much lower than in a *S. cerevisiae* S288c culture, which suggested the presence of a system that efficiently retains glycerol inside *Z. rouxii* cells. The karyotype analysis revealed that ATCC 42981 cells contain more chromosomes and have a bigger genome size than those of ATCC 2623.

Keywords: ***Zygosaccharomyces rouxii*; osmotolerance; halotolerance; glycerol accumulation; karyotype**

Introduction

Zygosaccharomyces rouxii belongs to the group of hemiascomycetous yeasts with a high tolerance to osmotic stress. This typical feature enables it to grow in environments with high concentrations of salts and/or sugars, i.e. under conditions restrictive to most other yeast species. Thus, *Z. rouxii* poses a major problem for the food industry — fruit juices and concentrates, jams and salad dressings are among the products commonly spoiled by this microorganism. On the other hand, it is employed in the fermentation processes of some salted oriental foodstuffs (e.g. Soy sauce, miso; Fleet, 1992; Onishi, 1963). The ability of *Z. rouxii* to grow in such concentrated environments is probably due to some sets of specific genes. Until now, relatively few *Z. rouxii* genes (and their products) involved in osmotolerance, e.g. *ZrGPD1* (Iwaki *et al.*, 2001; Watanabe *et al.*, 2004), *ZrHOG1* (Iwaki *et al.*, 1999; Kinclova *et al.*, 2001), *ZrFPS1* (Tang *et al.*, 2005), or in Na^+ extrusion from cells, e.g. *ZrSOD2*, *ZrSOD22*, *ZrSOD2–22* (Iwaki *et al.*, 1998; Kinclova *et al.*, 2001; Watanabe *et al.*, 1995), *ZrENA1* (Watanabe *et al.*, 1999), have been at least partially characterized. Isolated *Z. rouxii* genes corresponded to known homologues of *Saccharomyces*

cerevisiae, and most of them were obtained by complementation of *S. cerevisiae* functions or by screening of *Z. rouxii*'s genomic library with a probe against *S. cerevisiae* or *Schizosaccharomyces pombe* genes. None of them were *Z. rouxii*-specific. Most of these *Z. rouxii* genes have been isolated from two wild-type strains, ATCC 2623 (CBS 732) and ATCC 42981. Recently, the ATCC 2623 genome has been partially sequenced. The 18S and 25S rRNA sequence comparison and measurement of synteny placed *Z. rouxii* into the *Kluyveromyces* clade, close to the *Saccharomyces* clade (de Montigny *et al.*, 2000). This close relationship between *S. cerevisiae* and *Z. rouxii*, and thus the expected high homology of their genes, led to the identification of some other *Z. rouxii* genes by using *S. cerevisiae* GeneFilters (Schoondermark-Stolk *et al.*, 2002).

During our previous study, we observed a very different susceptibility of ATCC 2623 and ATCC 42981 derivatives to transformation by electroporation (Pribylova and Sychrova, 2003). Considering this fact, we have compared the two *Z. rouxii* wild-type strains regarding some of their morphological, physiological and genomic properties.

Materials and methods

Yeast strains

The *Z. rouxii* wild-type strains used in this study were ATCC 2623 (CBS 732; formerly ATCC 56077) and ATCC 42981. The *S. cerevisiae* strains were S288c, B31 (Banuelos *et al.*, 1998) and FL100 (ATCC 28383).

Media and growth experiments

Yeast cells were grown in the following media: YP (1% yeast extract, 2% peptone), YPD (YP supplemented with 2% glucose), YNB (0.17% yeast nitrogen base without amino acids and ammonium sulphate, 2% glucose, 0.1% nitrogen source is quoted in the text), YNB-NH_4 (0.67% yeast nitrogen base without amino acids, 2% glucose). For carbon source assimilation tests, media were prepared by substituting glucose in YNB-NH_4 medium for the corresponding carbon source (2% sugars, 3% alcohols and acetate). The growth on glycerol was tested on YP plates with various glycerol concentrations. To estimate the cell tolerance to alkali metal cations, media were supplemented with NaCl, KCl and LiCl, as indicated in the text. Solid media were firmed with 2% agar, media with a salt concentration higher than 2 M with 3% agar. For growth experiments on solid media, plates were inoculated with 5 µl drops of serial 15-fold dilutions of saturated yeast cultures. For growth curves, an overnight pre-inoculum was used; 20 ml of the medium in a 100 ml Erlenmayer flask was then inoculated with the pre-inoculum to a density of 6×10^5 cells/ml ($OD_{600} = 0.02$; spectrophotometer, Spekol 211 spectrophotometer, Carl Zeiss). The cultures were cultivated in a rotary shaker at 30 °C, 200 r.p.m. The increase of absorbance at 600 nm was measured. When OD_{600} of the liquid cultures reached 1:

1. The number of cells in 1 ml of the cell suspension was determined: 10 µl aliquots were taken, 10^4-fold diluted and 100 µl of the diluted cell suspension was spread on YPD plates (two experiments were performed in parallel). Three days later, colonies were counted and the number was then recalculated for 1 ml of the original culture.
2. The dry weight of biomass was estimated: 1 ml of the cell suspension (washed twice with water) was exposed to 90 °C for 4 h and the cooled dry biomass was subsequently weighed (two experiments were performed in parallel).

Total glycerol content measurement

To analyse the total glycerol content, yeasts were grown in the YPD media supplemented with various NaCl concentrations to $OD_{600} = 0.9–1.2$. Samples of 1 ml were then withdrawn, boiled for 10 min, mixed vigorously with a vortex and centrifuged (14000 × *g*, 10 min). The glycerol content was estimated in supernatants using the Free Glycerol Determination Kit (Sigma), following the instructions in the manual. Each measurement was taken twice in parallel, and each experiment was repeated three times.

Microscopy

Cells in the exponential phase of growth (in YNB-NH_4 medium) were viewed with an AX70 microscope (Olympus) in Nomarski contrast, magnification 100×, and photographed.

Isolation of genomic DNA and PCR

Genomic DNA was isolated (Hoffman and Winston, 1987) and used as a template for PCR reaction. PCR amplification was performed with Taq DNA polymerase (PPP Master Mix, Top-Bio) using a Gradient MasterCycler (Eppendorf).

Chromosomal DNA isolation and pulse-field gel electrophoresis (PFGE)

Chromosomal DNA in plugs was prepared according to the protocol designed for *S. cerevisiae* (Vezinhet *et al.*, 1990) with slight modifications. Lyticase from *Arthrobacter luteus* (Sigma) was used for cell treatment (100 μg/1.5 × 10^9 cells/ml). The 1% agarose gels were prepared from pulse-field certified agarose (Bio-Rad Laboratories) and run on a Bio-Rad CHEF (contour-clamped homogenous electric field) apparatus in 0.5× TBE at 14 °C. The operating conditions were: switch time, 300 s; run time, 100 h; angle, 106°; voltage, 3 V/cm.

Results

Osmotolerance

The tolerance of the two *Z. rouxii* strains to high osmotic pressure was first studied as the ability to grow in the presence of high salt concentrations in the media. To determine their halotolerance limits, *Z. rouxii* strains ATCC 2623 and ATCC 42981 were grown on solid YNB-NH_4 media with increasing concentrations of KCl, NaCl and LiCl. The highest salt concentrations allowing growth are summarized in Table 1. For comparison, the halotolerance of a *S. cerevisiae* wild-type strain is shown.

Both *Z. rouxii* strains grew up to the highest KCl concentrations tested (3 M). For ATCC 2623, this was the limiting concentration, as a deceleration in growth was observed. The growth of ATCC 42981 remained strong under these conditions. Both strains tolerated higher concentrations of KCl than *S. cerevisiae*. When their NaCl tolerance was tested, the growth of ATCC 2623 began to be inhibited at a concentration of 2 M; a 2.5 M salt concentration was its limit, allowing only very slow growth. ATCC 42981 grew well even at the highest concentration tested (3 M). When the strains were cultured on YPD (a medium with higher osmotic pressure than YNB-NH_4), the concentration limit for ATCC 2623 was 1 M NaCl; the ATCC 42981 strain grew well even in the presence of 2 M NaCl. Both strains proved to be more NaCl-tolerant than *S. cerevisiae*.

Table 1. The highest salt concentrations enabling growth of *Z. rouxii* and *S. cerevisiae* strains on solid YNB-NH_4 media

	Salt concentration (M)		
	KCl	NaCl	LiCl
Z. rouxii ATCC 2623	3.0*	2.5	0.1
Z. rouxii ATCC 42981	3.0*	3.0*	0.6
S. cerevisiae FL100	2.0	1.8	0.4

* Maximal concentration allowing solidification of plates.

In the case of LiCl, the toxicity of Li^+ cations influences growth more than the osmotic pressure caused by the presence of the salt. The growth of both strains began to reduce at much lower concentrations compared to KCl or NaCl. The growth of ATCC 2623 was reduced at a concentration of 0.06 M and limited at 0.1 M. The growth of ATCC 42981 was reduced at 0.2 M LiCl; it grew slightly up to 0.6 M, which we considered to be the limiting concentration for this strain. For *S. cerevisiae* FL100, the limiting concentration of LiCl in the medium was 0.4 M. Thus, in this case, the ATCC 2623 strain tolerated lower concentrations of lithium cations than *S. cerevisiae*, while the ATCC 42981 strain was again the most tolerant.

To avoid the effect of cation toxicity when testing osmotolerance, we examined the growth of the two strains in the presence of high concentrations of sorbitol or sucrose. Both strains grew well on YPD plates with 35% sorbitol or 20% sucrose (not shown). When the cells were grown in liquid YPD with 40% sucrose, the growth of ATCC 42981 was similar to in YPD. This was different from ATCC 2623, which was able to reach a higher OD in YPD than in YPD + 40% sucrose. In comparison with *S. cerevisiae*, both *Z. rouxii* strains grew better in the presence of high sorbitol or sucrose concentrations (not shown).

Thus, we confirmed the higher osmotolerance of *Z. rouxii* compared to *S. cerevisiae* but also revealed differences between the two *Z. rouxii*

strains concerning their osmotolerance and sensitivity to toxic lithium cations.

Glycerol content

To determine whether the observed difference in osmotolerance between the two *Z. rouxii* strains is related to different levels of accumulation of glycerol as a compatible solute upon osmotic stress, we measured glycerol production in the two strains. The total amount of glycerol was estimated in yeast cultures grown exponentially in YPD, YPD + 0.3 M NaCl (a low osmotic stress medium) and YPD + 1 M NaCl (a high osmotic stress medium; Figure 1). In the medium without osmotic stress (YPD), *Z. rouxii* ATCC 42981 and *S. cerevisiae* S288c produced similar amounts of glycerol (0.33 and 0.31 mg/ml, respectively); by contrast, the glycerol production of ATCC 2623 was about half (0.15 mg/ml). This difference between the strains remained in YPD + 0.3 M NaCl, where a slight increase in glycerol concentration (a factor of approximately 1.1–1.2) was apparent in all strains. In the medium with a high NaCl concentration (1 M), *S. cerevisiae* S288c strongly produced glycerol (3.91 times more than in YPD), i.e. a markedly higher amount than both the ATCC 2623 and ATCC 42981 strains, whose glycerol contents increased by a factor of 2.47 and 1.67, respectively, compared to YPD. Comparing the two *Z. rouxii* strains, the production of glycerol in ATCC 2623 increased more rapidly upon osmotic stress but the total concentration of glycerol always remained lower than in ATCC 42981.

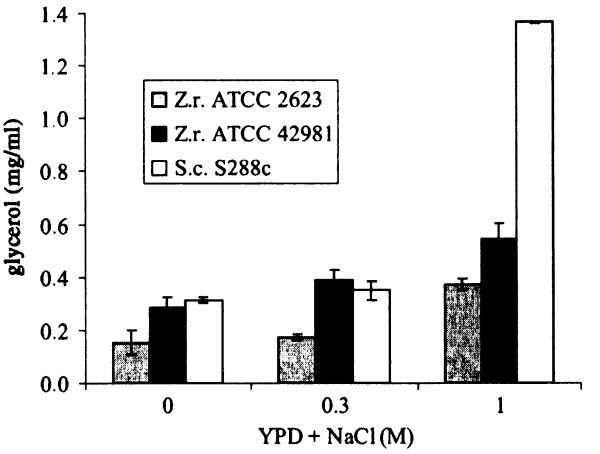


Figure 1. Total glycerol concentration in exponentially growing yeast cultures in YPD supplemented with different NaCl concentration. *S. cerevisiae* S288c was used for comparison. Z.r., *Z. rouxii*; S.c., *S. cerevisiae*

Colony and cell appearance

The colonies formed by cells of the two *Z. rouxii* wild-type strains were similar in appearance, with smooth surfaces, white to cream-white coloured, margins finely lobed (not shown); on the other hand, the cell sizes and shapes differed (Figure 2). The cells of ATCC 2623 were elongated and formed chains, which fully corresponded to the features shown in the taxonomic classification of Kurtzman and Fell (1998). The cells of the ATCC 42981 strain were bigger than those of ATCC 2623, ellipsoid or round and did not form chains. The cells of both *Z. rouxii* strains proved to be larger than those of *S. cerevisiae*.

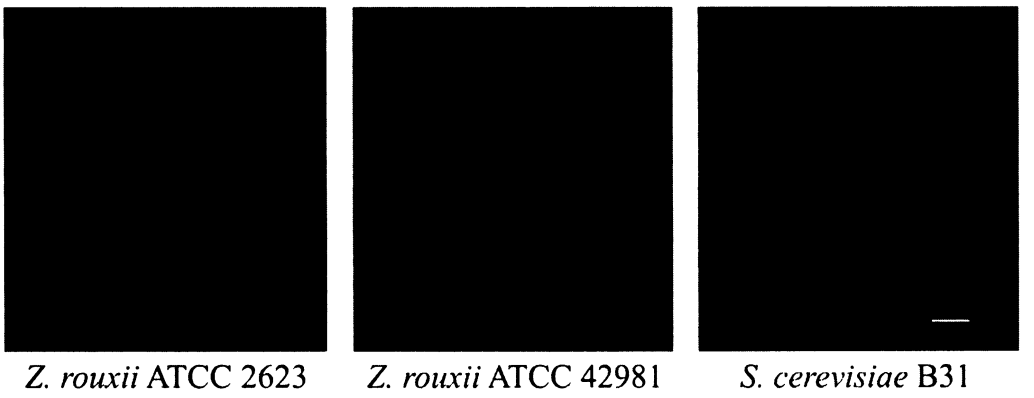


Figure 2. The appearance of *Z. rouxii* and *S. cerevisiae* cells from the exponential phase of growth in liquid YNB-NH_4 media. Bar, 5 μm

Growth characteristics in a rich medium

To compare the growth characteristics of the two *Z. rouxii* strains in medium abundant in nutrients, the strains were grown in liquid YPD. The growth curves were similar for both strains (not shown), except that ATCC 2623 was able to reach a higher OD_{600} (approx. 7.0) than ATCC 42 981 (approx. 4.0), suggesting a better utilization of nutrients by ATCC 2623. When the number of cells and the dry weight of biomass in 1 ml cell suspension was estimated, no significant differences were found (for both strains, $OD_{600} = 1$ corresponded to approx. 3×10^7 cells/ml, i.e. 1.16 mg dry weight/ml).

Utilization of carbon sources

The assimilation of 16 different carbon sources by the two *Z. rouxii* strains was tested on solid YNB-NH_4 medium, as described in Materials and methods. If it was difficult to interpret the results, we also tested the growth in a liquid culture in order to avoid the influence of agar as a possible carbon source (although the cells did not grow on YP-plates).

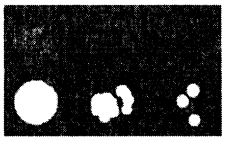
Of the sugars, eight monosaccharides or their derivatives (D-glucose, methyl α-D-glucoside, D-rhamnose, D-xylose, D-ribose, D-arabinose, D-galactose, D-fructose) and four disaccharides (sucrose, lactose, trehalose, maltose) were tested. Both strains were able to utilize almost all the sugars tested, except for D-rhamnose, D-ribose and lactose. No significant differences between the strains were found.

Concerning non-fermentable sources of carbon, we tested ethanol, methanol, acetate and glycerol. On solid media, both strains could utilize ethanol, methanol and acetate. Concerning the assimilation of glycerol, a difference was found — glycerol was assimilated only by ATCC 42 981. This observation was confirmed by growth tests of both *Z. rouxii* strains in liquid YP and YPD with various concentrations of glycerol (0–20%). Both strains grew well in the medium with both glucose and glycerol (YPD + 20% glycerol); the growth of ATCC 2623 was only slightly reduced. On the other hand, only ATCC 42 981 was able to grow in medium in which glycerol was substituted for glucose. Table 2 demonstrates the different assimilations of glycerol by the two *Z. rouxii* strains.

Nitrogen sources and osmotolerance

Osmotolerant yeasts grow on a broad range of nitrogen compounds. In some cases, the utilization of a nitrogen source is affected by a high external osmotic pressure. To explore the influence of a medium with increased osmotic activity on the assimilation of a nitrogen source, *Z. rouxii* ATCC 2623 and ATCC 42 981 cells were grown on YNB plates with six different sources of nitrogen in the presence/absence of 2 M KCl (Table 3). We observed a differing utilization of urea (the ATCC 2623 strain was able to grow, but the ATCC 42 981 strain was not) and α-aminoadipic acid (ATCC 2623 did not grow, while ATCC 42 981 grew weakly). High external osmotic pressure decreased the growth of ATCC 2623 on lysine or urea, but it improved the growth of both strains on α-aminoadipic acid.

Table 2. Growth of *Z. rouxii* strains in YP + 3% glycerol

Z. rouxii strain	Solid media after 5 days	OD_{600} in liquid media after 43 h
ATCC 2623		0.02*
ATCC 42981		2.36

* Value corresponding to inoculation.

Table 3. Dependence of growth of the two *Z. rouxii* strains on the various nitrogen sources and osmotic pressure

	Nitrogen source	NH_4^+		Pro		Lys		Trp		Urea		α-aa	
Z. rouxii strain	KCl (M)	0	2	0	2	0	2	0	2	0	2	0	2
ATCC 2623		+	+	+	+	+	±	+	+	±	−	−	±
ATCC 42981		+	+	+	+	+	+	+	+	−	−	w	±

+, positive; ±, positive but slow; w, weak; −, negative; Pro, proline; Lys, lysine; Trp, tryptophan; α-aa, α-aminoadipic acid.

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Conservation of synteny

To compare the two strains on a molecular level, we used PCR amplifications with primers designed to recognize several previously sequenced *Z. rouxii* DNA fragments. From the ATCC 42981 strain, we chose two DNA fragments containing the alleles of genes encoding for the Na^+/H^+ antiporters *ZrSOD2* and *ZrSOD22*. From the ATCC 2623 strain, we chose five previously sequenced DNA fragments: ADE2 (Sychrova *et al.*, 1999); HIS3 (Sychrova *et al.*, 2000b); LEU2 (Sychrova, 2001); HOG1 (Kinclova *et al.*, 2001) and Zr10-4 (Sychrova *et al.*, 2000a). Four of these seven fragments comprised more than one gene — the ADE2 fragment consisted of *ZrADE2*, *ZrRGA1* and part of the *ZrCPF1* gene; the HIS3 fragment of *ZrPET56*, *ZrHIS3* and part of the *ZrMRP51* gene; the LEU2 fragment of *ZrLEU2*, *ZrNSF1*, the Zr10-4 fragment of the *ZrRGD1* gene and two ORFs with homology to *S. cerevisiae* ORFs of unknown function. Three DNA fragments contained only one gene (*ZrHOG1*, *ZrSOD2* or *ZrSOD22*).

PCR reactions were run with combinations of primers amplifying DNA fragments that covered parts of adjacent genes and the corresponding intergenic regions (if the sequenced DNA fragment contained only one gene, at least one of the primers was designed to attach outside the coding sequence). An example of primer positions and the bands acquired by PCR is shown in Figure 3. Altogether, 11 combinations of primers were used to amplify different fragments of *Z. rouxii* DNA. The genomic DNA of *S. cerevisiae* FL100 was used as a control.

Except for the PCR amplification using primers for the *ZrSOD22*-containing fragment, the patterns shown by both *Z. rouxii* strains were identical for the same combinations of primers. In the case of *ZrSOD22*, no PCR product was obtained when

Figure 3. Conservation of synteny between the ATCC 42981 and the ATCC 2623 ADE2 fragments. (A) Positions of primers designed for the ADE2 fragment. (B) PCR reactions run with two combinations of primers: 1a + 1b and 2a + 2b. 2623, *Z. rouxii* ATCC 2623; 42981, *Z. rouxii* ATCC 42981; Sc, *S. cerevisiae* FL100; M, DNA Molecular Weight Marker III (Roche)

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the genomic DNA of ATCC 2623 was used. This fully corresponded to the previous observation that the ATCC 2623 strain contained only one copy of the *SOD2* gene (*ZrSOD2-22*; Kinclova *et al.*, 2001). When the DNA of *S. cerevisiae* was used as a template, either no DNA was amplified or the pattern was different compared to *Z. rouxii*. These results suggest the conservation of synteny between the two *Z. rouxii* strains.

Comparison of the karyotype by PFGE

The protocol designed for the isolation of chromosomal DNA (Vezinhet *et al.*, 1990) required treatment with Novozyme 234, a commercially prepared mixture of cell-wall degrading enzymes. Instead of Novozyme 234, we used Lyticase (Sigma), which works in a similar way. For the preparation of *Z. rouxii* chromosomal DNA, we followed the protocol designed for *S. cerevisiae* (Vezinhet *et al.*, 1990) but we prolonged the time of the cell treatment by Lyticase to approximately 5 h.

The karyotype of the two *Z. rouxii* strains is shown in Figure 4. Each strain had a different pattern. The ATCC 2623 strain seemed to have seven chromosomes, whereas ATCC 42981 appeared to contain eight. ATCC 2623 chromosomes VI and VII were also conserved in ATCC 42981 (chromosomes VII and VIII), and chromosome V from ATCC 2623 was missing in ATCC 42981. The other chromosomes of both strains were not conserved so far as their sizes were concerned. Altogether, ATCC 42981 seems to have a larger genome than ATCC 2623.

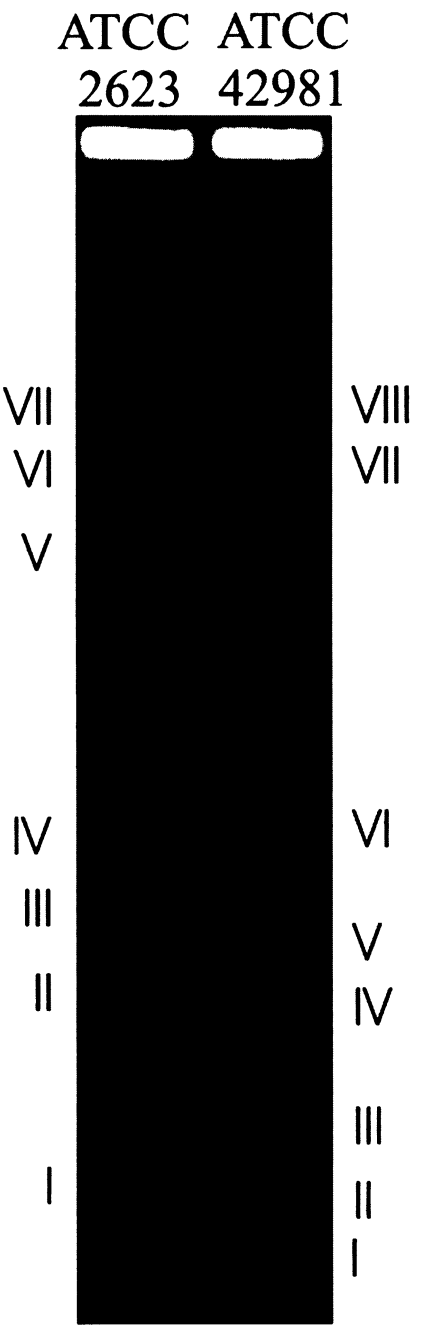

Figure 4. Karyotype of the two *Z. rouxii* strains. Chromosome numbers are indicated by roman numerals

Discussion

The cells of the two *Z. rouxii* strains differ in their size and shape. In our previous study, we revealed the necessity of using different protocols for their transformation (ATCC 2623 required LiAc treatment to weaken its cell surface structures; Pribylova and Sychrova, 2003). This, together with the different cell morphologies, suggested a different organization of the cell wall in the two strains. By contrast, the colonies look similar and are also similar in the number of cells and the dry weight of biomass corresponding to 1 ml of exponentially grown cells in YPD. However, ATCC 2623 seems to utilize the nutrients in YPD more effectively than ATCC 42981.

Concerning salt tolerance, both *Z. rouxii* strains supported higher KCl or NaCl concentrations in the media than *S. cerevisiae*. On the other hand

ATCC 2623, of the three strains tested, was the most sensitive to Li^+ cations (even to concentrations without osmotic effect), which inhibit the activity of RNA modifying enzymes (Dichtl *et al.*, 1997). The partial toxicity of sodium cations could also be observed in our experiments — the highest acceptable concentration of NaCl was always lower than that of KCl. In all cases, ATCC 42981 proved to be more resistant than ATCC 2623. The question arises, whether *Z. rouxii* is halophilic rather than just halotolerant. Our experiments proved *Z. rouxii* to grow better in the absence than in the presence of salts on solid media, as previously observed in a study focused on the ATCC 42981 strain (Watanabe and Takakuwa, 1984). However, in liquid cultures, ATCC 42981 grew better in the presence of of 1 or 2 M NaCl than in a medium without salt (Watanabe and Takakuwa, 1984). The discrepancy between the effects of NaCl on cell growth in static vs. shaken cultures could be explained by different aeration levels. A conditions-dependent halophilic behaviour has already been shown for another salt-resistant yeast, *Debaryomyces hansenii*, whose growth was stimulated by NaCl at higher temperatures (Almagro *et al.*, 2000).

When we cultured the two *Z. rouxii* strains in the presence of high concentrations of osmotically active compounds, which do not exhibit the additional toxic effect as salt ions do (sorbitol, sucrose), the growth of both *Z. rouxii* strains was similar, and better than *S. cerevisiae* growth.

The difference in salt tolerance between the two *Z. rouxii* strains can be related to the conditions in which they naturally evolved. ATCC 2623 was isolated from grape must, which is from an environment high in sugars but not in salts. Thus, this strain had no need to be highly halotolerant. The ATCC 42981 strain was isolated from the Japanese traditional salty seasoning miso. Consequently, this strain either had to adapt its metabolism to high cytoplasmic concentrations of salts or develop efficient efflux systems to maintain a low intracellular level of alkali metal cations that enter the cells along their concentration gradient.

The ATCC 42981 strain is known to contain two gene alleles encoding a Na^+/H^+ antiporter (Iwaki *et al.*, 1998; Watanabe *et al.*, 1995), while the ATCC 2623 strain has only one (Kinclova *et al.*, 2001). Also, the genes involved in the regulation of cell response to osmotic shock are usually found in two copies in the ATCC 42981 strain, e.g. *HOG1* (Iwaki *et al.*, 1999), *GPD1* and *GCY1* (Iwaki *et al.*, 2001). This doubling of genes involved in adaptation to osmotic shock could be the reason for the high halotolerance of ATCC 42981. Besides only one copy of the Na^+/H^+ antiporter gene, the ATCC 2623 strain only contains one copy of the *HOG1* gene (Kinclova *et al.*, 2001); no data are available for *GPD1* or *GCY1* genes in ATCC 2623. Recent findings referred to *Z. rouxii* ATCC 42981 as a possible hybrid, as it possesses two copies of many nuclear-encoded genes, one copy closely related to *Z. rouxii* ATCC 2623 and the other showing a higher sequence divergence (James *et al.*, 2005). Our results concerning the different karyotypes of the two strains tend to favour this hypothesis. *Z. rouxii* ATCC 2623 contains seven chromosomes with a total nuclear genome size of about 12.8 Mb (Sychrova *et al.*, 2000a), while ATCC 42981 seems to have a bigger genome size with eight chromosomes. For *Z. rouxii*, several attempts to estimate the number and size of its chromosomes have been published (de Jonge *et al.*, 1986; Johnston *et al.*, 1989; Oda and Tonomura, 1995; Torok *et al.*, 1993). The number of chromosomes ranged from six (de Jonge *et al.*, 1986) to nine (Johnston *et al.*, 1989), depending on the strain and conditions of electrophoretic separation. Polymorphism in chromosome number and/or length could result from the hybrid nature of some *Z. rouxii* strains, as already confirmed for NCYC 1682, NCYC 3060 and NCYC 3061 and suggested for ATCC 42981 (James *et al.*, 2005). Our results of synteny conservation between the two strains support the hypothesis that ATCC 42981 is a hybrid of *Z. rouxii* ATCC 2623 and another *Zygosaccharomyces* strain.

Concerning the growth characteristics in a rich medium and utilization of different sources of carbon or nitrogen, the two *Z. rouxii* strains behaved very similarly but one interesting difference was found — the strains differed in their ability to utilize glycerol. According to its taxonomic classification (Kurtzman and Fell, 1998), *Z. rouxii* is able to assimilate this source of carbon. Our experiments showed that this was only true for the ATCC 42981 strain. As glycerol is an important factor affecting osmotolerance, this difference in glycerol metabolism (different biosynthesis and/or degradation) could be related to the different osmotolerance that each strain exhibited. The total

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glycerol measurements showed that under non-stressing conditions, the ATCC 42 981 strain produced higher amounts of glycerol than ATCC 2623. This higher glycerol production obviously enables ATCC 42 981 to support higher concentrations of osmotically active compounds (as was shown for salts) than the ATCC 2623 strain. When compared to *S. cerevisiae*, although the glycerol contents of ATCC 42 981 and S288c under non-stressing conditions are similar, the glycerol production differs strongly when cultured in a high concentration of NaCl. *S. cerevisiae* produces glycerol at a much higher level than both of the *Z. rouxii* strains, but at the same time it is only able to survive a much lower salt concentration. This supports the hypothesis that *Z. rouxii* may possess a system that enables it to either retain glycerol inside the cells, which would be normally lost by diffusion, or to retransport it back into the cell. Such a transporter in *Z. rouxii* was already predicted by kinetic studies (van Zyl *et al.*, 1990); but no gene has been identified so far.

Our data showed that besides the predicted differences in cell wall properties (Pribylova and Sychrova, 2003), marked differences between the two most-studied *Z. rouxii* wild-type strains exist in crucial properties, such as osmotolerance and glycerol production and metabolism. The abilities of the ATCC 42 981 strain to tolerate high salt concentrations and to produce and assimilate glycerol were higher than those of ATCC 2623, which suggested a different gene content or regulation. The karyotype analysis supports the hypothesis that ATCC 42 981 is a hybrid.

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5.2.2. Publikace č. 6: Differences in osmotolerant and cell wall properties of two *Zygosaccharomyces rouxii* strains

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Na základě rozdílných vlastností buněčných povrchů kmenů CBS 732^T a ATCC 42981 zjištěných v publikaci č. 1 (odlišné podmínky pro rozvolnění buněčných stěn před elektroporací) a publikaci č. 5 (rozdíly v morfologii) jsme se rozhodli porovnat složení a strukturu buněčných stěn obou kmenů. Fakt, že k úspěšné elektroporaci kmenů odvozených od CBS 732^T a ATCC 42981 bylo zapotřebí buňky kultivovat za mírného solného stresu (v přítomnosti 300 mM NaCl; viz publikace č. 1), naznačoval, že se budou vlastnosti osmotolerantní a vlastnosti buněčné stěny obou kmenů *Z. rouxii* navzájem ovlivňovat, jak již bylo prokázáno u několika druhů kvasinek (viz kap. 2.1.2.). U obou kmenů jsme proto sledovali vliv osmotického stresu na složení, odolnost a strukturu buněčné stěny.

U buněk jsme sledovali rezistenci k enzymům lyzujícím buněčnou stěnu (Lyticasa, Zymolyasa), obsah polymerů tvořících buněčnou stěnu a mikromorfologii buněčných stěn. Vlastnosti jsme porovnali jak za růstu v nestresových podmínkách (bohaté médium YPG), tak v podmínkách mírně zvýšené osmolarity (YPG + 300 mM NaCl). Rozdíly nalezené mezi kmeny naznačují, že buňky méně osmotolerantního kmene CBS 732^T mají rigidnější buněčnou stěnu než buňky osmotolerantnějšího ATCC 42981, jejichž buněčná stěna byla dokonce méně odolná k působení lytických enzymů než stěna buněk osmosenzitivní kvasinky *S. cerevisiae*. Elastičtější buněčná stěna může pravděpodobněji flexibilněji reagovat na změny osmotického tlaku, a tak přispívat k vyšší osmotoleranci kmene ATCC 42981.

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Differences in Osmotolerant and Cell-Wall Properties of Two *Zygosaccharomyces rouxii* Strains

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ABSTRACT. The osmotolerant and cell wall properties of the two most studied wild-type *Zygosaccharomyces rouxii* strains (CBS 732 and ATCC 42981) were examined. Differences in their (*1*) tolerance to high salt content in the medium, (*2*) resistance to the lysing enzymes lyticase and zymolyase, (*3*) cell-wall polymer content and (*4*) cell wall micromorphology suggested that the less osmotolerant CBS 732 strain possesses a more rigid cell wall than the more osmotolerant ATCC 42981, whose cell wall seems to be more flexible and elastic.

Abbreviations

CFU	colony-forming units	ETL	electron-transparent layer
CWP	cell-wall protein	GlcNAc	*N*-acetyl-D-glucosamine
DTT	1,4-dithiothreitol	YPD	YPD broth
EOL	electron opaque layer	YPD+NaCl	YPD + 0.3 mol/L NaCl

Zygosaccharomyces rouxii is a hemiascomycetous yeast known for its high tolerance to osmotic stress. For this unique feature, it is employed in the food industry (fermentations of concentrated Japanese seasonings) but it is also known as a spoilage yeast of concentrated food products (jams, salad dressings *etc.*). So far, the reason for its high osmotolerance remains unknown. Considering the close phylogenetic relationship of *Z. rouxii* and *Saccharomyces cerevisiae* (de Montigny *et al.* 2000), identifying the genes that are responsible for *Z. rouxii* osmotolerance would be of a great interest to the food industry – their heterologous expression in industrial strains of *S. cerevisiae* could improve the growth capacity of these strains under adverse conditions.

Properties of the cell wall are one of the factors influencing the osmotolerance of the yeast cell (Klis *et al.* 2006). Four classes of macromolecules, which are all interconnected by covalent bonds, form the *S. cerevisiae* cell wall: the mannosylated CWPs, 1,3-β-D-glucan, 1,6-β-D-glucan and chitin (a polymer of GlcNAc). The cell wall consists of an inner (electron transparent) layer of load-bearing polysaccharides, acting as a scaffold for a protective outer (electron dense) layer of CWPs that extend into the medium. This apparently rigid structure maintains a high degree of flexibility needed for adapting to different developmental programs and to different environmental conditions (Klis *et al.* 2006; Farkaš 2003).

So far, little is known about *Z. rouxii* cell wall. The derivatives of the two most studied *Z. rouxii* wild-type strains, CBS 732 and ATCC 42981, responded differently to Li^+ treatment before transformation by electroporation (Přibylová and Sychrová 2003), which suggested different cell wall composition or organization (Ito *et al.* 1983). The cultivation of both *Z. rouxii* strains in the presence of 0.3 mol/L NaCl enhanced the transformation efficiency, which indicated that a mild salt stress affected the cell wall by rendering it more permeable for DNA.

Here we compare the halotolerant and cell wall properties (*i.e.* sensitivity to the lysing enzymes lyticase and zymolyase, cell-wall polymer content and cell wall micromorphology) of the two *Z. rouxii* wild-type strains. The effect of salt stress on cell wall properties is examined.

MATERIALS AND METHODS

Strains. The *Z. rouxii* wild-type strains were CBS 732 (ATCC 2623) and ATCC 42981. Standard laboratory *S. cerevisiae* wild-type strains (FL100 and S288C) were used for comparison of *Z. rouxii* and *S. cerevisiae* properties.

Media and growth experiments. Cells were grown in YPD (5 %; *Difco*) medium at 30 °C. For growth experiments on solid media (drop tests), plates were inoculated with 5 mL drops of serial 15-fold dilutions of saturated yeast cultures. Halotolerance was tested on minimal YNB-NH_4 (0.67 % yeast nitrogen base without amino acids, *Difco*, 2 % glucose) medium, as this medium has lower osmotic pressure than YPD, and thus enables better determination of the halotolerance limits.

Lyticase and zymolyase treatment. Lyticase from *Arthrobacter luteus*, activity ≥33 μkat/mg protein (*Sigma*, cat. no. L2424) and zymolyase-100 T, activity 1.7 μkat/mg protein (*Seikagaku America*, cat. no. 120493-1) were used as lysing enzymes. 1.3×10^8 cells in the mid-exponentional phase of growth in YPD or YPD+NaCl were washed with EDTA (50 mmol/L, pH 8.0), resuspended in 1 mL CPE (mmol/L: Na_2HPO_4 120, citric acid 40, EDTA 20), incubated with 33 nkat/mL of lyticase or zymolyase for 20 min, appropriately diluted and plated on YPD. After 3 d, the survival percentage was estimated.

Cell-wall content analysis. Exponential-phase cells were washed with cold water and disrupted by ballotini beads in a rotary disintegrator (Novotný 1964) immersed in an ice-bath until >96 % of the cells were broken. The slurry was decanted with water, centrifuged (1200 *g*, 10 min) and the pellet was washed 20× with 1 mol/L NaCl and finally with water. The isolated cell walls were lyophilized. For the determination of β-glucan, 10 mg of the cell walls was suspended in 0.2 mL of ice-cold 70 % (*V/V*) sulfuric acid and the tubes kept in the ice overnight. After that, 2 mL of water was added to each tube; the tubes were sealed and heated for 8 h at 105 °C. The hydrolysates were neutralized with 5 mol/L NaOH, the volume was adjusted with water to 5 mL and glucose was determined in aliquots with a glucose oxidase–peroxidase kit (*Lachema*, Czechia). GlcNAc was determined in the enzymic digest of native (representing the total chitin) and/or alkali-extracted (representing the crystalline chitin) cell walls. Ten mg of lyophilized cell walls were suspended in 2 mL of 1 mol/L NaOH and heated for 1 h at 80 °C. The cell walls were then collected by centrifugation (1500 *g*, 10 min) and the supernatant was used for the determination of CWP by the Lowry method. The pellet was subsequently washed with 1 mol/L acetic acid and 2× with water. Ten mg of the native cell walls or their alkali-extracted equivalent was suspended in 0.5 mL of incubation mixture containing 25 mmol/L acetate buffer (pH 5.5), 0.2 mg of *Serratia marcescens* chitinase (*Sigma*), 1 mg of dialyzed glusulase (*Sigma*) and 200 ppm sodium azide. The mixtures were incubated for 2 d at 30 °C. GlcNAc in the hydrolyzates was determined with the Ehrlich reagent (Davidson 1966). All analyses were done 2× and the determinations performed in duplicate.

Electron microscopy. Cells grown in YPD or YPD+NaCl were harvested and pelleted by centrifugation. Pellets were washed 3× with H_2O and fixed in 3 % glutaraldehyde solution in cacodylate buffer (0.13 mol/L, pH 7.4) for 3 h at room temperature. Cells were then washed in 0.1 mol/L cacodylate buffer for 30 min 3× and post-fixed in 1 % osmium tetroxide in 0.1 mol/L cacodylate buffer for 1 h at room temperature. After rapid dehydration in a graded series of ethanol, cells were embedded in an "LR-White" mixture. Ultrathin sections, obtained with a diamond knife on an Ultracut *Reichert-Jung* ultramicrotome, were placed on Formvar coated copper grids, stained with 2.5 % uranyl acetate (6 min) and lead(IV) citrate (3 min) and observed with a *Philips* Morgagni transmission electron microscope.

RESULTS

Halotolerance. The ATCC 42981 strain proved to be more salt tolerant than the CBS 732 strain. It grew well in the presence of 3 mol/L KCl or NaCl (*not shown*), while for the CBS 732 strain, 3 mol/L KCl and 2.5 mol/L NaCl were the limiting concentrations allowing growth (Fig. 1). Compared with the *S. cerevisiae* FL100 wild type (which supported no more than 1.8 mol/L NaCl or 2.0 mol/L KCl in the media), both *Z. rouxii* strains were shown to be significantly more salt-tolerant. On the other hand, CBS 732 was the most sensitive to Li^+ cations, even to a concentration without osmotic effect: it could not grow if the concentration of LiCl was >0.1 mol/L, while the limiting concentration for ATCC 42981 was 0.6 and 0.4 mol/L for *S. cerevisiae* FL100.

Resistance to lysing enzymes. In order to see if the cell walls of the two *Z. rouxii* wild-type strains differ, we tested their sensitivity to lyticase and zymolyase and compared it to that of *S. cerevisiae* S288C wild type. The two lysing enzymes affected the yeast strains differently (Table I). For all strains, zymolyase was more effective in lysing than lyticase; the survival of cells after zymolyase treatment was always lower

than their survival rate after lyticase treatment. The ATCC 42981 strain was the most sensitive of the three strains tested. Lyticase affected only *S. cerevisiae* S288C and *Z. rouxii* ATCC 42981 cell survival, whereas it did not lyse *Z. rouxii* CBS 732 cells. The effect of lyticase on strains S288C and ATCC 42981 was enhanced by cultivating the cells in NaCl.

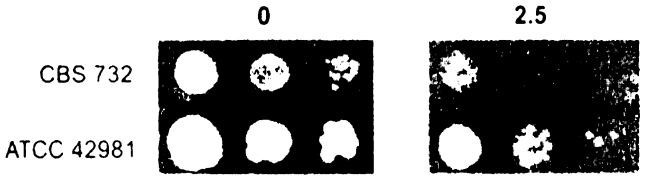

Fig. 1. Growth of the two *Z. rouxii* wild-type strains on YNB-NH_4 medium without (0) or with (2.5 mol/L) NaCl after 5 d of cultivation.

Table I. Survival of cells (% CFU) cultivated in YPD and/or YPD+NaCl after 20-min exposure to lyticase or zymolyase[a,b]

Strain		Lyticase		Zymolyase	
Z. rouxii	CBS 732	101	110	65.3	72.9
	ATCC 42981	62.3	43.0	10.9	9.30
S.cerevisiae	S288c	90.8	78.2	73.6	54.9

[a] *1st columns* - YPD, *2nd columns* - YPD+NaCl [b] Averages from 3 independent analyses.

Visualization of the cell walls by electron microscopy. The cell wall of the *Z. rouxii* strains consisted of two layers, the inner layer being ETL, the outer one EOL. In ATTC 42981 cells cultivated in YPD, the ETL (inner) was more or less constant in thickness and the EOL (outer) was thin (Fig. 2A). In contrast, in CBS 732 cells cultivated in YPD, both layers were of varying thickness; where the ETL was thin, the EOL

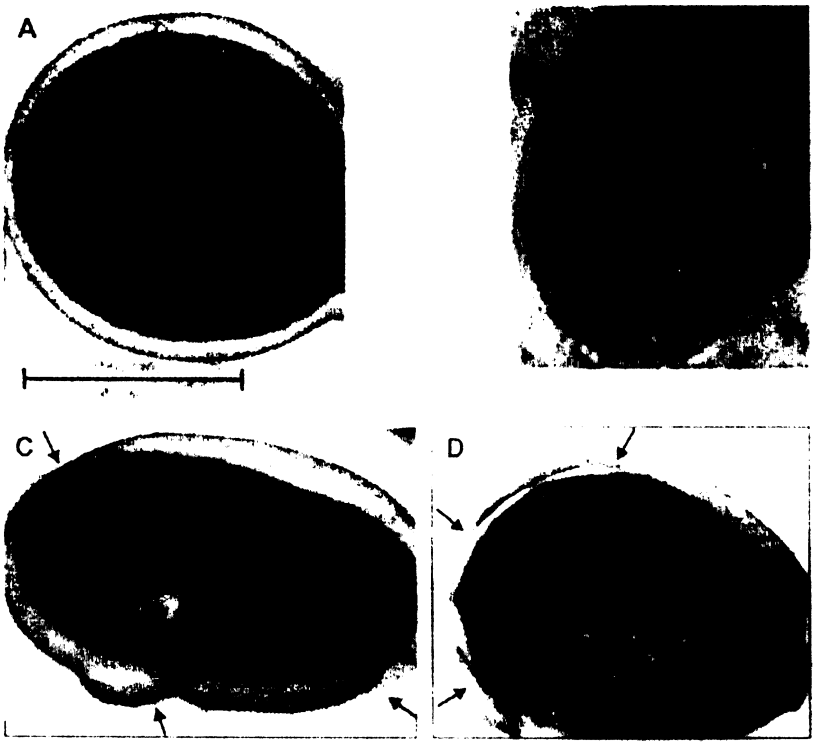

Fig. 2. Visualization of ultrathin sections of *Z. rouxii* cell walls by electron microscopy. Strains were either cultivated in YPD (**A**, ATCC 42981; **C**, CBS 732), or in YPD+NaCl (**B**, ATCC 42981; **D**, CBS 732). *arrows* - regions with distorted cell wall; *bar* = 2 μm.

was thicker. At some locations, only a thin EOL was detected and the ETL was missing (Fig. 2C). The cells of both strains cultivated in YPD+NaCl showed abnormality in their cell walls (Fig. 2B,D). This abnormality was more pronounced in CBS 732 cells than in ATTC 42981 cells. The cell wall of ATCC 42981 cultivated in YPD+NaCl was also thinner compared to ATCC 42981 cultivated in YPD.

Cell wall polymer content in cells cultivated in YPD or in YPD+NaCl was determined (Table II). Compared to CBS 732, the ATCC 42981 strain contained more CWP, more chitin (both crystalline and total), its crystalline chitin content being less affected by cultivation in NaCl. Slight influence of NaCl on β-glucan and CWP was observed: in both strains, the β-glucan content was slightly reduced, and, on the other hand, the CWP content was slightly increased.

Table II. Polymer content (%) in cell walls of the two *Z. rouxii* wild-type strains[a,b]

Strain	GlcNAc				CWP		Glucans[c]	
	NaOH-extracted		native					
CBS 732	2.38	1.52	2.92	2.14	5.17	5.77	57.9	54.5
ATCC 42981	2.91	2.71	3.36	3.52	5.51	6.06	55.4	52.5

[a]*1st columns* – YPD, *2nd columns* – YPD+NaCl. [b]Averages from 2 independent analyses. [c]Both 1,3- and 1,6-β-D-glucan.

DISCUSSION

All strains of *Z. rouxii* and *S. cerevisiae* were more sensitive to zymolyase than to lyticase. Possible explanations might be that the declared activity units of the two β-glucan-digesting enzymes are not of the same quality and/or there are more proteinases present in zymolyase, which can facilitate the access of the glucanase to the polysaccharide network. As far as differences among the strains are concerned, the CBS 732 strain was the most resistant and its resistance was not affected by the presence of NaCl in the cultivation medium. ATCC 42981 was on the other hand the most sensitive, even more than *S. cerevisiae*. This finding was interesting together with the observation that the ATCC 42981 strain was (compared to CBS 732) more halotolerant, contained more chitin and its cell wall architecture, as visualized by electron microscope, seemed to be more stable and less disorganized when cultivated with NaCl (although thinner than when cultivated without NaCl).

Evidently, the organization of the cell walls of the two *Z. rouxii* strains differs. This is supported by the cell sizes and shapes of the two strains. *Z. rouxii* CBS 732 cells were smaller and ellipsoid, while ATCC 42981 cells were bigger and more spherical (*not shown*). ATCC 42981 was much more sensitive to lysing enzymes than CBS 732, though it contained more chitin and CWP and similar amounts of β-glucan. This could be due to the degree and nature of association of the polymers into a 3-D network, which plays a crucial role in cell wall functionality. The glucan–chitin-CWP network could be more rigid in CBS 732, which may lead to a higher resistance to lysing enzymes but also to a lower elasticity and flexibility, which could then cause the distorted cell-wall pattern seen under the electron microscope and possibly also its lower osmotolerance. When yeast cells are transferred to a hypertonic medium, they rapidly shrink because of the loss of internal water. They recover volume by accumulating glycerol to levels that equilibrate the inner and outer osmolarity (Hohmann 2002). These changes in cell volume represent a stress for the cell wall and it can be assumed that an elastic and flexible cell wall can deal with these rapid volume changes more efficiently. The ATCC 42981 cell wall may thus adapt to the osmotic stress better than the apparently more rigid cell wall of CBS 732. The necessity of additional Li^+ treatment of CBS 732 cells before transformation compared to ATCC 42981 (Přibylová and Sychrová 2003) also pointed to a more resistant cell wall in CBS 732. It is also obvious that the presence of NaCl in the cultivation medium affects the *Z. rouxii* cell wall by lowering the chitin content (as observed by Tomita *et al.* 1996) though it has no effect on CBS 732 resistance to lysing enzymes.

In *S. cerevisiae* and *Candida albicans*, coordinated activity of the high osmolarity glycerol (HOG; Hohmann 2002) and protein kinase C (PKC; Gustin *et al.* 1998) pathways, mediating adaptive rearrangements in cell wall composition and architecture, are coping with osmotic stress (Wojda *et al.* 2003; Eisman *et al.* 2006). With *Z. rouxii*, the differing cell wall properties and osmotolerance of the two strains may be related to the presence of differing numbers of *HOG1* genes in each strain (ATCC 42981 contains two copies; Iwaki *et al.* 1999, while CBS 732 only one; Kinclová *et al.* 2001) and also to the fact that the CBS 732 strain is not able to assimilate glycerol, in contrast to ATCC 42981 (Přibylová *et al.* 2007).

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5.3. Na^+/H^+-transportní proteiny

Na^+/H^+-transportní proteiny patří mezi membránové přenašeče kationtů alkalických kovů, které se podílejí na iontové homeostázi buňky, a tedy i na osmotoleranci (viz kap. 2.1.3.). Z tohoto důvodu jim v této práci byla věnována zvýšená pozornost.

Abychom zjistili, jak jsou jednotlivé skupiny kvasinkových přenašečů alkalických kovů fylogeneticky konzervované, provedli jsme srovnávací analýzu sekvencí kvasinkových přenašečů homologních k *Sc*Nha1p, *Sc*Nhx1p a *Sc*Kha1p. Výsledky jsme shrnuli do publikace č. 7 (kap. 5.3.1.).

Co se týče Na^+/H^+-transportních proteinů kvasinky *Z. rouxii*, zatím byl v typovém kmeni *Z. rouxii* CBS 732^T identifikován pouze přenašeč s úzkou substrátovou specifitou (*Zr*Sod2-22p, schopný eliminovat z cytoplasmy ionty Na^+ a Li^+, ale nikoli K^+; viz kap. 2.2.1.), jehož funkce byla zatím studována pouze prostřednictvím heterologní exprese v *S. cerevisiae* (Kinclová *et al.*, 2001a, 2002). V jiném kmeni *Z. rouxii* (ATCC 42981) byly identifikovány dva geny kódující Na^+/H^+-transportní proteiny (*ZrSOD2* a *ZrSOD22*), oba vykazující vysoký stupeň podobnosti s genem *ZrSOD2-22*, z nichž pouze *Zr*Sod2p byl popsán jako podílející se na eliminaci sodných kationtů z buněk, *ZrSOD22* zřejmě není v buňkách vůbec přepisován (Iwaki *et al.*, 1998; Watanabe *et al.*, 1995). V *Z. rouxii* tak dosud nebyl identifikován Na^+/H^+-transportní protein se specifitou pro transport draselných kationtů.

V této dizertační práci se nám v genomu kmene *Z. rouxii* CBS 732^T podařilo identifikovat dosud neznámý gen kódující transportní protein s primární strukturou velmi podobnou rodině kvasinkových Na^+/H^+-antiportérů plasmatické membrány, který jsme nazvali *Zr*Nha1p. Protein jsme funkčně charakterizovali (jak přímo v *Z. rouxii*, tak prostřednictvím heterologní exprese v *S. cerevisiae*), a určili jej jako přenašeč sodných, lithných a zároveň draselných kationtů. Za pomoci nástrojů genového inženýrství vyvinutých pro *Z. rouxii* jsme dále studovali i funkci Na^+/H^+-transportního proteinu *Zr*Sod2-22p přímo v této kvasince. Funkce obou přenašečů jsme porovnali. Tyto výsledky popisuje kap. 5.3.2.

5.3.1. Publikace č. 7: Exploration of yeast alkali metal cation/H^+ antiporters: Sequence and structure comparison

Přibylová, L., Papoušková, K., Zavřel, M., Souciet, J.-L., Sychrová, H. (2006) *Folia Microbiol* 51(5): 413 – 424

Pro zjištění významu konkrétních oblastí Na^+/H^+-antiportérů kvasinek pro jejich funkci jsme ve veřejně dostupných databázích obsahujících alespoň částečné sekvence genomů patnácti různých kvasinek lokalizovali proteiny homologní k Na^+/H^+-transportním systémům *Sc*Nha1p, *Sc*Nhx1p a *Sc*Kha1p, a jejich sekvence porovnali na úrovni DNA i proteinů. Identifikovali jsme konzervované aminokyselinové zbytky či celé motivy, které tak vypovídají o funkčním významu těchto oblastí. Fylogenetická analýza příbuznosti identifikovaných přenašečů s Na^+/H^+-transportními proteiny ostatních organismů ukázala, že proteiny Nhx1 jsou příbuzné savčím a rostlinným přenašečům plasmatické membrány i vnitrobuněčných membrán, proteiny Kha1 jsou podobné bakteriálním přenašečům a proteiny Nha1 tvoří skupinu, jejíž představitelé byly zatím identifikovány pouze v houbových organismech a nedávno i v lidském genomu (Brett *et al.*, 2005).

Zajímavé bylo, že dva kvasinkové druhy, *Y. lipolytica* a *S. pombe*, obsahovaly hned dva geny kódující Na^+/H^+-antiportéry homologní k *ScNHA1*, z nich pouze *Spsod2* byl charakterizovaný (Jia *et al.*, 1992; Kinclová *et al.*, 2002). Kolegyně Mgr. Klára Papoušková identifikované antiportéry charakterizovala a zjistila, že tyto kvasinky obsahují každá dva Na^+/H^+-antiportéry lišící se substrátovou specifitou (jeden transportující pouze Na^+ a Li^+, druhý přednostně K^+, a také Na^+ a Li^+), které patrně hrají rozdílné role v buněčné fyziologii. Ostatní kvasinky obsahovaly jen jeden gen kódující přenašeč Na^+/H^+; ve druzích, kde již byl charakterizován, plnil vždy funkci transportéru všech tří kationtů (tj. Na^+, Li^+ i K^+; Banuelos *et al.*, 2002; Kinclová *et al.*, 2002; Velková a Sychrová, 2006). Bylo důvodné předpokládat, že i kvasinka *Z. rouxii*, ve které byl dosud identifikován pouze přenašeč Na^+ a Li^+ (ZrSod2-22p), bude obsahovat antiportér typu Na^+/H^+ rozeznávající jako svůj substrát K^+. Jelikož přenašeč *Zr*Sod2-22p byl dosud studován pouze heterologní expresí v *S. cerevisiae*, rozhodli jsme se sledovat jeho funkci přímo v *Z. rouxii*. Zároveň jsme využili přístupu do zatím neveřejné databáze Génolevures 3 obsahující kompletní sekvenci genomu *Z. rouxii* pro vyhledání možných dalších genů kódujících přenašeče typu Na^+/H^+. Získané výsledky shrnuje kap. 5.3.2.

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Exploration of Yeast Alkali Metal Cation/H^+ Antiporters: Sequence and Structure Comparison

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ABSTRACT. The *Saccharomyces cerevisiae* genome contains three genes encoding alkali metal cation/H^+ antiporters (Nha1p, Nhx1p, Kha1p) that differ in cell localization, substrate specificity and physiological function. Systematic genome sequencing of other yeast species revealed highly conserved homologous ORFs in all of them. We compared the yeast sequences both at DNA and protein levels. The subfamily of yeast endosomal/prevacuolar Nhx1 antiporters is closely related to mammalian plasma membrane NHE proteins and to both plasma membrane and vacuolar plant antiporters. The high sequence conservation within this subfamily of yeast antiporters suggests that Nhx1p is of great importance in cell physiology. Yeast Kha1 proteins probably belong to the same subfamily as bacterial antiporters, whereas Nha1 proteins form a distinct subfamily.

Abbreviations

aa amino acid
ORF open reading frame
tmd(s) transmembrane domain(s)

Microorganisms in antiporter names

Cal	*Candida albicans*	*Sca*	*Saccharomyces castellii*
Cgl	*Candida glabrata*	*Sce*	*Saccharomyces cerevisiae*
Ctr	*Candida tropicalis*	*Sku*	*Saccharomyces kudriavzevii*
Dha	*Debaryomyces hansenii* var. *hansenii*	*Smi*	*Saccharomyces mikatae*
Kla	*Kluyveromyces lactis*	*Spa*	*Saccharomyces paradoxus*
Pan	*Pichia (Hansenula) anomala*	*Spo*	*Schizosaccharomyces pombe*
Pso	*Pichia sorbitophila*	*Yli*	*Yarrowia lipolytica*
Sba	*Saccharomyces bayanus*	*Zro*	*Zygosaccharomyces rouxii*

The maintenance of intracellular alkali-metal-cation homeostasis is a crucial task for the survival of a yeast cell. In yeast cells, potassium is the major cytoplasmic cation involved, among others, in the regulation of cell volume and intracellular pH, whereas sodium is toxic. Thus, yeast cells spend energy to accumulate high intracellular concentrations of K^+, on the one hand, and efficiently eliminate surplus Na^+ on the other. To maintain an optimum cytoplasmic concentration of potassium and a stable high intracellular K^+/Na^+ ratio, yeast cells employ transport systems mediating cation efflux and influx with different substrate specificities and using diverse mechanisms, *e.g.*, ATPases, symporters, antiporters and channels (Rodriguez-Navarro 2000; Sychrová 2004).

For effective Na^+ transport, the cells of most organisms use Na^+/H^+ antiporters as well as ATPases (Brett *et al.* 2005*a*). Although alkali metal cation/H^+ antiporters represent conserved transport systems existing in almost all types of organisms (archaea, bacteria, fungi, parasites, insects, plants, and mammals), their structure, substrate specificity and probable cell function have diverged during their evolution. While most microorganisms and plants use the inward gradient of protons created by the plasma membrane H^+-ATPase as a driving force to pump alkali metal cations out, animal cells usually consume the Na^+ gradient resulting from Na^+/K^+-ATPase activity in order to force the excess protons out and regulate intracellular pH. Cells of higher eukaryotes usually express several Na^+/H^+ antiporters in parallel, *e.g.*, the 9 isoforms in human cells (Orlowski and Grinstein 2004) or 3–5 isoforms in many plants (Blumwald 2000; Mansour *et al.* 2003). Yeast cells usually express only a few types of alkali metal cation/H^+ antiporters and thus become a suitable model to study their molecular properties and diverse functions.

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Systematic sequencing of the *Saccharomyces cerevisiae* genome revealed three ORFs encoding putative Na^+/H^+ antiporters – YDR456w, YJL094c and YLR138w (Andre 1995; Nelissen *et al.* 1997; Paulsen *et al.* 1998). None of these genes is essential, and a triple mutant harboring deletions of all three genes is viable though extremely sensitive to external salts of alkali metal cations (Marešová and Sychrová 2005).

YDR456w encodes Nhx1p (TC2.A.36) which is an endosomal/prevacuolar Na^+/H^+ exchanger participating in salt tolerance by the compartmentation of toxic cations inside the cell (Nass *et al.* 1997; Nass and Rao 1998). The physiological role of Nhx1p is more complex, since it was found to participate in intracellular protein trafficking (Bowers *et al.* 2000; Ali *et al.* 2004), to contribute to cell resistance to hyperosmotic shock (Nass and Rao 1999), to participate in the regulation of intracellular pH (Brett *et al.* 2005*b*) and also to transport potassium cations (Fukuda *et al.* 2004). Antiporters with a high similarity to yeast Nhx1p were found in nematodes (Nehrke and Melvin 2002), mammals and plants. In mammalian cells, the NHE family has at least 9 members localized either in the plasma or intracellular membranes (Orlowski and Grinstein 2004). Nhx1 homologues were found in the plasma membrane of *Arabidopsis thaliana* (Shi *et al.* 2000), in the tonoplast membrane in many different plant species (Blumwald *et al.* 2000) and some of them (*e.g.*, *Os*Nhx1p from *Oryza sativa*) were shown to complement *nhx1*Δ defects in *S. cerevisiae* (Kinclová-Zimmermannová *et al.* 2004).

The product of YJL094w, named Kha1p (TC2.A.37) is the least-characterized antiporter in *S. cerevisiae* cells. Though it was thought to mediate K^+/H^+ exchange at the plasma membrane level (Ramirez *et al.* 1998), it has been shown to be localized intracellularly, probably in the Golgi apparatus, and play an important role in the growth of the cell at alkaline pH levels (Marešová and Sychrová 2005). Similar sequences can be found in the genomes of many organisms (*e.g.*, bacteria, *Neurospora crassa*, *Aspergillus nidulans*, *Xenopus laevis*, *Drosophila melanogaster*) but their corresponding products have not been characterized.

The *NHA1* gene (YLR138w) encodes an antiporter (TC 2.A.37) that is localized in the plasma membrane and has a broad substrate specificity for at least 4 alkali metal cations (K^+, Li^+, Na^+, Rb^+) (Bañuelos *et al.* 1998; Kinclová *et al.* 2001*b*). Nha1p is also involved in the regulation of intracellular pH (Sychrová *et al.* 1999; Brett *et al.* 2005*b*), in the response of cells to osmotic shock (Kinclová *et al.* 2001*b*; Proft and Struhl 2004), and its importance for the regulation of the cell cycle has been demonstrated (Simon *et al.* 2001).

So far, only genes encoding Nha1-type Na^+/H^+ antiporters have been identified and characterized in other yeast species, namely *Schizosaccharomyces pombe* (Jia *et al.* 1992), *Zygosaccharomyces rouxii* (Watanabe *et al.* 1995), *Candida albicans* (Soon *et al.* 2000), *Pichia sorbitophila* (Bañuelos *et al.* 2002) and *Debaryomyces hansenii* (Velková and Sychrová 2006). Here we present an inventory of all alkali metal cation/H^+ antiporters resulting from a detailed search in yeast genome databases, together with a comparison of their deduced protein sequences and structures.

MATERIALS AND METHODS

The following databases and programs were used to search for the sequences encoding antiporters, to compare the DNA and protein sequences and to predict protein hydropathy profiles:

Biotools: **http://saier-144-37.ucsd.edu/** (Saier 2000)
Candida database: **http://genolist.pasteur.fr/CandidaDB/**
ClustalX: **http://www.hgmp.mrc.ac.uk/Registered/Option/clustalx.html** (Thompson *et al.* 1997)
EBI: **http://www.ebi.ac.uk/**
Génolevures: **http://cbi.labri.fr/Genolevures/Genolevures.php** (Dujon *et al.* 2004)
HMMTOP: **http://www.enzim.hu/hmmtop/** (Tusnady and Simon 2001)
MEGA2.2: **http://www.megasoftware.net/** (Kumar *et al.* 2001)
MEMSAT: **http://www.cs.ucl.ac.uk/staff/D.Jones/memsat.html** (McGuffin *et al.* 2000)
MIPS: **http://mips.gsf.de/genre/proj/yeast/index.jsp** (Mewes *et al.* 1998)
S. castellii DB: **http://www.genetics.wustl.edu/saccharomycesgenomes/** (Cliften *et al.* 2003)
SGD: **http://www.yeastgenome.org/** (Cherry *et al.* 1998; Kellis *et al.* 2003)
TMHMM Server 2.0: **http://www.cbs.dtu.dk/services/TMHMM/** (Sonnhammer *et al.* 1998)
TMPred: **http://www.ch.embnet.org/software/TMPRED_form.html** (Hofmann and Stoffel 1993)
TransportDB: **http://66.93.129.133/transporter/wb/index2.html** (Ren *et al.* 2004)

Table 1. Yeast alkali metal cation/H^+ antiporters (Nha1, Kha1, Nhx1)

Yeast	Strain	Gene name	Protein length, aa	Introns	Substrate specificity	Reference/Database
		Antiporter	**Nha1**			
Candida albicans	MEN	*CNH1*	800	0	Na^+, Li^+, K^+, Rb^+	Kinclová *et al.* 2001a
glabrata	CBS 138		945	0	–	Dujon *et al.* 2004
tropicalis	pK233	*NHA1*	975	0	–	Kamauchi *et al.* 2002
Debaryomyces hansenii var. *hansenii*	CBS 767	*NHA1*	940	1	Na^+, Li^+, K^+, Rb^+	Velková and Sychrová 2006
Kluyveromyces lactis	CLIB 210		901	0	–	Dujon *et al.* 2004
Pichia (Hansenula) anomala	LKBY-1	*NHA1*	954	1	–	Kamauchi *et al.* 2002
sorbitophila	CBS 7064	*NHA1*	927	1	Na^+, Li^+, K^+, Rb^+	Bañuelos *et al.* 2002
Saccharomyces castellii	NRRL Y-12630		957	0	–	Cliften *et al.* 2003
cerevisiae	S288c	*NHA1*	985	0	Na^+, Li^+, K^+, Rb^+	Prior *et al.* 1996
paradoxus	NRRL Y-17217		985	0		Kellis *et al.* 2003
Schizosaccharomyces pombe	972	*sod2*	468	1	Na^+, Li^+	Dibrov *et al.* 1997
Yarrowia lipolytica	CBS 57504		853	0	–	Dujon *et al.* 2004
Zygosaccharomyces rouxii	CBS 732	*SOD2-22*	806	0	Na^+, Li^+	Kinclová *et al.* 2001b
		Antiporter	**Kha1**			
Candida glabrata	CBS 138		876	0		Dujon *et al.* 2004
Debaryomyces hansenii var. *hansenii*	CBS 767		822	0	–	*ditto*
Kluyveromyces lactis	CLIB 210		781	0	–	*ditto*
Saccharomyces bayanus	623-6c		875	0	–	Kellis *et al.* 2003
castellii	NRRL Y-12630		879	0	–	*ditto*
cerevisiae	S288c	*KHA1*	873	0	–	Marešová and Sychrová 2005
paradoxus	NRRL Y-17217		873	0	–	Kellis *et al.* 2003
Schizosaccharomyces pombe	972		898	3		EBI (acc. no. CAB76234)
		Antiporter	**Nhx1**			
Candida albicans	MEN	*NHX1*	663	0	–	*Candida* database (acc. no. CA2932)
glabrata	CBS 138		618	0	–	Dujon *et al.* 2004
Debaryomyces hansenii var. *hansenii*	CBS 767		671	0	–	*ditto*
Kluyveromyces lactis	CLIB 210		614	0	–	*ditto*
Saccharomyces bayanus	MCYC623		631	0	–	Kellis *et al.* 2003
castellii	NRRL Y-12630		653	0	–	*ditto*
cerevisiae	S288c	*NHX1*	633	0	Na^+, Li^+, K^+	Nass *et al.* 1997
kudriavzevii	IFO 1802		633	0	–	Kellis *et al.* 2003
mikatae	IFO 1815		632	0	–	*ditto*
paradoxus	NRRL Y-17217		633	0	–	*ditto*
Schizosaccharomyces pombe	972		569	3	–	EBI (acc. no. CAB10103)
Yarrowia lipolytica	CBS 57504		600	1	–	Dujon *et al.* 2004

Transport proteins: `http://www.biology.ucsd.edu/~msaier/transport/` (Saier 2000)
Lasergene99 (*DNASTAR Inc.*, USA)
UWGCG package version 8.1 (Devereux *et al.* 1984)

RESULTS AND DISCUSSION

DNA sequences highly homologous to *S. cerevisiae NHA1* and corresponding to the entire ORF size were found in 12, mainly hemiascomycetous, yeast species (Table I). In 6 species, Nha1p activity was characterized in detail. Antiporters from *S. cerevisiae*, *C. albicans*, *P. sorbitophila* and *D. hansenii* transport at least four substrates whereas those of *Z. rouxii* CBS732 and *S. pombe* seem to be specific to toxic sodium and lithium cations (Kinclová *et al.* 2002). In some species, two ORFs homologous to Nha1p were found in the databases. The *S. pombe* genome contains two candidates, but only one of them has been characterized (Dibrov *et al.* 1997). Also *Y. lipolytica* chromosomes encode two putative Nha1 antiporters, one of them being much shorter (516 aa) than the other (853 aa). The function and transport properties of both proteins remain to be established. The number of genes encoding Nha1 antiporters can vary within a species depending on the strain. For example, one *Z. rouxii* strain contains two similar genes, *SOD2* and *SOD22* (Watanabe *et al.* 1995; Iwaki *et al.* 1998) whereas only one copy (*SOD2-22*) encoding the Nha1-type antiporter exists in another strain (Kinclová *et al.* 2001*a*). *Zro*Sod2-22p (806 aa) is highly similar to *Zro*Sod2p (791 aa) but it contains a segment of 15 aa residues specific to *Zro*Sod22p (808 aa) (Kinclová *et al.* 2001*a*). When both *NHA1* alleles of a diploid *P. sorbitophila* strain were sequenced and analyzed, their sequences were only 93 % identical at the protein level (Bañuelos *et al.* 2002).

Single copies of *SceNHX1* homologues were found in the genomes of 11 species, but none of them has been characterized so far. In the case of *KHA1*, homologous ORFs were identified in only 7 species (*see* Table I). Whereas the Génolevures database predicts an intron for the *KHA1* gene of *K. lactis*, we propose no introns in this sequence, according to the DNA translation and protein sequence comparison. Surprisingly, *KHA1* has not been found in the genome of *Y. lipolytica*, and this is the only example of an alkali metal cation/H^+ antiporter missing in a yeast species with a completely sequenced genome.

In the partially sequenced genomes of *Kluyveromyces thermotolerans* and some *Saccharomyces* species, parts of the sequences corresponding to *NHA1*, *KHA1* and *NHX1* were also found, suggesting that all three antiporters exist in these yeast species as well.

Synteny. Studying the conservation of synteny using complete genome sequences permits a high level of precision. When species of the same genus were compared (*S. cerevisiae*, *S. paradoxus*, *S. mikatae*, *S. bayanus*), a high conservation of synteny was found for DNA fragments containing the genes for all the respective antiporters. The SGD and Génolevures databases allowed us to perform a simple search for synteny among DNA fragments with genes encoding the alkali metal cation/H^+ antiporters of *S. cerevisiae*, *C. glabrata*, *K. lactis*, *D. hansenii* and *Y. lipolytica*. Our search revealed that a DNA block of ≈45 kb, composed of 20 genes and containing the *NHA1* coding sequence, shares almost the same gene order and orientation in both *S. cerevisiae* (YLR126c to YLR148w) and *C. glabrata* (CAGL0M04213g to CAGL0M04653g). In *K. lactis*, *D. hansenii* and *Y. lipolytica*, the synteny of the *NHA1* locus is absent or almost undetectable. For *KHA1*, synteny is well conserved between *C. glabrata* and *S. cerevisiae*, this long block of ≈33 kb is also conserved in *K. lactis* but the *KHA1* gene is localized on another chromosome. As far as the *NHX1* locus is concerned, synteny of flanking genes was observed between *S. cerevisae* and *C. glabrata* but to a lower extent, and is absent in *K. lactis* and *D. hansenii*. The presence of syntenic clusters of variable size illustrates that numerous rearrangements have occurred during the evolution of the various lineages and that the processes of chromosomal dynamics by segmental duplication play a key role in genome evolution (Dujon *et al.* 2004). Syntenic clusters diminish in size and number as the phylogenetic distances between the studied species increases.

Protein similarity. Table II summarizes the levels of similarity and identity between the antiporters from the different yeast species. The most similar proteins exist in the *Saccharomyces* family together with the antiporters of *C. glabrata*. *D. hansenii* Nha1p appears to be very close to the Nha1p of two other osmotolerant species, *C. albicans* (70.6 % identity) and *P. sorbitophila* (70.2 %), whereas the level of identity with the third osmotolerant yeast *Z. rouxii* is much lower (49.4 %). Besides the phylogenetically distant *S. pombe*, also the longer *Y. lipolytica* Nha1 protein seems to be less related to the others (*cf.* Table II). In spite of their high sequence homology, the group of kinetically characterized Nha1 antiporters can be divided, as far as substrate specificity and probable cell function are concerned, into two distinct subgroups (Bañuelos *et al.* 2002; Kinclová *et al.* 2002; Velková and Sychrová 2006): (*1*) the group of antiporters with substrate specificity only for Na^+ and Li^+ (*S. pombe*, *Z. rouxii* antiporters) and primarily a detoxication function in cells, and (*2*) the

group of antiporters (*S. cerevisiae*, *C. albicans*, *P. sorbitophila*, *D. hansenii*) mediating the transport of all alkali metal cations that, besides the elimination of toxic cations, have a role in other cell functions (regulation of intracellular pH, cell volume, the cell cycle). The distribution of yeast antiporters in the subfamilies does not reflect their level of protein identity, as the *S. cerevisiae* Nha1p and *Z. rouxii* Sod2-22p share a high degree of similarity (Table II) but belong by their substrate specificity to different subfamilies.

Table II. Level of protein identity (%) and *similarity* (%, *italics*) between yeast alkali metal cation/H^+ antiporters of the three subfamilies (Nha1, Kha1, Nhx1)[a]

Antiporter Nha1

	Cal	*Cgl*	*Ctr*	*Dha*	*Kla*	*Pan*	*Pso*	*Sca*	*Sce*	*Spa*	*Spo*	*Yli*	*Zro*
Cal	**100**	55.5	81.6	70.6	56.4	58.5	70.7	60.1	53.6	54.1	44.5	51.3	49.5
Cgl	*66.0*	**100**	52.5	52.5	59.4	58.4	51.4	67.3	*78.9*	66.9	43.0	58.2	59.5
Ctr	*85.5*	*62.6*	**100**	63.0	51.8	56.7	63.2	53.4	52.0	51.0	44.4	51.0	53.2
Dha	*77.8*	*62.8*	*70.1*	**100**	50.4	57.0	70.2	51.2	52.5	53.2	43.1	55.7	49.4
Kla	*66.2*	*67.0*	*59.8*	*59.9*	**100**	57.6	51.0	60.6	59.3	58.8	41.3	59.7	55.0
Pan	*66.2*	*66.6*	*63.5*	*66.9*	*66.1*	**100**	56.2	57.1	54.9	57.2	43.2	46.3	51.4
Pso	*78.1*	*62.0*	*69.4*	*77.7*	*59.1*	*64.2*	**100**	49.0	52.7	53.9	44.0	48.0	46.8
Sca	*69.1*	*74.4*	*62.4*	*61.1*	*69.0*	*65.1*	*58.1*	**100**	68.6	68.3	44.3	44.4	60.5
Sce	*71.4*	65.2	*60.4*	*62.0*	*74.1*	*72.3*	*70.2*	*74.7*	**100**	96.3	41.9	41.6	56.0
Spa	*64.3*	*74.4*	*59.7*	*63.2*	*67.1*	*65.9*	*63.2*	*74.8*	*97.4*	**100**	43.0	54.7	58.6
Spo	*56.7*	*53.6*	*56.8*	*55.8*	*53.9*	*53.7*	*55.1*	*55.0*	*65.9*	*53.8*	**100**	42.5	43.3
Yli	*62.3*	*69.2*	*60.4*	*65.5*	*69.3*	*56.7*	*57.6*	*54.9*	*64.2*	*65.1*	*53.9*	**100**	47.4
Zro	*60.7*	*68.0*	*62.2*	*58.1*	*64.0*	*60.2*	*56.7*	*68.9*	*73.8*	*68.6*	*53.7*	*58.3*	**100**

Antiporter Kha1

	Cgl	*Dha*	*Kla*	*Sba*	*Sca*	*Sce*	*Spa*	*Spo*
Cgl	**100**	45.8	47.1	65.3	64.6	63.7	64.3	46.6
Dha	*56.1*	**100**	45.0	51.6	45.1	42.7	49.5	49.5
Kla	*69.7*	*55.5*	**100**	54.9	54.9	47.1	54.4	45.7
Sba	*73.1*	*62.6*	*64.9*	**100**	66.6	87.5	87.5	44.9
Sca	*72.5*	*56.3*	*65.5*	*73.1*	**100**	69.0	68.6	46.3
Sce	*79.0*	*64.8*	*69.7*	*91.3*	*75.5*	**100**	95.8	30.2
Spa	*72.8*	*59.9*	*65.0*	*91.0*	*75.3*	*96.9*	**100**	46.2
Spo	*61.5*	*63.1*	*60.1*	*59.8*	*61.7*	*54.0*	*61.4*	**100**

Antiporter Nhx1

	Cal	*Cgl*	*Dha*	*Kla*	*Sba*	*Sca*	*Sce*	*Sku*	*Smi*	*Spa*	*Spo*	*Yli*
Cal	**100**	66.9	76.4	73.7	67.2	66.0	66.7	66.7	66.8	67.6	64.8	66.8
Cgl	*72.9*	**100**	66.8	75.8	76.5	76.5	74.4	78.4	78.4	78.3	56.9	65.6
Dha	*80.9*	*73.1*	**100**	75.0	68.2	67.3	64.7	67.4	68.2	68.1	63.7	72.2
Kla	*80.4*	*81.1*	*81.7*	**100**	74.5	75.0	74.7	73.7	74.9	74.7	59.9	67.6
Sba	*73.2*	*82.6*	*75.0*	*79.9*	**100**	79.2	92.2	93.5	91.6	93.2	56.1	65.9
Sca	*72.4*	*80.7*	*74.3*	*81.3*	*83.4*	**100**	79.1	78.8	78.0	79.3	58.5	67.7
Sce	*73.5*	*85.4*	*78.5*	*80.0*	*93.5*	*82.7*	**100**	94.0	95.2	96.8	54.6	62.6
Sku	*73.7*	*84.6*	*74.9*	*79.8*	*95.1*	*82.6*	*94.9*	**100**	93.8	95.1	56.3	66.1
Smi	*73.2*	*83.9*	*75.1*	*80.4*	*93.2*	*81.5*	*96.4*	*95.2*	**100**	96.0	56.2	66.4
Spa	*74.0*	*83.5*	*74.7*	*80.0*	*94.3*	*83.1*	*97.2*	*95.7*	*96.8*	**100**	57.1	*72.9*
Spo	*77.0*	*66.4*	*76.0*	*68.4*	*67.0*	*68.2*	*73.9*	*66.8*	*65.8*	*67.5*	**100**	60.0
Yli	*73.2*	*72.8*	*78.6*	*74.7*	*71.9*	*73.5*	*77.1*	*72.6*	*72.9*	66.6	*68.8*	**100**

[a]For species abbreviation *see* p. 413.

The Nhx1 proteins have higher levels of identity, compared to the subfamilies of Nha1 and Kha1, respectively. The most distant member, Nhx1 in *S. pombe*, shares >54 % identity with other Nhx antiporters whereas some Nha1 or Kha1 proteins share <45 % identity (Table II). The highest degree of sequence disparity exists between *S. cerevisiae* and *S. pombe* Kha1p (30.2 % identity).

Table III. Prediction of *S. cerevisiae* Nha1p secondary structure

Prediction	tmd total	N-terminus[a]	Transmembrane					
			1	*2*	*3*	*4*	*5*	*6*
Kyte–Doolittle	12	1–13	14–33	37–57	71–91	105–126	135–153	168–192
SGD	11	1–13	14–32	36–62	69–97	97–125	176–194	202–229
TMHMM 2.0	9	1–11 ic	12–33	38–60	103–125	204–226	246–265	293–315
MEMSAT2	10	1–102 ic	103–126	135–153	178–194	204–227	245–261	268–284
HMMTOP	13	1–13 ec	14–33	42–61	70–89	102–126	135–154	173–192
TMPred	11	1–13 ic	14–33	37–60	75–93	107–129	178–195	208–227

[a]ic – intracellular, ec – extracellular.

Hydropathy profiles. Alkali metal cation/H^+ antiporters should, similarly to other transporters, consist of hydrophilic N- and C-termini, hydrophobic segments embedded in the membrane and hydrophilic connecting loops. Most yeast transporters are believed to consist of an even number (usually 10 or 12) of tmds with N- and C-termini facing the cytosol, though there are only a few examples of experimental confirmation of the predicted structure (*e.g.*, Gap1, general amino acid permease with 12 tmds; Gilstring and Ljungdahl 2000). A number of programs and/or methods exist for the prediction of protein tmds. We have tried six of them (Table III) to predict the number, length and position of the tmds of *Sce*Nha1p as a model alkali metal cation/H^+ antiporter. Surprisingly, each method gave a different result as to the number of tmds (9–13) and/or their position within the protein structure. We have chosen the Kyte–Doolittle-based prediction method (Kyte and Doolittle 1982) for further analysis of antiporters. This program predicted 12 tmds for Nha1p (Fig. 1), and best fitted when used for the prediction of Gap1p's secondary structure (*not shown*). For all other antiporters from the Nha1, Nhx1 and Kha1 families, the Kyte–Doolittle method also predicted 12 transmembrane segments. As far as mammalian Nhx1 homologues (the NHE family) are concerned, most of them are believed also to consist of 12 tmds (Orlowski and Grinstein 1997). Bacterial NhaA proteins were reported to form dimers of monomers composed of 12 transmembrane helices each (Williams 2000; Gerchman *et al.* 2001). On the other hand, vacuolar *A. thaliana* Nhx1p was shown to consist of 9 tmds. Three hydrophobic regions do not appear to span the tonoplast membrane; yet appear to be membrane-associated. And whereas the N-terminus of *A. thaliana* Nhx1p faces the cytosol, almost the entire C-terminal hydrophilic region resides in the vacuolar lumen (Yamaguchi *et al.* 2003). Altogether, comparison of the methods for the prediction of membrane protein secondary structure revealed that the prediction is very approximate and no definitive conclusions can be drawn without accompanying experimental data.

The prediction and comparison of the secondary structure of all antiporters from the three families revealed that they have relatively short hydrophilic N-termini, 12 tmds and C-termini of widely ranging sizes. The hydrophobic transmembrane segments and connecting loops have a conserved size in all antiporters (from 397 aa for *Spo*Kha1p to 426 aa for several Nha1p; Table IV), and also the distribution of tmds within this section (*i.e.* the length of the loops) is very similar with the members of all three families (*not shown*).

Nha1 antiporters have the shortest and highly similar N-termini (12 or 13 aa; Table IV) with conserved residues W4 and H13 in all antiporters, and Q6 in all of them except for *P. anomala*. The N-termini of Kha1 proteins are longer compared to Nha1 antiporters, but they contain several conserved residues (N13, Y17, P22 in all antiporters, and G7, P14, S21 in all except for *S. pombe*). The longest and least conserved are the N-termini of Nhx1 proteins. Their size ranges from 30 to 61 aa residues and, though some conserved stretches exist in the N-termini of several Nhx1 antiporters, the only conserved residues are E57, immediately preceding the first tmd in all Nhx1p, and P53 conserved in all except *S. pombe*.

The length of tmds and connecting loops is smallest for *S. pombe* antiporters in all three families (Table IV). The membrane section (tmd + loops) of Nha1p from other yeasts is 425–426 aa residues long (*Spo*sod2 only 421 aa) and highly conserved regions exist in both the tmd and loops. For example, the 11th tmd shares >50 % identity among all Nha1p (5th and 6th tmds >40 % identity), and the extracellular loop between the 9th and 10th tmd is >40 % identical in all Nha1p. As for Nhx1 proteins, their transmembrane segments seem to be more conserved compared to Nha1 antiporters. Only three tmds exhibit <50 % identity, and the 3rd transmembrane segments have 20 out of 23 aa residues identical (87 %). On the other hand, Nhx1p's loops are less conserved. The cytoplasmic loop between the 4th and 5th tmd is only 6 aa long but none of these residues is conserved in all members of the family. Similarly to Nha1, the most conserved loop

domain							C-terminus
7	8	9	10	11	12	13	
205–227	245–275	293–313	323–349	362–382	411–439	–	440–985
249–277	295–318	324–351	357–384	410–438	–	–	438–985
327–349	364–381	412–434	–	–	–	–	435–985 ec
296–315	323–340	362–382	411–429	–	–	–	430–985 ec
205–224	245–263	268–285	296–314	323–340	363–382	413–437	438–985 ic
246–265	293–315	330–349	366–386	411–429	–	–	430–985 ec

is between the 9th and 10th tmd (67 % identity). Kha1p tmds and loops have the lowest level of identity compared to Nha1 or Nhx1 proteins.

Charged amino-acid residues conserved in transmembrane domains and loops. As the substrates of antiporters are positively charged cations, charged aa residues should be involved in substrate binding and passage through the protein molecule. Therefore the charged aa residues indispensable for antiporter activity should be conserved in the antiporter subfamilies. Altogether, 21 charged residues are conserved in the tmd (16) and loops (5) of all yeast Nha1 proteins (Fig. 1). Detailed studies of mutagenized *Spo*sod2 antiporters revealed that five Asp (corresponding to D145, D177, D241, D266 and D267 in *Sce*Nha1p: Fig. 1) and one His (H367) residues are important for alkali metal cation and/or proton translocation (Dibrov *et al.* 1998; Wiebe *et al.* 2003). Three negatively charged residues from tmds (E90, D145, D17) and one positively charged residue from a loop (R166) are conserved not only within the yeast species but also in bacterial Na^+/H^+ antiporters. Kha1 and Nhx1 proteins have more conserved charged residues in the connecting loops than in the transmembrane segments (10 in the loops, 7 in the tmds for Kha1p: 15 in the loops, 9 in the tmds for Nhx1p). As far as Nhx1p is concerned, 10 residues, both in the tmds and loops, are conserved in plant homologues as well, and 4 conserved charged residues (D500, E502, D601, K606) can also be found in Nhx1p C-termini.

The high importance of two conserved charged residues in one of the loops of a bacterial Na^+/H^+ antiporter has been recently reported (Tzubery *et al.* 2004). Residues K249 and E252 in the intracellular loop between tmds 8 and 9 are directly involved in *E. coli* NhaA activity. Lysine at three amino acids away from a glutamate is present in the loop connecting the 8th and 9th tmds of almost all yeast Kha1 antiporters (except for that of *K. lactis* with alanine instead of glutamate, but a sequencing error in this case cannot be ruled out) and in most Nha1 proteins. In Nhx1 antiporters, two lysines are conserved in this loop, but neither of the negatively charged residues (E or D) is.

If the tmds and loops of Nha1 and Nhx1 proteins are compared, two highly conserved motifs can be found. The first of them, **ATDP** (aa 143–146; Fig.1) is conserved in the 5th tmd of all yeast Nha1 and Nhx1 antiporters, except for *S. pombe* (STDP). The ATDP motif is fully conserved also in some plants (*A. thaliana* Sos1 Na^+/H^+ plasma membrane antiporter) and partly in mammalian NHE antiporters (AVDP in *Rattus norvegicus* NHE1, NHE2, NHE3 proteins). The neighboring 6th tmd contains the conserved motif **ES**GC**ND** (aa 172–177; Fig. 1) in all Nha1 antiporters and a similar motif **ES**LL**ND** exists in the 6th tmd of Nhx1 antiporters (ESILND for *S. pombe*). The difference between the two motifs is the presence of two highly hydrophobic residues in the middle of the Nhx1p motif. The same motif (ESLLND) can be found in rat NHE proteins and with some modifications also in plant Na^+/H^+ antiporters (ESLMND in *A. thaliana* Sos1p, EGVVND in *Oryza sativa* Nhx1p). Part of this motif also exists in Kha1 proteins, where the ND preceded by at least one hydrophobic residue is present in the 6th tmd. The highly conserved aspartate at the end of the motif was shown to be indispensable for proton translocation *via* the *S. pombe* sod2 antiporter (Wiebe *et al.* 2003), and its conservation across the family of alkali metal cation/H^+ antiporters from different organisms confirms its importance for protein activity.

Conserved motifs in C-termini. The C-termini of yeast antiporters are less conserved than other regions, and differ significantly both in length (Table IV) and primary structure. In spite of this, C-termini play an important role not only in the regulation of antiporter transport activity but are probably also involved in other cell functions. The long hydrophilic *Sce*Nha1 C-terminus that forms 55 % of the antiporter (Fig. 1) was shown to be important in several cell processes, besides its role in the regulation of Nha1p transport activity. Its observed importance for cell survival upon hyperosmotic shock caused by solutes other than salts (*e.g.*, glucitol) suggests a role in the immediate cell response to osmotic shock (Kinclová *et al.*

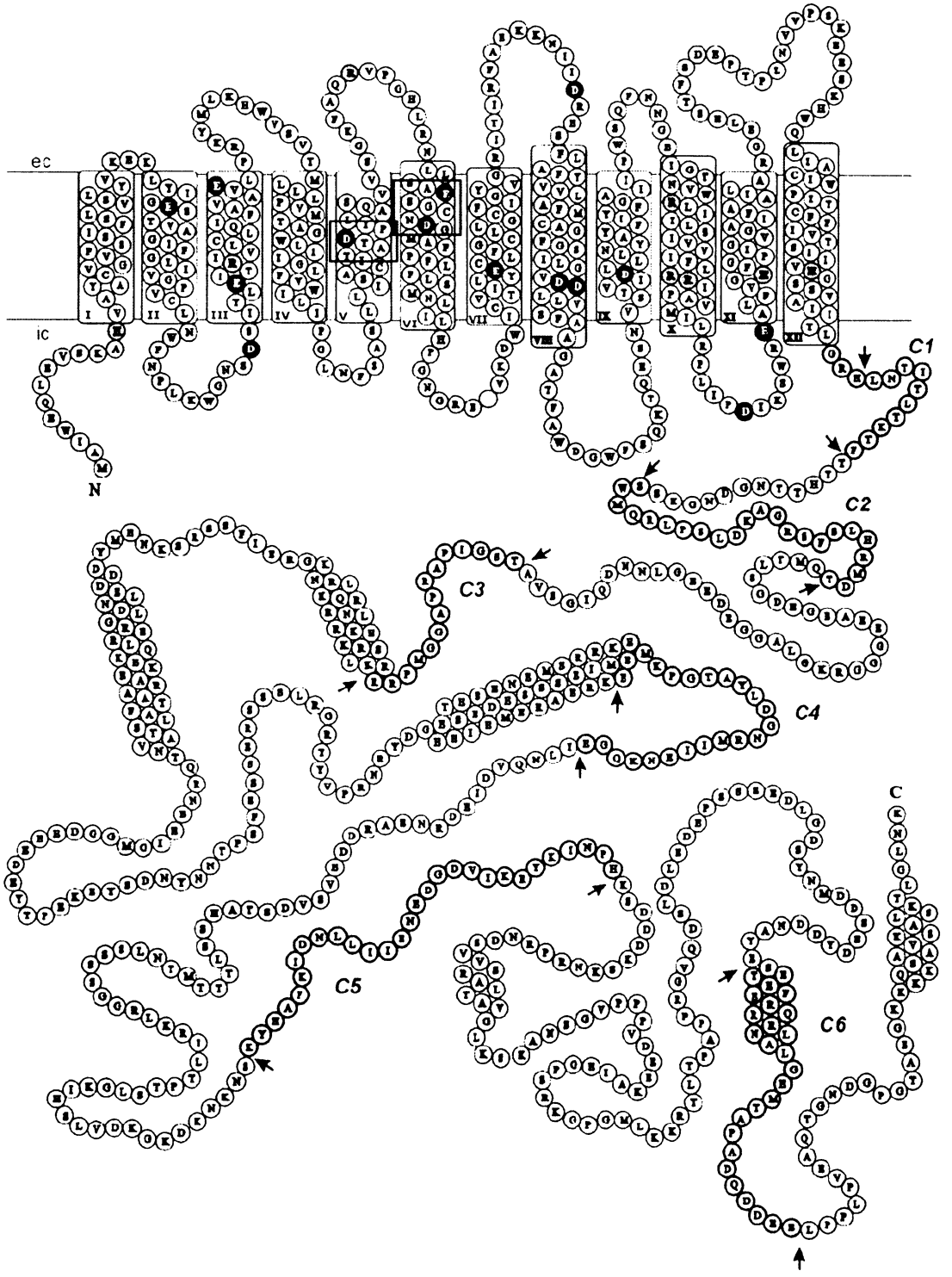

Fig. 1. Model for the membrane topology of the *S. cerevisiae* plasma membrane Na^+/H^+ antiporter Nha1 with 12 transmembrane domains (I–XII) and a long hydrophilic C-terminus. Charged amino-acid residues conserved in the transmembrane domains and connecting loops of all yeast plasma membrane Na^+/H^+ antiporters are *accented* (negative in *black*, positive in *grey*), conserved regions in the C-terminus are marked with *arrows* (*C1–C6*), and conserved motifs in the transmembrane helices are *boxed*; ec – extracellular, ic – intracellular.

2001*b*). *Sce*Nha1p and its long C-terminus may play an important role in the regulation of the cell cycle, since *NHA1* expression was able to clear the blockage at the G_1–S transition in cells with conditional *sit4*

hal3 mutations (Simon *et al.* 2001). This function was independent of the role of the antiporter in improving the tolerance to sodium cations, and it required the integrity of a relatively large region (aa 800–948) of its C-terminus. A screening for loss-of-function mutations in the C-terminus revealed a number of residues required for Nha1p functionality in the cell cycle, most of them clustering in two regions, from residues 869 to 876 and 918 to 927 (Simon *et al.* 2003). The importance of different regions of the Nha1p C-terminus for its functionality was also confirmed in a study comparing the structure and activity of four Na^+/H^+ antiporters (Kamauchi *et al.* 2002). Six distinct conserved regions were found in Nha1p C-termini (*C1–C6*; Fig. 1) and the first one (*C1*, 16 aa) was found to be important both for transport activity and plasma membrane targeting of the protein (Mitsui *et al.* 2004*a,b*). A comparison of all yeast Nha1 antiporters showed that these six conserved regions exist in all of them, except for the very short C-terminus of *Spo*sod2p that contains only *C1*, and for *Zro*Sod2-22p in which *C6* is missing.

Table IV. Comparison of the number of amino-acid residues in three subfamilies of yeast alkali metal cation/H^+ antiporters

Antiporter	Number of amino acids			
	total	in N-terminus	in tmd and loops	in C-terminus
Antiporter Nha1				
*Cal*Cnh1	800	12	426	362
*Cgl*Nha1	945	13	426	506
*Ctr*Nha1	975	12	426	537
*Dha*Nha1	940	12	426	502
*Kla*Nha1	901	12	425	464
*Pan*Nha1	954	13	426	515
*Pso*Nha1	927	12	426	489
*Sca*Nha1	957	13	425	519
*Sce*Nha1	985	13	426	546
*Spa*Nha1	985	13	426	546
*Spo*sod2	468	12	421	35
*Yli*Nha1	853	12	425	416
*Zro*Sod2-22	806	12	425	369
Antiporter Kha1				
*Cgl*Kha1	876	21	405	450
*Dha*Kha1	822	25	405	392
*Kla*Kha1	781	22	401	358
*Sba*Kha1	875	22	405	448
*Sca*Kha1	879	21	405	453
*Sce*Kha1	873	22	405	446
*Spa*Kha1	873	22	405	446
*Spo*Kha1	898	29	397	472
Antiporter Nhx1				
*Cal*Nhx1	663	43	424	196
*Cgl*Nhx1	618	43	420	155
*Dha*Nhx1	671	46	424	201
*Kla*Nhx1	614	51	422	141
*Sba*Nhx1	631	57	420	154
*Sca*Nhx1	653	58	420	175
*Sce*Nhx1	633	57	420	156
*Ski*Nhx1	633	57	420	156
*Smi*Nhx1	632	57	420	155
*Spa*Nhx1	633	57	420	156
*Spo*Nhx1	569	30	409	130
*Yli*Nhx1	600	61	414	125

The search for conserved motifs in the C-termini of Nhx1 proteins revealed two highly conserved regions. The first domain **TTAGMLEVLNIKTGCISEEDTSDDEFDIE** (aa 478–507 in *Sce*Nhx1p) is in close proximity to the last tmd, as in Nha1 proteins, but these two *C1* domains share no significant level of homo-

logy. The highly conserved *C2* domain (**QWF**QN**FDEQVLKPV**F**LD**) spans the region of aa 595–611 in *Sce*Nhx1p. Regions homologous to *C1* and *C2* can also be found in plant Nhx1 proteins (*e.g.*, *A. thaliana*, *Zea mays*, *Lycopersicon esculentum*) but not in mammalian NHE proteins. In the case of Kha1 antiporters, their C-termini have a low level of similarity and conserved domains can only be found in the *Saccharomyces* genus.

Conclusion. Yeast alkali metal cation/H^+ antiporters belong to a broad family of secondary active transport systems that possess 12 transmembrane hydrophobic segments with their N- and C-termini most probably facing the cytosol. Their primary protein structures show sequence similarity with sodium-specific antiporters from both prokaryotes and higher eukaryotes. The fact that such transport systems were conserved through the evolution from bacteria to humans, confirms the general need of a cell to efficiently regulate the intracellular concentration of potassium and toxic sodium cations. Fig. 2 shows a phylogenetic tree of 25 fungal, 2 bacterial, 3 plant and 3 mammalian alkali metal cation/H^+ antiporters. It is evident that the sub-

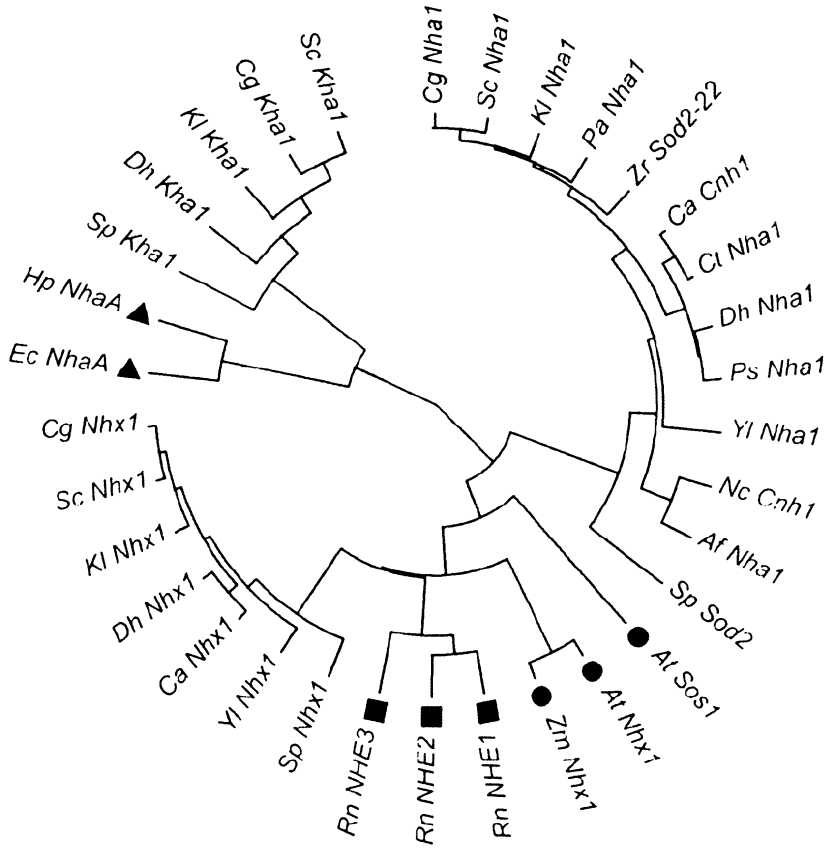

Fig. 2. Phylogenetic tree of Na^+/H^+ antiporters. The set of 33 transporters contains 25 fungal members (23 yeast), 2 bacterial (*triangles*), 3 plant (*circles*) and 3 mammalian (*squares*) representatives of both plasma membrane and intracellular antiporters; the tree is based on multiple-sequence alignment (ClustalX) and drawn with the Mega2 2 program; abbreviations in protein names: *Af* – *Aspergillus fumigatus*, *At* – *Arabidopsis thaliana*, *Ca* – *Candida albicans*, *Cg* – *Candida glabrata*, *Ct* – *Candida tropicalis*, *Dh* – *Debaryomyces hansenii* var. *hansenii*, *Ec* – *Escherichia coli*, *Hp* – *Helicobacter pylori*, *Kl* – *Kluyveromyces lactis*, *Nc* – *Neurospora crassa*, *Pa* – *Pichia* (*Hansenula*) *anomala*, *Ps* – *Pichia sorbitophila*, *Rn* – *Rattus norvegicus*, *Sc* – *Saccharomyces cerevisiae*, *Sp* – *Schizosaccharomyces pombe*, *Yl* – *Yarrowia lipolytica*, *Zm* – *Zea mays*, *Zt* – *Zygosaccharomyces rouxii*.

family of yeast prevacuolar/endosomal Nhx1 antiporters is closely related to mammalian plasma membrane NHE proteins and to both plasma membrane and vacuolar plant antiporters. The highest sequence conservation within this subfamily of yeast antiporters suggests its high importance for cell physiology. Yeast Kha1 proteins seem to belong to the same subfamily as bacterial antiporters, and Nha1 proteins form a distinct subfamily that has so far only been characterized in fungi, though some evidence appears (Brett *et al.* 2005*a*) that homologous genes could also exist in the human genome.

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5.3.2. Charakterizace Na^+/H^+-transportních proteinů *Z. rouxii*

Autorce této dizertační práce byl vzhledem k její účasti na projektu Génolevures 3 umožněn přístup do databáze obsahující dosud neanotovanou sekvenci genomu *Z. rouxii*. Tohoto přístupu jsme využili a databázi prohledali ve snaze identifikovat geny, které by mohly představovat dosud neznámé přenašeče patřící do rodiny Na^+/H^+-transportních proteinů. Identifikovali jsme gen, který jsme nazvali *ZrNHA1*. Funkci *Zr*Nha1p jme studovali jak heterologně v *S. cerevisiae*, tak přímo v *Z. rouxii*.

Dále jsme sledovali, jakou funkci má v *Z. rouxii* *Zr*Sod2-22p. Tento Na^+/H^+-transportní protein byl totiž doposud studován pouze prostřednictvím heterologní exprese v *S. cerevisiae*, kde umožnil buňkám postrádajícím vlastní Na^+/H^+-antiportér a ATPasy Ena růst v přítomnosti sodných a lithných ale nikoli draselných kationtů, a buňky jej exprimující byly schopny transportovat sodné, nikoli draselné kationty (viz kap. 2.2.1.). Zajímalo nás, zda se i v buňkách *Z. rouxii* *Zr*Sod2-22p podílí pouze na trasportu sodných a lithných kationtů, a ne na trasportu kationtů draselných, a dále zda existují rozdíly ve funkci *Zr*Nha1p a *Zr*Sod2-22p. Ke studiu jsme využili nástroje pro genové manipulace *Z. rouxii* vytvořené v této práci.

5.3.2.1. Identifikace a izolace *ZrNHA1*

V dosud neanotované databázi Génolevures 3 obsahující sekvenci genomu *Z. rouxii* CBS 732^T jsme na základě homologie s genem *ZrSOD2-22* identifikovali gen kódující patrně přenašeč typu Na^+/H^+, který jsme vzhledem k jeho podobnosti s *ScNHA1* nazvali *ZrNHA1*.

Gen *ZrNHA1* jsme izolovali prostřednictvím PCR a vložili do mnohokopiového vektoru YEp352 za promotor *ScNHA1*, a připravili tak plasmid YEp352_*ZrNHA1*. Pro potvrzení správnosti izolované sekvence byl úsek plasmidu obsahující vložený gen *ZrNHA1* sekvenován. Sekvence námi izolovaného genu i jím kódovaného proteinu vykazovala mírné rozdíly oproti sekvenci nalezené v databázi. Jelikož byla pro izolaci genu použita polymerasa s tzv. „proofreading" aktivitou (tedy schopná opravit případnou nukleotidovou záměnu při procesu polymerace), je rozdíl v sekvenci přítomné v databázi a sekvence izolované možno vysvětlit tím, že klon kmene CBS 732^T použitý pro izolaci genu *ZrNHA1* nebyl totožný s klonem CBS 732^T použitým pro sekvenaci (Prof. Jacky de Montigny, osobní sdělení). V sekvenci DNA námi izolovaného genu bylo nalezeno sedm nukleotidových substitucí, čtyři z nich vedly k záměně aminokyselinového zbytku v kódovaném proteinu (R658 za G, V694 za

A, H806 za R a T938 za S, viz Příloha 10.1.). Proteinovou sekvenci nalezenou v databázi jsme spolu s proteinovou sekvencí námi izolovaného genu porovnali se sekvencemi přenašečů *Zr*Sod2-22 a ScNha1p a zjistili jsme, že všechny čtyři nalezené aminokyselinové záměny se nacházejí mimo transmembránové oblasti, v hydrofilním C-konci. Vzhledem k tomu, že na funkci přenašečů Na^+/H^+ se podílí hlavně oblast transmembránových domén. ve kterých se sekvence produktu námi izolovaného gene neliší od produktu genu v databázi, lze učinit závěr, že protein kódovaný námi izolovaným genem *ZrNHA1* by měl mít velmi podobné nebo i totožné transportní vlastnosti jako protein vzniklý přepisem a překladem sekvence v databázi.

5.3.2.2. Sekvenční analýza *Zr*Nha1p

Produkt námi izolovaného genu, *Zr*Nha1p, je tvořen 994 aminokyselinovými zbytky (viz Příloha 10.1.). Porovnáme-li jej s homologními *Zr*Sod2-22p a *Sc*Nha1p, zjistíme, že svou délkou se protein *Zr*Nha1p blíží spíše proteinu *Sc*Nha1p, tj. má, stejně jako *Sc*Nha1p a na rozdíl od *Zr*Sod2-22p, poměrně dlouhý C-konec (tab. 2 a viz Příloha 10.2.).

Tab. 2. Struktura přenašečů *Zr*Nha1p, *Zr*Sod2-22p a *Sc*Nha1p podle modelu navrženého pro *Sc*Nha1p (Kinclová *et al.*, 2001b).

Antiportér	Počet aminokyselinových zbytků			
	Celkem	N-konec	TMS+smyčky	C-konec
ScNHA1	985	12	419	554
ZrSOD2-22	806	11	418	377
ZrNHA1	994	11	419	564

TMS, transmembránová oblast (transmembrane segment).

Srovnáme–li sekvenční identitu celkových proteinů a sekvenční identitu konzervovaných transmembránových oblastí antiportérů, které jsou klíčové pro transportní specifitu proteinů, je *Zr*Nha1p v obou případech podobnější spíše *Sc*Nha1p než *Zr*Sod2-22p (tab. 3). Na základě pozorované homologie můžeme odhadnout, že protein *Zr*Nha1p obsahuje, stejně jako *Sc*Nha1p, krátký cytoplasmatický N-konec, 12 transmembránových domén a dlouhý cytoplasmatický C-konec. Tyto výsledky naznačovaly, že tento dosud neznámý přenašeč by mohl v buňkách *Z. rouxii* plnit funkce obdobné funkci *Sc*Nha1p v *S. cerevisiae*.

Tab. 3. Sekvenční identita (%) celých proteinů/transmembránových oblastí + smyček antiportérů.

Antiportér	*Sc*Nha1p	*Zr*Sod2-22p	*Zr*Nha1p
*Zr*Sod2-22p	45,6/76,6	-	47,5/76,6
*Zr*Nha1p	52,6/79,5	47,5/76,6	-

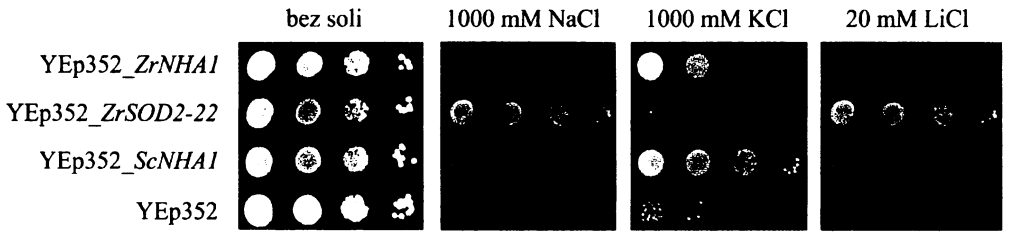
K porovnání sekvencí byl použit program Vector NTI (Invitrogen).

5.3.2.3. Heterologní exprese *Zr*Nha1p v *S. cerevisiae* BW31

Abychom zjistili substrátovou specifitu, transportní vlastnosti a pravděpodobnou funkci identifikovaného přenašeče, byl izolovaný gen přítomný v plasmidu YEp352 pod kontrolou promotoru *ScNHA1* heterologně exprimován v buňkách kmene *S. cerevisiae* postrádajícího vlastní systémy pro výstup kationtů alkalických kovů (kmen BW31, *nha1Δ ena1-4Δ*; Kinclová-Zimmermannová *et al.*, 2005). Takto bylo možno porovnat vlastnosti proteinu *Zr*Nha1p s vlastnostmi přenašečů *Zr*Sod2-22p a *Sc*Nha1p, které byly charakterizovány za stejných experimentálních podmínek (Kinclová *et al.*, 2002).

5.3.2.3.1. Vliv *Zr*Nha1p na toleranci *S. cerevisiae* BW31 k solím v médiu

Tolerance buněk BW31 exprimujících *ZrNHA1* ke zvýšené koncentraci solí NaCl, KCl a LiCl v médiu byla porovnána s tolerancí buněk exprimujících *ZrSOD2-22* nebo *ScNHA1* (pozitivní kontroly) a buněk obsahujících prázdný vektor (negativní kontrola). Přítomnost funkčního přenašeče byla detekována jako zvýšení tolerance, podmíněné schopností proteinu eliminovat nadbytečné kationty z cytoplasmy. Protein *Zr*Nha1p umožnil buňkám růst za vyšší koncentrace všech tří testovaných solí, a je tedy zřejmě schopen eliminovat z cytoplasmy všechny tři typy kationtů (Na^+, Li^+ i K^+; obr. 5). Na médiu s NaCl nebo LiCl byla schopnost proteinů *Zr*Nha1p a *Sc*Nha1p eliminovat kationty srovnatelná, nejvyšší toleranci vykazovaly buňky exprimující *Zr*Sod2-22p. Tyto však, na rozdíl od buněk exprimujících *Zr*Nha1p nebo *Sc*Nha1p, nebyly schopny růstu za vyššího obsahu KCl (v souladu s již publikovanými výsledky; Kinclová *et al.*, 2002). Nejvyšší tolerance ke KCl v médiu dosahovaly buňky exprimující *Sc*Nha1p.

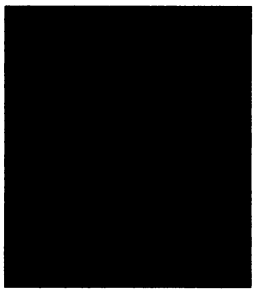


Obr. 5. Růst buněk *S. cerevisiae* BW31 exprimujících různé přenašeče na médiu YNB-NH_4 obsahujícím zvýšené koncentrace různých solí.

5.3.2.3.2. Lokalizace *Zr*Nha1p v buňkách *S. cerevisiae* BW31

Abychom ověřili, že *Zr*Nha1p je přenašečem plasmatické membrány a ne vnitrobuněčných organel, byla na 3' konec genu *ZrNHA1* připojena sekvence GFP tak, aby při expresi genu v buňkách vznikl fúzní protein. Toto bylo provedeno tak, že byl gen vložen do plasmidu pGRU1 pod kontrolou promotoru *ScNHA1*. Buňky *S. cerevisiae* nesoucí plasmid pGRU1_*ZrNHA1* pak obsahovaly fúzní protein *Zr*Nha1p-GFP.

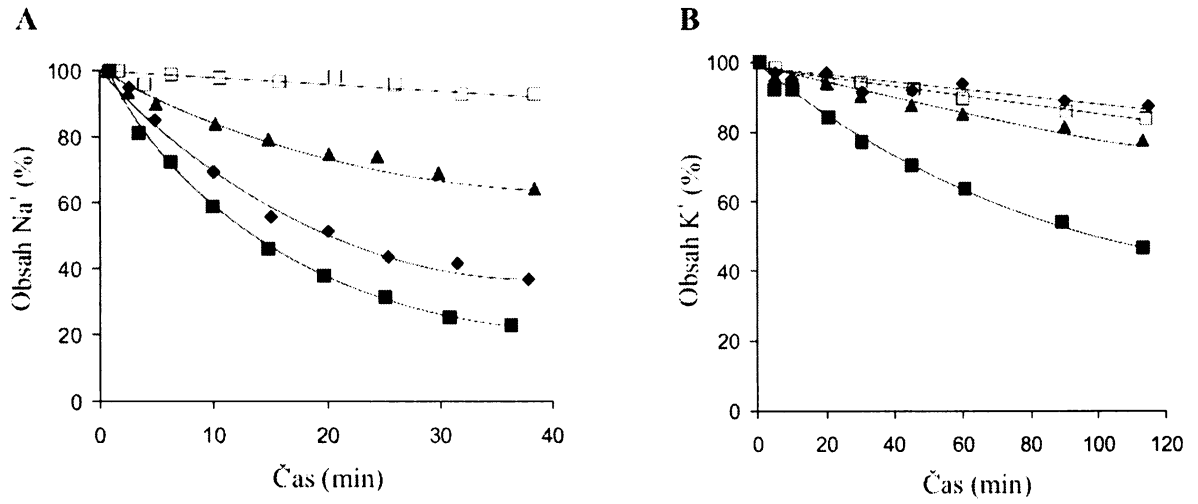
Bylo potvrzeno, že GFP neovlivnil aktivitu proteinu, protože tolerance buněk exprimujících fúzní protein byla totožná s tolerancí buněk exprimujících neznačený *Zr*Nha1p. Lokalizace *Zr*Nha1p-GFP v buňkách rostoucích v médiu YNB-NH_4 bez přidaných solí byla zjištěna fluorescenční mikroskopií. Obr. 6 ukazuje, že protein je lokalizován poblíž buněčného povrchu, a nikoli v membránách vnitrobuněčných. Tento výsledek je potvrzením, že protein *Zr*Nha1p je přenašečem plasmatické membrány.



Obr. 6. Lokalizace proteinu ZrNha1p značeného GFP v buňkách *S. cerevisiae*.

5.3.2.3.3. Výstup kationtů z buněk *S. cerevisiae* BW31 exprimujících *ZrNHA1*

Pro ověření, že zvýšená tolerance k solím buněk BW31 produkujících přenašeč *Zr*Nha1p, patrná z obr. 5, byla skutečně způsobena exportem kationtů z buněk, jsme sledovali výstup sodných a draselných kationtů z buněk BW31 produkujících *Zr*Nha1p. Funkčnost proteinu byla porovnána s aktivitou druhého antiportéru *Z. rouxii* (*Zr*Sod2-22p), majícího úzkou substrátovou specifitu, a proteinu *Sc*Nha1p kvasinky *S. cerevisiae*, transportujícího z buněk i draselné kationty. Měření výstupu a analýzu obsahu kationtů provedla Mgr. Klára Papoušková prostřednictvím metody plamenové atomové absorpční spektrofotometrie (Kinclová *et al.*, 2001b; obr. 7).



Obr. 7. Výstup sodných (A) a draselných (B) kationtů z buněk *S. cerevisiae* BW31 produkujících různé Na^+/H^+-antiportéry plasmatické membrány. □ Buňky transformované YEp352 (negativní kontrola), ■ buňky produkující *Sc*Nha1p, ♦ buňky produkující *Zr*Sod2-22p, ▲ buňky produkující *Zr*Nha1p.

Z obr. 7A je patrné, že v buňkách exprimujících kterýkoli z antiportních proteinů dochází k výraznému exportu sodných kationtů (obsah Na^+ v buňkách postupně klesá). Aktivita *Zr*Nha1p je přitom nižší než aktivita druhých dvou přenašečů, nejvyšší aktivitu vykazuje přenašeč *Sc*Nha1p. Nižší výstup sodných kationtů z buněk exprimujících *ZrSOD2-22* než z buněk exprimujících *ScNHA1* je v souladu s již publikovanými výsledky (Kinclová *et al.*, 2002).

Z obr. 7B je patrné, že nepatrný výstup draselných kationtů z buněk byl naměřen i v případě buněk exprimujících *ZrSOD2-22*, jehož produkt nerozpoznává K^+ jako substrát, a z buněk obsahujících pouze prázdný plasmid YEp352. Mgr. Papoušková sestavila tabulku, která ukazuje průměrný úbytek draselných kationtů z buněk exprimujících různé přenašeče za dobu měření 120 min (tab. 4).

Tab. 4. Průměrná ztráta K^+ z buněk *S. cerevisiae* BW31 produkujících různé antiportéry během 120 min.

Přenašeč	Průměrná ztráta K^+ (nmol/mg suché váhy)
Žádný (YEp352)	96,63 ± 2,54
*Sc*Nha1p	290,07 ± 26,29
*Zr*Sod2-22p	70,85 ± 3,70
*Zr*Nha1p	160,00 ± 10,78

Tab. 4 dokazuje, že buňky neobsahující systém pro výstup K^+ (obsahující prázdný plasmid YEp352 nebo exprimující *ZrSOD2-22*) ztrácí v průběhu 120 min přibližně 70 – 100 nmol K^+/mg suché váhy buněk. V tomto případě se zřejmě na úbytku K^+ z buněk podílejí neznámé nespecifické transportéry. Buňky exprimující *ZrNHA1* exportují za 120 min přibližně 1,7 až 2,3krát více draselných kationtů než obě negativní kontroly. Nejvyšší výstup draselných kationtů byl v souladu s výsledky kapkových testů naměřen u buněk exprimujících *ScNHA1*. I zdánlivě pomalý výstup K^+ z buněk naměřený u buněk obsahujících *Zr*Nha1p je zřejmě zcela dostačující pro umožnění růstu buněk za přítomnosti vyšších koncentrací K^+ v médiu (viz obr. 5).

Získané výsledky potvrzují, že zvýšená tolerance k přítomnosti NaCl a KCl kmene BW31 produkujícího *Zr*Nha1p v porovnání s negativní kontrolou (obr. 5) je dána aktivním exportem sodných resp. draselných kationtů z buněk *S. cerevisiae*.

Výše uvedené výsledky ukazují funkci přenašečů exprimovaných heterologně v *S. cerevisiae*. Abychom zjistili, zda tyto proteiny zastávají v *Z. rouxii* stejné funkce, jako když jsou heterologně exprimovány v *S. cerevisiae*, studovali jsme podíl přenašečů na halotoleranci buněk *Z rouxii* prostřednictvím sledování fenotypu delečních mutantů.

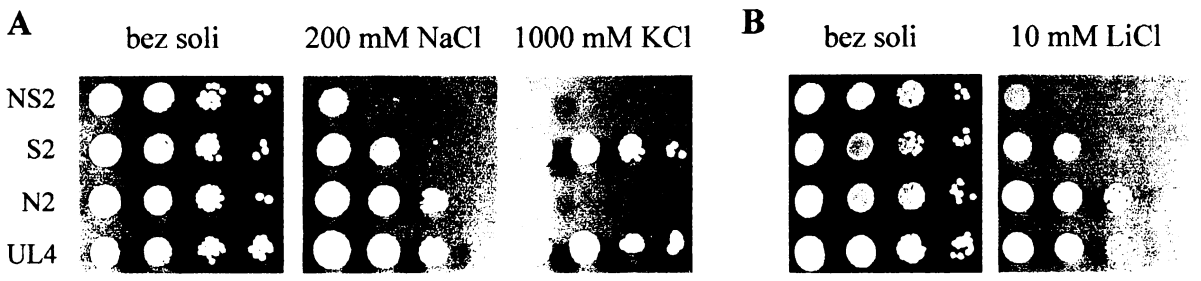
5.3.2.4. Delece genů *ZrSOD2-22* a *ZrNHA1* v *Z. rouxii*

Ve kmeni *Z. rouxii* UL4 byly pomocí metody využívající deleční kazety *loxP-kanMX-loxP* (viz publikace č. 4) vytvořeny delece genů *ZrSOD2-22* a/nebo *ZrNHA1*, a byly tak připraveny mutantní kmeny *ura3 Zrsod2-22Δ::loxP* (S2), *ura3 Zrnha1Δ::loxP* (N2) a *ura3 Zrsod2-22Δ::loxP Zrnha1Δ::loxP* (NS2). Pro přípravu delečních kazet byly využity oligonukleotidy SOD2-22kanf a SOD2-22kanr (pro deleci *ZrSOD2-22*) a NHA1kanf a NHA1kanr (pro deleci *ZrNHA1*; viz kap. 4.2.2. tab. 1). Delece genů byly ověřeny prostřednictvím PCR.

5.3.2.5. Vliv delecí *ZrSOD2-22* a *ZrNHA1* na halotoleranci buněk *Z. rouxii*

Podíl přenašečů *Zr*Sod2-22p a *Zr*Nha1p na halotoleranci buněk *Z. rouxii* byl sledován prostřednictvím růstu delečních mutantů S2, N2 a NS2 za přítomnosti solí NaCl a KCl (200 – 2000 mM) na bohatém médiu YPG a na minimálním médiu YNB-NH_4 s uracilem. Růst mutantů v přítomnosti LiCl (10 – 60 mM) byl sledován pouze na médiu YNB-NH_4 s uracilem, protože již velmi mírná koncentrace LiCl (10 mM) v médiu YPG znemožňovala růst rodičovského kmene *Z. rouxii* UL4, a tedy i od něj odvozených mutantních kmenů. Tento rozdíl v toleranci k LiCl buněk rostoucích v médiu YPG oproti buňkám rostoucím v médiu YNB-NH_4 spočívá zřejmě v tom, že médium YPG má vyšší pH (cca 6.2) než YNB-NH_4 (cca 4.8), tj. ve vnějším prostředí buněk rostoucích v médiu YPG se nachází nižší koncentrace protonů, a proteiny tak nemohou fungovat efektivně v důsledku snížené hnací síly gradientu protonů přes membránu, již ke své funkci využívají.

Obr. 8 ilustruje toleranci kmenů S2, N2 a NS2 ke zvýšené koncentraci solí v médiu. Delece *ZrSOD2-22* snížila schopnost buněk růst v médiu obsahujícím zvýšenou koncentraci NaCl nebo LiCl, ale neměla vliv na růst buněk v přítomnosti KCl (viz růst S2). Samotná delece *ZrNHA1* zhoršila schopnost růstu buněk v přítomnosti zvýšené koncentrace KCl, a do menší míry také NaCl nebo LiCl (viz růst N2; na vybraném obrázku není zhoršení růstu v médiu s LiCl patrné; rozdíl oproti kontrole byl zřejmý až za vyšších koncentrací LiCl, viz dále). Delece obou přenašečů zároveň růst buněk v médiích s NaCl nebo LiCl v porovnání s jednotlivými delecemi ještě více omezila (viz růst NS2).



Obr. 8. Růst kmenů *Z. rouxii* postrádajících *Zr*Sod2-22p (S2), *Zr*Nha1p (N2) nebo oba přenašeče (NS2) a kmene UL4 (pozitivní kontrola) za přítomnosti zvýšené koncentrace solí na médiu YPG (A) a YNB-NH_4 (B).

Delece *ZrSOD2-22* zhoršila schopnost růstu buněk v přítomnosti NaCl na obou typech médií (YPG i YNB-NH_4) obdobně – i na médiu YNB-NH_4 bylo pozorováno obdobné omezení růstu jako na YPG (viz obr. 8). Na růst buněk v přítomnosti KCl neměla delece vliv ani na jednom typu médií, v médiu s LiCl (testován byl pouze růst v YNB-NH_4) byl růst buněk *Zrsod2-22Δ* výrazně omezen (viz obr. 8).

Samotná delece *ZrNHA1*, na rozdíl od jejího zásadního vlivu na toleranci buněk ke KCl (na médiu YNB-NH_4 bylo pozorovnáno obdobné omezení růstu jako na médiu YPG, viz obr. 8), ovlivnila toleranci buněk k NaCl nebo LiCl jen mírně. Vliv na toleranci buněk k NaCl byl pozorován pouze na médiu YPG (buňky nerostly za vyšší než 400 mM koncentrace NaCl, oproti buňkám kontrolního rodičovského kmene UL4, který slabě roste až do koncentrace 1 M NaCl); na médiu YNB-NH_4 rostly buňky stejně dobře jako kontrolní kmen. V médiu YNB-NH_4 s LiCl bylo omezení růstu buněk se samotnou delecí *ZrNHA1* pozorováno až za velmi vysokých koncentrací LiCl (40 mM). Na médiu YNB-NH_4 s NaCl nebo LiCl je zřejmě efekt mutace *Zrnha1Δ* komplementován druhým z přenašečů. Médium YPG již vzhledem k vyššímu pH nedovoluje přenašeči *Zr*Sod2-22p pracovat tak účinně jako médium YNB-NH_4, a delece *ZrNHA1* se pak projeví zhoršeným růstem buněk v přítomnosti soli. Vliv delece *ZrNHA1* na růst buněk v médiích s NaCl a LiCl byl zásadní v buňkách, které již postrádaly i *ZrSOD2-22* (viz růst NS2, obr. 8).

Pro růst buněk v přítomnosti KCl byl tedy důležitý pouze *Zr*Nha1p, nikoli *Zr*Sod2-22p, a delece *ZrSOD2-22* měla větší vliv na toleranci buněk vůči Na^+ a Li^+ než delece *ZrNHA1* (což je v souladu s výsledky prezentovanými na obr. 5, kde je patrná vyšší schopnost proteinu

*Zr*Sod2-22 eliminovat sodné nebo lithné kationty z cytoplasmy). Proteiny *Zr*Nha1p a *Zr*Sod2-22p se tedy ve funkci eliminace sodných a lithných kationtů z buněk navzájem doplňují.

Pro ověření, že zjištěný fenotyp mutantních kmenů *Z. rouxii* N2 a S2 byl způsoben právě chybějícím přenašečem, jsme provedli komplementaci mutace opětovným vložením genu na plasmidu.

5.3.2.6. Komplementace mutací *Zrsod2-22Δ* a *Zrnha1Δ* vložením genu na plasmidu

Vzhledem k tomu, že ani jeden z plasmidů YEp352 nebo pGRU1 nesoucí příslušné geny nebylo možno použít pro transformaci kmenů *Z. rouxii*, protože nejsou schopny se v buňkách *Z. rouxii* pomnožovat (Ushio *et al.*, 1988), byly geny *ZrSOD2-22* a *ZrNHA1* pod kontrolou promotoru *ScNHA1* vloženy do plasmidu pZEU (episomální plasmid *Z. rouxii* připravený pro v rámci této práce, viz publikace č. 4). Plasmidem pZEU_*ZrNHA1* byl pak transformován kmen *Z. rouxii* N2 a plasmidem pZEU_*ZrSOD2-22* kmen S2.

Byly provedeny kapkové testy růstu transformantů na médiích YNB-NH_4 se zvýšenou koncentrací solí. Transformované kmeny na těchto médiích rostly stejně dobře nebo i mírně lépe než kmen kontrolní (UL4 transformovaný plasmidem pZEU). Tento mírný rozdíl mohl být způsoben tím, že geny byly exprimované z mnohokopiového plasmidu pZEU, takže se v buňkách trasformantů nalézalo více produktů genů kódujících přenašeče než v rodičovském kmeni.

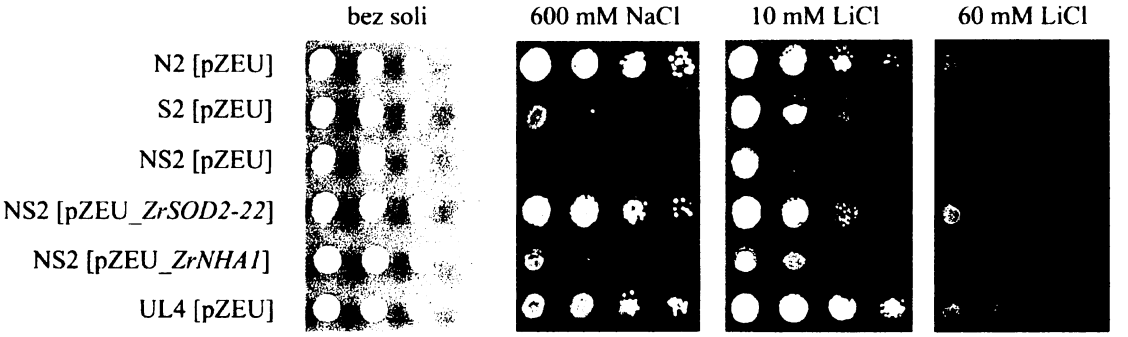
Tento výsledek potvrdil, že geny vnesené na plasmidu plně komplementovaly mutace *Zrsod2-22Δ* resp. *Zrnha1Δ*, a tedy že pozorovaný fenotyp citlivosti kmenů S2 a N2 vůči solím (viz obr. 8 a kap. 5.3.2.5.) je způsoben právě delecí genů *ZrSOD2-22* resp. *ZrNHA1*.

*Zr*Sod2-22p byl důležitý pro růst buněk *Z. rouxii* v přítomnosti zvýšené koncentrace NaCl nebo LiCl, ale nikoli KCl. Protein *Zr*Nha1p naopak udává buňkám toleranci ke všem třem testovaným kationtům, i když na toleranci buněk k Na^+ a Li^+ se podílí v menší míře než *Zr*Sod2-22p. Totéž bylo pozorováno i při heterologní expresi obou proteinů v *S. cerevisiae* (viz obr. 5). Proteiny tak mají v *Z. rouxii* shodné substrátové specifity jako při heterologní expresi v *S. cerevisiae*.

5.3.2.7. Vzájemná komplementace funkcí *Zr*Sod2-22p a *Zr*nha1p

Abychom zjistili, do jaké míry jsou přenašeče schopny komplementovat své funkce eliminace sodných nebo lithných kationtů, provedli jsme pokus, ve kterém jsme pozorovali, do jaké míry zvýšená exprese jednoho z přenašečů umožní komplementovat halosenzitivní fenotyp mutanta *Z. rouxii* NS2 (tj, postrádajícího oba přenašeče). Geny jsme v mutantním kmeni NS2 exprimovali z mnohokopiového plasmidu pZEU.

Kmen NS2 transformovaný plasmidem pZEU_*ZrNHA1* tak obsahoval několik kopií genu *ZrNHA1* a žádnou *ZrSOD2-22*, a naopak kmen NS2 transformovaný plasmidem pZEU_*ZrSOD2-22* obsahoval několik kopií genu *ZrSOD2*-22 a žádnou *ZrNHA1*. Obr. 9. ukazuje schopnost růstu transformantů za vyšší koncentrace solí v médiu.



Obr. 9. Růst buněk kmene NS2 (*Zrnha1Δ Zrsod2-22Δ*) exprimujících *ZrNHA1* nebo *ZrSOD2-22* z plasmidu pZEU nebo obsahujících prázdný vektor pZEU (negativní kontrola) a kmenů N2 (*Zrnha1Δ*), S2 (*Zrsod2-22 Δ*) a UL4 obsahujících prázdný vektor pZEU (pozitivní kontroly) na médiu YNB-NH_4 obsahujícím zvýšené koncentrace NaCl nebo LiCl.

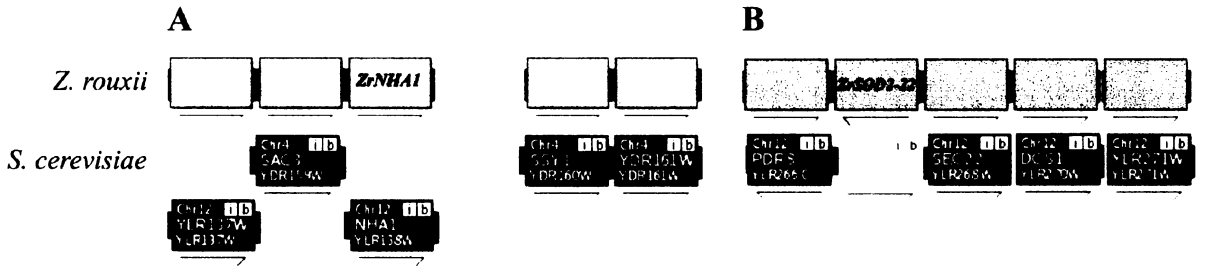
Z obr. 9 je zřejmé, že přenašeč *Zr*Nha1p je schopen částečně komplementovat mutaci *Zrsod2-22Δ*: růst kmene NS2 obsahujícího plasmid pZEU_*ZrNHA1*, tj. několik kopií genu *ZrNHA1* a žádnou *ZrSOD2-22*, je totiž v médiu obsahujícím 600 mM NaCl mírně lepší než růst kmene s delecí *ZrSOD2-22* (obsahujícího tedy pouze gen *ZrNHA1* na chromosomu; viz růst S2 [pZEU]). Obdobný efekt přítomnosti několika kopií *Zr*Nha1p na růst kmene S2 [pZEU] za přítomnosti LiCl však nebyl pozorován. Přenašeč *Zr*Nha1p tak fenotyp mutace *Zrsod2-22Δ* na médiu s LiCl není zřejmě vzhledem ke své nízké kapacitě schopen komplementovat, nicméně při absenci *ZrSOD2-22* je pro růst buněk v přítomnosti i nižších

koncentrací LiCl jako je 10 mM vyžadován (viz rozdíl růstu kmenů S2 [pZEU] a NS2 [pZEU], obr. 9, a též viz obr. 8). Přítomnost více kopií genu *ZrSOD2-22* na druhou stranu do velké míry komplementuje mutaci *Zrnha1Δ*, což je vidět na růstu buněk za vysoké koncentrace LiCl (60 mM; srovnej růst kmenů NS2 [pZEU_*ZrSOD2-22*] a UL4 [pZEU]). Při růstu na médiu s KCl vzhledem ke své substrátové specifitě omezené na Na^+ a Li^+ *Zr*Sod2-22p fenotyp mutace *Zrnha1Δ* nekomplementoval.

Tyto výsledky ukazují, že zvýšená exprese *Zr*Nha1p je schopna částečně komplementovat mutaci *Zrsod2-22Δ* při růstu buněk za vyšší koncentrace NaCl a zvýšená exprese *Zr*Sod2-22p zase mutaci *Zrnha1Δ* při růstu buněk za vyšší koncentrace LiCl. Přenašeče jsou tak schopny se nejen funkčně vzájemně doplňovat, ale při růstu za zvýšené koncentrace NaCl a LiCl i alespoň částečně komplementovat své funkce. K přesnému určení funkcí přenašečů v buňkách *Z. rouxii* bude zapotřebí podrobnější analýzy (např. studium exprese genů za různých podmínek).

5.3.2.8. Fylogenetický vztah *ZrSOD2-22* a *ZrNHA1*

Abychom zjistili fylogenetický vztah genů *ZrNHA1* a *ZrSOD2-22*, porovnali jsme uspořádání (synteny) ORFů je obklopujících s uspořádáním homologů těchto ORFů a genu *ScNHA1* na chromosomech blízce příbuzné kvasinky *S. cerevisiae* (obr. 10).



Obr. 10. Srovnání synteny ORFů obklopujících geny *ZrNHA1* (A) a *ZrSOD2-22* (B) s uspořádáním jejich pravděpodobných homologů na chromosomech *S. cerevisiae*. Systém zobrazení byl převzat z Yeast Gene Order Browser (http://wolfe.gen.tcd.ie/ygob). Obdélníky představují ORFy - velmi tmavě šedá barva představuje ORFy *S. cerevisiae* vykazující syntenické uspořádání se svými homology v *Z. rouxii*, šedá barva ORFy *Z. rouxii* a světle šedá barva gen *S. cerevisiae* nevykazující syntenické uspořádání se svým homologem v *Z. rouxii*; šipky znázorňují orientaci čtecích rámců; šedé spojnice obdélníků (ORFů) představují nekódující oblasti DNA.

Zjistili jsme, že uspořádání ORFů na úseku DNA obsahujícím *ZrNHA1* je podobné uspořádání pravděpodobných homologů kvasinky *S. cerevisiae* nacházejících se na chromosomech IV a XII. Fakt, že se homology ORFů nacházející se v *Z. rouxii* na jednom chromosomu nacházejí v *S. cerevisiae* na chromosomech dvou, je v souladu s hypotézou, že genom *Z. rouxii* ve své historii neprodělal celkovou duplikaci (viz kap. 2.4.1.)

ORFy nacházející se v okolí genu *ZrSOD2-22* vykazují stejné uspořádání jako jejich homology nacházející se v *S. cerevisiae* na chromosomu XII na jiném místě než je gen *ScNHA1*. Obr. 10 tedy naznačuje, že gen *ZrSOD2-22* patrně vznikl duplikací předka genu *ZrNHA1*, a inzercí kopie na jiné místo genomu. Nejnovější data analýzy genomu *Z. rouxii* (veřejnosti zatím nepřístupná databáze Génolevures 3) potvrzují, že geny *ZrNHA1* a *ZrSOD2-22* leží každý na jiném chromosomu.

5.3.2.9. Závěr

V této kapitole dizertační práce jsme zjistili, že Na^+/H^+-transportní protein *Z. rouxii* *Zr*Sod2-22p, který byl dosud studován pouze prostřednictvím heterologní exprese v *S. cerevisiae*, umožňuje buňkám *Z. rouxii* růst za vyšších koncentrací NaCl nebo LiCl, ale nemá vliv na růst buněk za vyšších koncentrací KCl. Tím jsme potvrdili jeho funkci v eliminaci sodných nebo lithných, ale nikoli draselných kationtů z buněk, v souladu s dříve popsanou aktivitou tohoto přenašeče heterologně exprimovaného v buňkách *S. cerevisiae* (Kinclová *et al.*, 2002).

Prohledáním dosud neanotované databáze Génolevures 3 obsahující kompletní sekvenci genomu *Z. rouxii* CBS 732^T jsme nalezli gen kódující nový Na^+/H^+-transportní protein *Z. rouxii*. Gen jsme nazvali *ZrNHA1* na základě sekvenční podobnosti jím kódovaného proteinu s *Sc*Nha1p. Protein *Zr*Nha1p jsme funkčně charakterizovali jako přenašeč plasmatické membrány, který umožňuje růst buněk *Z. rouxii* za vyšších koncentrací KCl, NaCl i LiCl. Analýza transportní kapacity a fenotypu buněk *S. cerevisiae* exprimujících *ZrNHA1* potvrdila funkci přenašeče v eliminaci všech třech typů kationtů (tj. K^+ , Na^+ i Li^+) z buněk.

Analýza delečních mutantů *Z. rouxii* postrádajících *Zr*Sod2-22p a/nebo *Zr*Nha1p ukázala, že na toleranci buněk *Z. rouxii* k NaCl či LiCl se podílí *Zr*Sod2-22p do větší míry než *Zr*Nha1p. Totéž ukázalo i sledování fenotypu buněk *S. cerevisiae* exprimujících tyto přenašeče

a měření transportní kapacity pro Na^+ buněk *S. cerevisiae* obsahujících *Zr*Nha1p, která se ukázala být slabší než kapacita buněk *S. cerevisiae* obsahujících *Zr*Sod2-22p.

Porovnáme-li transportní kapacitu obou přenašečů *Z. rouxii* a Na^+/H^+-antiportéru *S. cerevisiae Sc*Nha1p, jako nejúčinnější systém transportující sodné i draselné kationty se ukázal být *Sc*Nha1p (*Zr*Sod2-22p netransportuje K^+ vůbec). I zdánlivě pomalý výstup kationtů z buněk naměřený u buněk obsahujících *Zr*Nha1p však umožnil růst buněk za přítomnosti vyšších koncentrací solí v médiu. *Zr*Sod2-22p ze všech tří transportérů nejúčinněji zajistil růst buněk *S. cerevisiae* ve vysoké koncentraci NaCl nebo LiCl.

Sledování fenotypu delečních mutantů dále ukázalo, že oba přenašeče *Z. rouxii* se funkčně doplňují a mohou částečně komplementovat své funkce v eliminaci sodných nebo lithných kationtů z buněk.

Analýza uspořádání genů na chromosomech naznačila, že *ZrSOD2-22* patrně vznikl duplikací předka genu *ZrNHA1*, a jeho inzercí na jiné místo genomu.

Kvasinka *Z. rouxii* (kmen CBS 732^T) tedy obsahuje kromě proteinu *Zr*Sod2-22p eliminujícího z buněk toxické kationty Na^+ a Li^+ (tj. s úzkou substrátovou specifitou) i další přenašeč z rodiny Na^+/H^+-antiportérů plasmatické membrány (*Zr*Nha1p), který transportuje kromě Na^+ a Li^+ i K^+ (tj. má širokou substrátovou specifitu), a může se tak v buňkách podílet na udržování stabilní cytoplasmatické koncentrace K^+, stálého buněčného objemu nebo pH cytoplasmy.

Přítomnost dvou typů Na^+/H^+-antiportních systémů (jednoho s úzkou a druhého se širokou substrátovou specifitou) byla již detekována ve dvou druzích kvasinek - *Y. lipolytica* (Papoušková a Sychrová, 2006) a *S. pombe* (Papoušková a Sychrová, 2007). Přenašeče těchto dvou druhů však byly studovány pouze prostřednictvím heterologní exprese v *S. cerevisiae*. *Z. rouxii* tak představuje první kvasinku, ve které byla funkce obou antiportních systémů charakterizována.

5.4. Sekvenace a anotace genomu *Z. rouxii*

Během projektu Génolevures 3 byla sekvenována genomová DNA čtyř kvasinkových druhů, *Z. rouxii*, *S. kluyveri*, *K. thermotolerans* a *K. lactis*. Pro každý druh byly vytvořeny plasmidové knihovny obsahující inzerty o velikosti 3 - 5 kbp, které byly sekvenovány metodou „shotgun" (Dujon *et al.*, 2004). Na základě překrývajících se sekvencí inzertů byly fragmenty uspořádány do úseků odpovídajících chromosomům. Kde toho bylo zapotřebí (např. v případě úseků obsahujících repetitivní sekvence), bylo pro zjištění návaznosti sekvencí využito knihoven připravených v umělých bakteriálních chromosomech (BAC). Byly identifikovány geny a funkční elementy DNA. Veškeré predikce vytvářel počítačový program vyvinutý speciálně pro potřeby projektu. K predikci funkce translačních produktů program využíval srovnání se známými proteiny *S. cerevisiae* a dalších kvasinek, jejichž DNA byla sekvenována v projektu Génolevures 2. Nalezené homologie pak vedly k seřazení nově identifikovaných ORFů představujících pravděpodobně strukturní geny do funkčních rodin (spolu s již známými geny). Poté následovala anotace manuální, tj. informace získané prostřednictvím počítačového programu byly ověřovány a doplňovány konkrétní osobou, členem konsorcia. Tyto manuální anotace byly rozděleny do několika fází. Fáze jedna se zabývala anotacemi funkčních rodin vytvořených programem. Fáze dva se zabývala anotací identifikovaných ORFů, které netvořily funkční rodiny. Nezávisle na těchto dvou fázích probíhaly anotace funkčních elementů DNA (tj. funkčních úseků nekódujících proteiny). V době sepsání této dizertační práce byl proces anotací v závěrečné fázi.

Součástí práce bylo nejprve připravit genomovou DNA *Z. rouxii* pro potřeby sekvenování. Samotná sekvenace pak byla provedena v rámci centra Génoscope v Paříži (partner projektu Génolevures). Získané sekvence byly členům konsorcia Génolevures zpřístupněny v neveřejné databázi. Z následného procesu anotací se autorka této dizertační práce podílela na fázi jedna, tj. na úpravě anotací funkčních rodin navržených programem.

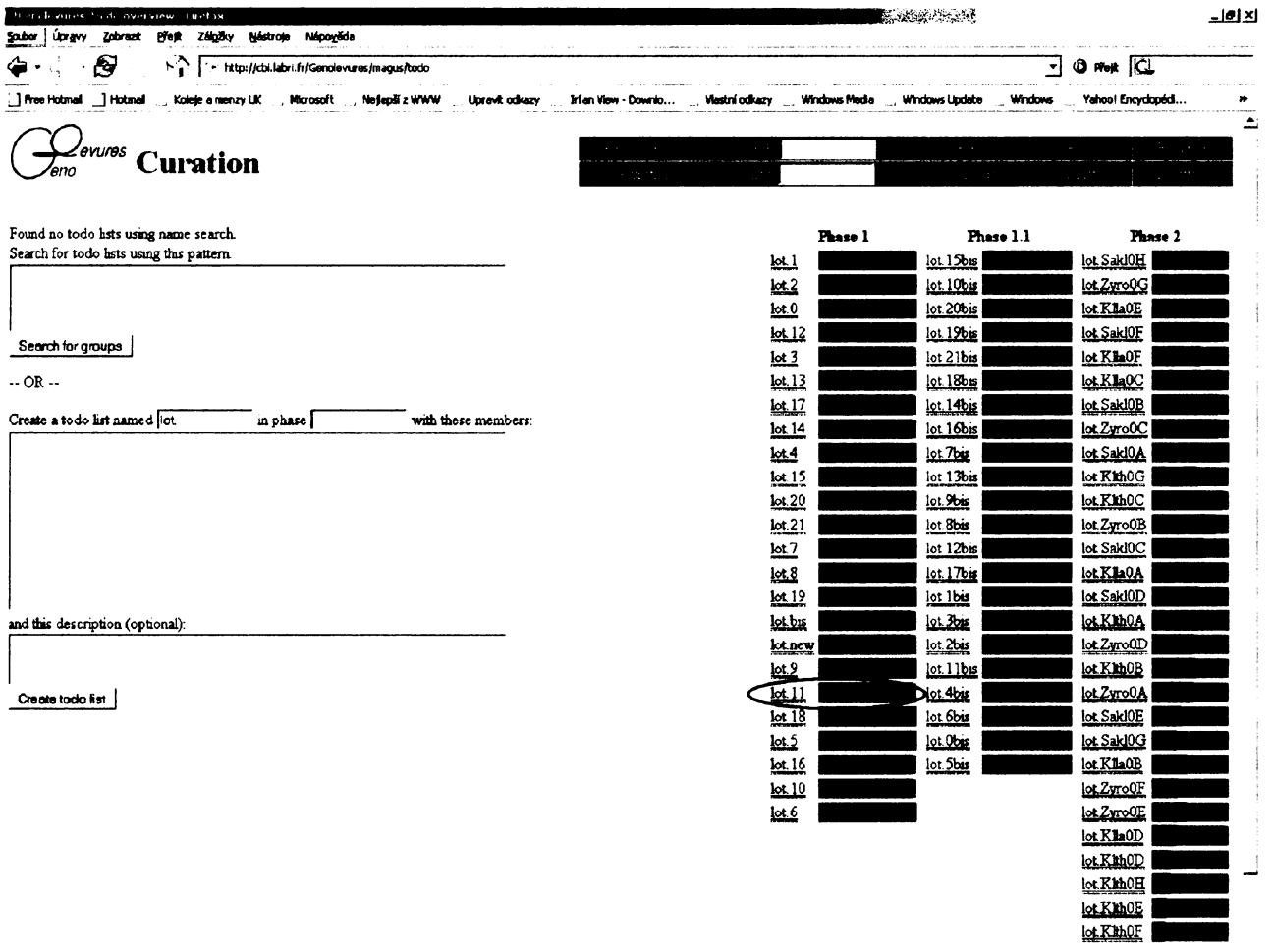
5.4.1. Příprava DNA *Z. rouxii* CBS 732^T pro sekvenaci

Buňky kmene *Z. rouxii* CBS 732^T byly napěstovány v bohatém médiu YPG a byla izolována genomová DNA v agarozových bločcích metodou přípravy DNA k analýze karyotypu prostřednictvím PFGE, tj. způsobem, který umožňuje izolaci kompletních

nepoškozených chromosomů (nedochází ke zlomům). Připravená DNA byla odeslána do centra Génoscope k sekvenování.

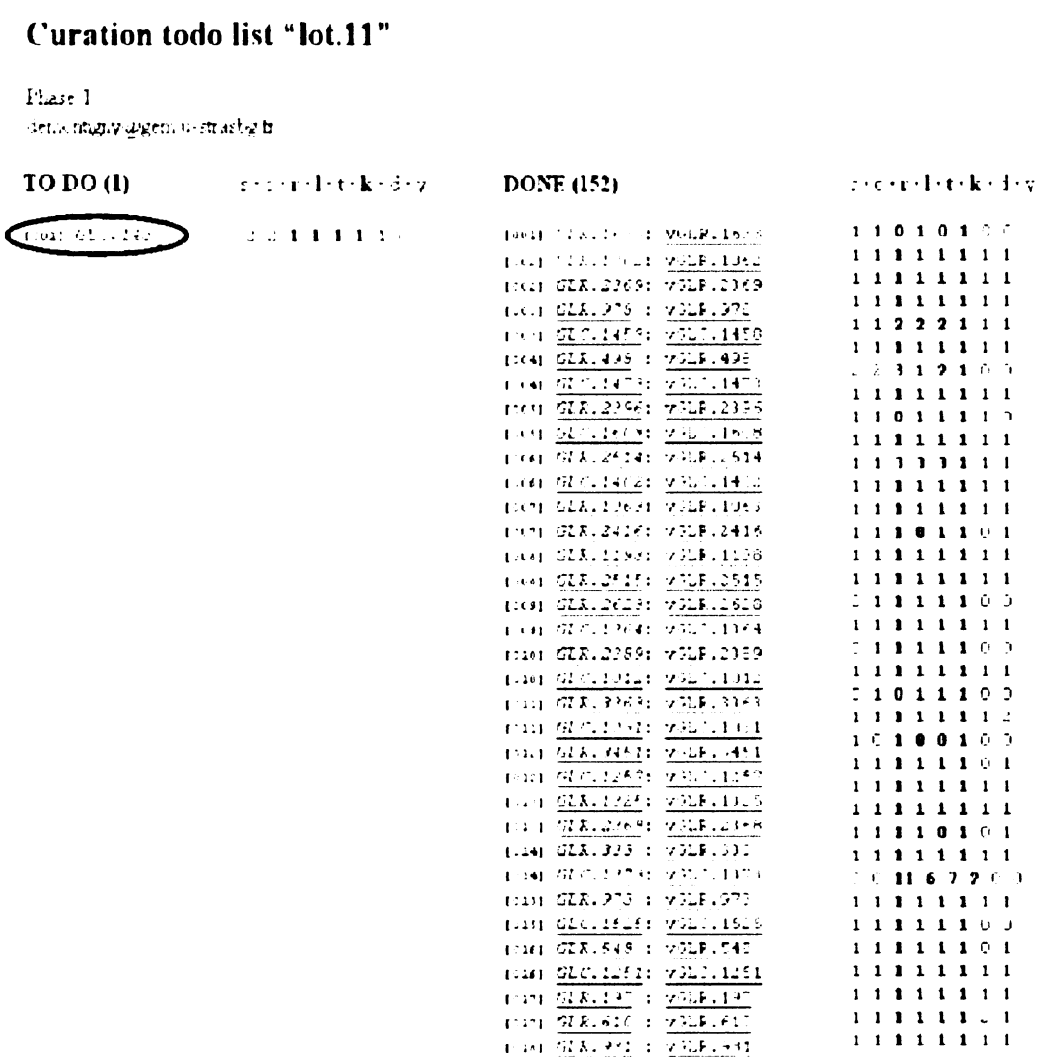
5.4.2. Anotace

Manuální anotace genomů kvasiek *Z. rouxii*, *S. kluyveri*, *K. lactis* a *K. thermotholerans* byly prováděny na serveru Génolevures, k němuž byl přístup chráněn heslem. Každý člen konsorcia Génolevures, který se účastnil anotací fáze 1, měl přiděleno několik desítek funkčních rodin, dohromady tvořících tzv. „lot" (obr. 11).



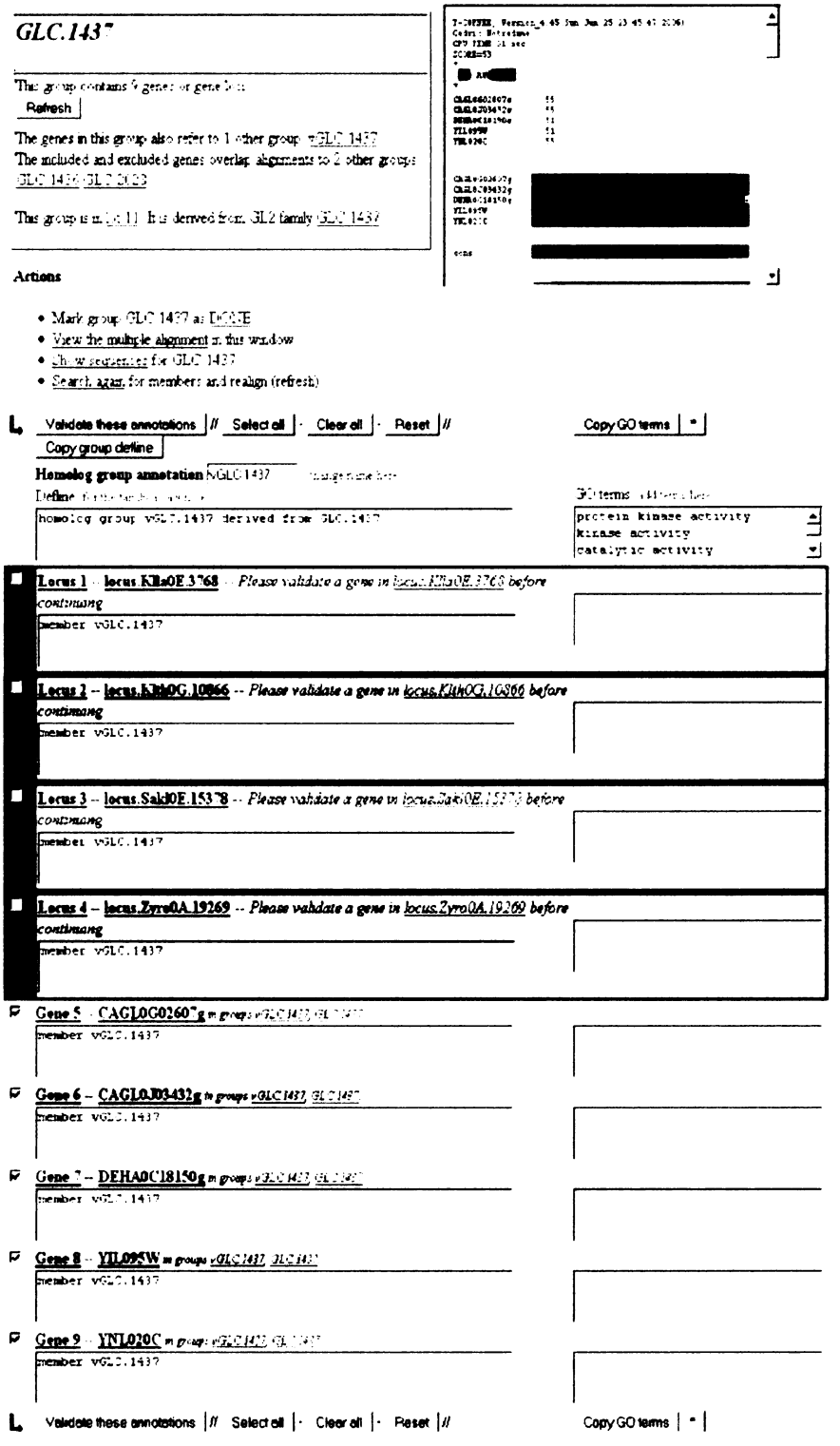
Obr. 11. Anotace fáze 1 - úvodní strana obsahující grafické zobrazení probíhajících anotací. Sloupec Phase 1 odpovídá anotacím prováděným ve fázi 1. Sloupce Phase 1.1. a Phase 2 odpovídají následujícím etapám anotací Génolevures 3, které nebudou v této práci diskutovány. Poměr zelené a červené barvy symbolizuje poměr manuálně ověřených (zeleně) a dosud neověřených (červeně) anotací navržených programem. Autorka této práce se podílela na anotacích sekvencí zahrnutých do lot 11 (v červeném rámečku).

Poklepáním na odpovídající lot (v případě této dizertační práce č. 11) se zobrazil seznam genových rodin určených k manuální anotaci (Obr 12.).



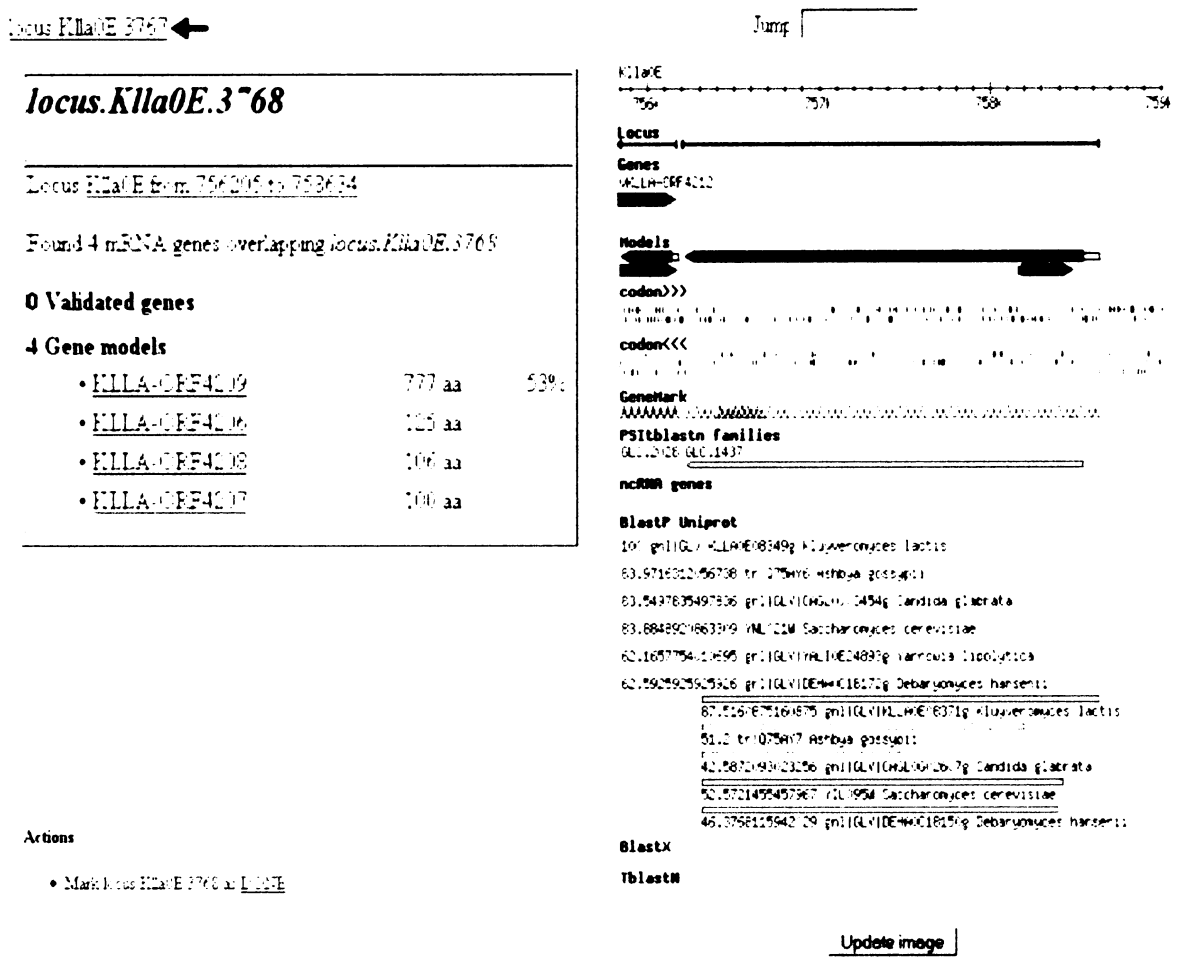
Obr. 12. Seznam funkčních rodin připravených k anotacím patřících do lot 11. Levý sloupeček „TO DO" je seznamem funkčních rodin, jež dosud nebyly manuálně anotovány, pravý „DONE" je soupisem již ověřených anotací. Sloupečky s·c·r·l·t·k·d·y. značí, v kolika oblastech (lokusech) DNA v daném organismu (s, *S. cerevisiae*; c, *C. glabrata*, r., *Z. rouxi*; l, *S. [Lachancea] kluyveri*; t, *K. thermotolerans*, k, *K. lactis*, d, *D. hansenii*, y, *Y. lipolytica*) byly programem identifikovány ORFy kódující protein patřící do příslušné funkční rodiny. V tomto konkrétním případě zbývala jediná rodina, u jejíchž členů nebyla manuálně ověřena správnost anotací navržených programem (GLC.1437, v červeném rámečku).

Při poklepání na název rodiny se zobrazil seznam úseků DNA, ve kterých byly identifikovány ORFy patřící do rodiny, a srovnání proteinových sekvencí již známých členů rodiny (obr. 13.).



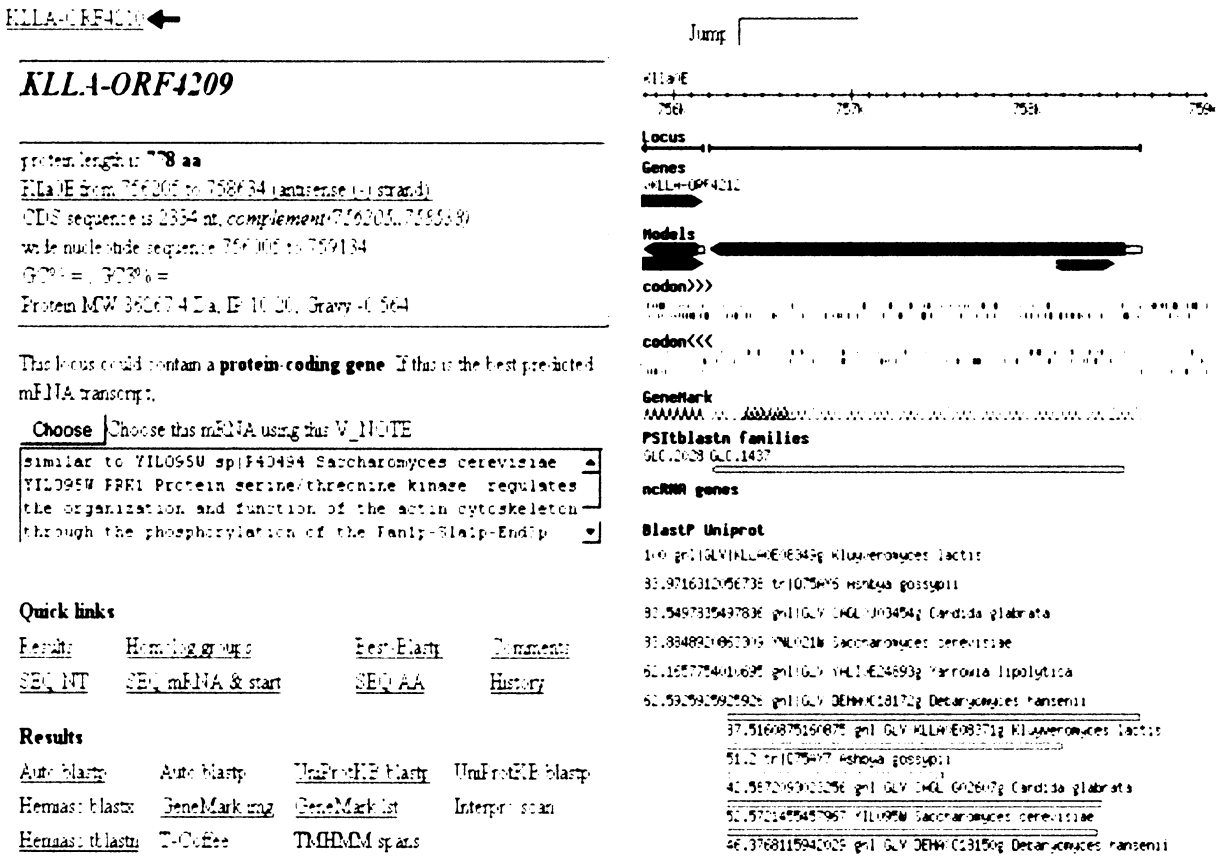
Obr. 13. Funkční rodina GLC.1437. V levé horní části je stručný popis rodiny, v pravém horním rámečku pak srovnání sekvencí translačních produktů již známých genů zařazených do rodiny. V dolní části je seznam členů rodiny. Červeně jsou zvýrazněny rámečky informující o oblastech DNA, ve kterých program identifikoval ORFy, jejichž anotace nebyly dosud manuálně ověřeny (Locus 1 – 4). Název lokusu obsahuje zkratku názvu druhu, ze kterého pochází, a pozici na chromosomu. Zbývajících 5 členů rodiny jsou již známé geny ostatních druhů kvasinek, jejichž genomy byly anotovány v projektu Génolevures 2 (Gene 5 - 9). V případě této skupiny byl tedy v každém ze čtyř druhů analyzovaných v Génolevures 3 identifikován jeden lokus DNA obsahující ORF zařazený na základě homologie sekvencí do této funkční rodiny.

Při poklepání na název lokusu určeného k manuální anotaci se zobrazila informace o jeho umístění na chromosomu a o nalezených „modelech" genu, tj. nalezených ORFech. Program o přítomných ORFech referuje jako o nalezených mRNA. V mnoha případech program identifikoval pouze jeden ORF, a tedy navrhl jeden model genu (jednu mRNA), v mnoha jiných případech však program identifikoval dva a více modelů; např. navrhl přítomnost intronu a několik možností jeho umístění (navržena tedy byla přítomnost několika různých mRNA). Úkolem pracovníka provádějícího manuální anotace pak bylo vybrat „správný" model genu. Pokud program identifikoval více možností vytvoření mRNA, a tedy i více možností utvoření proteinu, byla vybrána ta mRNA, jež pokrývala co možná nejdelší oblast lokusu, a jejíž translační produkt byl nejpodobnější ostatním členům rodiny. Proces anotací bude v této práci detailně popsán na příkladě, ve kterém program identifikoval v lokusu mRNA bez intronů. Takovým příkladem je lokus Klla0E.3768 (obr. 14. a viz též obr. 13., kde se tento lokus nachází jako první v seznamu členů funkční rodiny GLC.1437).



Obr. 14. Lokus Klla0E.3768. V levém rámečku je seznam navržených modelů genů (Gene models), tj. mRNA, které program identifikoval jako nacházející se v lokusu či do něj zasahující (celkem 4). Je udána také délka jimi kódovaných proteinů. Vpravo je pak zobrazení umístění modelů genů na DNA. Vpravo nahoře je graficky znázorněn chromosom, na kterém se lokus nachází. Anotovaná oblast lokusu je zvýrazněna žlutě. Pod ní jsou seřazeny identifikované a již manuálně anotované geny zasahující do lokusu (zde Klla-ORF4212), následují návrhy modelů genů. Modrá barva značí model nacházející se na řetězci 5'→3', červená na řetězci komplentárním (3'←5'). Údaje níže informují o rozložení všech ORFů v lokusu, o pravděpodobnosti, zda určitý ORF kóduje protein (grafické znázornění vytvořené programem GeneMark; http://exon.gatech.edu/GeneMark), a o nalezených homologiích v rámci genomů ostatních kvasinek (BlastP Uniprot). V tomto případě se ve žlutě vyznačeném úseku určeném k manuální anotaci nacházejí dva modely genu (dvě mRNA), jeden pokrývající celý úsek komplementárního řetězce, druhý, podstatně kratší, pokrývající část řetězce 5'→3'. Grafická informace z programu GeneMark naznačuje, že ona dlouhá mRNA (na rozdíl od krátké) bude pravděpodobně kódovat protein.

Poklepáním na konkrétní model genu (ve vybraném případě mRNA pokrývající celou délku lokusu Klla0E.3768, viz obr. 14) se zobrazil název ORFu (ve vybraném případě Klla-ORF4209) a informace o proteinu, který by vznikl translací kódující sekvence (coding sequence, CDS). Program na základě srovnání proteinové sekvence s již známými proteiny ostatních druhů kvasinek navrhl, k jakému genu je identifikovaný ORF homologní , tj. jakou funkci by protein mohl v organismu zastávat (obr. 15.).



Obr. 15. Klla-ORF4209. Rámeček vlevo nahoře podává informace o vlastnostech CDS a kódovaného proteinu. Rámeček pod ním informuje, k jakému známému genu byla srovnáním sekvencí translačních produktů nalezena nejvyšší homologie (zde byl gen vyhodnocen jako podobný YIL095w *S. cerevisiae*). Níže jsou pak odkazy na vlastnosti sekvence a její srovnání s jinými sekvencemi. V pravé část obrázku je žlutě zvýrazněn model genu, jinak je pravá část totožná s pravou částí obr. 14.

Úkolem pracovníka provádějícího anotace bylo vybrat správný model genu a ověřit, zda je počítačem určená podobnost ORFu se známým genem vskutku korektně stanovená, popřípadě data opravit. K tomu sloužily informace poskytnuté srovnáním sekvence identifikovaného proteinu se známými proteiny z databáze UniProtKB. Tímto srovnáním bylo např. možno zjistit, zda program korektně navrhl začátek proteinové sekvence (tj. např. nezaměnil počáteční aminokyselinu methionin [Met] za jiný Met nacházející se uvnitř proteinového řetězce, např. kvůli tomu, že „přehlédl" intron na 5' konci). Dále bylo možno zjistit, zda jsou si proteiny podobné v celé své délce, a zastávají tak patrně v organismu podobnou funkci, nebo zda nalezené homologie pokrývají jen určité oblasti, a jinak se jedná o proteiny odlišné (případ funkčních domén, např. transmembránových). Procentuální vyčíslení shody (identity) aminokyselinových sekvencí nově identifikovaného proteinu a jemu nejvíce podobného známého proteinu z jiné kvasinky určovaly, jakým způsobem bude nalezená podobnost zanesena do databáze. Pokud byla shoda vyšší než 80 %, do rámečku infrormujícího o nalezených homologiích se zapsalo „highly similar to" (velmi podobný); v případě, že shoda byla mezi 50 - 80 %, bylo uvedeno„similar to" (podobný) a při shodě 30 - 50 % bylo zapsáno „weakly similar to" (vykazující malou míru podobnosti s). Pokud byla shoda nalezena jen v určitých oblastech proteinu (případ funkčních domén), byla nalezená homologie uvedena jako „some similarities with" (zčásti podobný).

Nově identifikované sekvence v rámci funkčních rodin obvykle nacházely své homologní protějšky hned v několika ze známých druhů kvasinek. Jako referenční byl v takovém případě brán protein (resp. gen) kvasinky *S. cerevisiae*. Pouze pokud byl anotovaný protein výrazně podobnější proteinu z jiné kvasinky než *S. cerevisiae*, byl v rámečku popisujícím nalezenou homologii uveden gen této kvasinky.

Po ověření (případně opravení) anotací provedených programem byla informace o nově identifikovaném genu uložena (poklepáním na tlačítko „choose" v rámečku informujícím o nalezené homologii, viz obr. 15.). Anotace genu tak byla ukončena, a mohlo být přikročeno k anotaci dalšího člena rodiny. Po ověření anotací u všech členů funkční rodiny byly pak uloženy informace o celé rodině (poklepáním na tlačítko „validate these annotations",viz obr. 13. úplně dole vlevo). Ta se pak na úvodní stránce přesunula ze sloupečku „TO DO" do sloupečku „DONE" (viz obr. 12.).

Po dokončení anotací fáze 1 (anotace genových rodin) proběhla fáze 2 (anotace ORFů, které netvořily rodiny). Anotace ostatních funkčních elementů probíhaly nezávisle na těchto dvou fázích. V době sepsání dizertační práce byl proces anotací v závěrečné fázi. Po dokončení procesu anotací bude databáze veřejně zpřístupněna a informace o sekvencích obdržené v průběhu anotací budou konsorciu Génolevures sloužit jako data pro vypracování srovnávacích studií majících za cíl odhalit mechanismy podílející se na molekulární evoluci kvasinkových genomů, a potažmo tak evoluci eukaryotického genomu vůbec.