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Physiological Basis for the High Salt Tolerance of *Debaryomyces hansenii*

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The effects of KCl, NaCl, and LiCl on the growth of *Debaryomyces hansenii*, usually considered a halotolerant yeast, and *Saccharomyces cerevisiae* were compared. KCl and NaCl had similar effects on *D. hansenii*, indicating that NaCl created only osmotic stress, while LiCl had a specific inhibitory effect, although relatively weaker than in *S. cerevisiae*. In media with low K^+, Na^+ was able to substitute for K^+, restoring the specific growth rate and the final biomass of the culture. The intracellular concentration of Na^+ reached values up to 800 mM, suggesting that metabolism is not affected by rather high concentrations of salt. The ability of *D. hansenii* to extrude Na^+ and Li^+ was similar to that described for *S. cerevisiae*, suggesting that this mechanism is not responsible for the increased halotolerance. Also, the kinetic parameters of Rb^+ uptake in *D. hansenii* (V_{max}, 4.2 nmol mg [dry weight]$^{-1}$ min^{-1}; K_m, 7.4 mM) indicate that the transport system was not more efficient than in *S. cerevisiae*. Sodium (50 mM) activated the transport of Rb^+ by increasing the affinity for the substrate in *D. hansenii*, while the effect was opposite in *S. cerevisiae*. Lithium inhibited Rb^+ uptake in *D. hansenii*. We propose that the metabolism of *D. hansenii* is less sensitive to intracellular Na^+ than is that of *S. cerevisiae*, that Na^+ substitutes for K^+ when K^+ is scarce, and that the transport of K^+ is favored by the presence of Na^+. In low K^+ environments, *D. hansenii* behaved as a halophilic yeast.

In recent years considerable effort has been put in the study and understanding of the mechanisms mediating salt tolerance in cell-walled eukaryotic organisms (9, 21). *Saccharomyces cerevisiae*, a moderately tolerant yeast, has been used as a model in these studies (9).

The stress resulting from the presence of high salt concentrations can be explained by two different processes, one associated with osmotic problems and the other dependent on the toxicity of the specific ions. In *S. cerevisiae*, specific sodium toxicity has been shown to be exerted by intracellular sodium (15), whose concentration is the net result of the amount of ion entering and leaving the cell. To prevent excessive sodium accumulation, *S. cerevisiae* has the ability to decrease influx and to favor efflux. This yeast does not have a specific sodium uptake system, and sodium crosses the membrane through the potassium transport system(s) coded by the *TRK* genes (3, 5, 24). Since TRK proteins have a much higher affinity for potassium than for sodium, potassium is preferentially accumulated by the cells (7). On the other hand, *ENA* genes play an important role in sodium and lithium tolerance. They code for P-type ATPases, which mediate sodium and lithium efflux and prevent sodium and lithium accumulation. *ENA* genes are found as a tandem array of four to five copies (6, 32) and are tightly regulated by several transduction pathways (13, 14, 32). Very recently a Na^+/H^+ antiporter that may play a role in Na^+ tolerance, as known in *Zygosaccharomyces rouxii* (30, 31), has been proposed (18).

Debaryomyces hansenii is a spoilage yeast usually found contaminating brine food and low-water-activity products. It can also be isolated from salty water and solar saltworks and is considered a halotolerant yeast (11, 28). Most previous studies of *Debaryomyces* deal with compatible solutes which accumulate inside the cells (1, 2), although some information on ion fluxes is also available (12, 17).

Norkrans and Kylin, in 1969, described the regulation of the K^+/Na^+ ratio in relation to salt tolerance in *D. hansenii* and *S. cerevisiae* (17). Based on the results of long-term transport experiments (several hours) they proposed that salt tolerance in *D. hansenii* was partially dependent on the ability to extrude Na^+ and to take up K^+. To our knowledge, no further information is available on the role of ion transport in mechanisms involved in salt tolerance in this yeast.

Our objective was to compare salt tolerance and alkali cation transport in *D. hansenii* and *S. cerevisiae* in an attempt to understand the physiological basis underlying the relatively high halotolerance of *D. hansenii*.

MATERIALS AND METHODS

Strains, growth media, and culture conditions. *D. hansenii* IGC 2968 (CBS 767) and *S. cerevisiae* IGC 4455 (CBS 1171) were routinely kept on solid YPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] glucose, 2% [wt/vol] agar). Unless otherwise stated, cells were grown at 28°C in an orbital shaker (200 rpm) in flasks (250 to 1,000 ml) containing mineral medium (29) with ammonium as the nitrogen source, vitamins, trace elements, and 2% (wt/vol) glucose as the carbon and energy source. When necessary, NaCl was added at the desired concentration. For K^+ limiting and starvation media, a modification of the medium was used in which H_3PO_4 was substituted for KH_2PO_4 at the same concentration. KCl was added to obtain the required amount of K^+. All media were adjusted to pH 4.5 with NH_4OH. For cation flux assays, cells were grown to mid-exponential phase, centrifuged at 4,250 × *g* for 8 min at 4°C, and washed twice with cold water.

Specific growth rates. Growth assays were performed in 250-ml flasks with 50 ml of medium at 28°C in an orbital shaker. Growth was monitored by optical density at 550 nm (OD_{550}) up to the end of exponential phase in a Beckman DU640 spectrophotometer.

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Intracellular volume. The classic method of Rottenberg (25) was modified, with polyethylene glycol (PEG) 4000 as the external marker. Cells were grown in mineral medium containing different salt concentrations; when the OD_{640} reached a value of 1, cells were harvested, washed twice with cold water, and resuspended in 10 ml of the same medium (OD_{640}, 30 to 50) containing PEG 4000 (5 mg ml^{-1}). Aliquots (1 ml) were distributed in Eppendorf tubes in quadruplicate and, after addition of 2.5 μCi of ^3H_2O and 0.25 μCi of [^{14}C]PEG, were incubated for 5 min. After centrifuging at 16,000 × *g* for 16 min, 20 μl of supernatant and the pellet (removed by cutting the bottom tip of the tube) were counted in a liquid scintillation system. The remaining 6 ml of cell suspension was used to determine the dry weight.

Cation intracellular contents. Cation contents of the cells were determined as described previously (19). Samples of cells were filtered, washed with 20 mM $MgCl_2$, and treated with acid, and the cations were analyzed by atomic absorption spectrophotometry.

Cation fluxes. (i) Influx experiments. Influx assays were performed as described previously (19, 20). Cells were potassium starved for 6 h in medium without K^+, harvested by centrifugation, washed with cold water, and resuspended at an OD of 0.2 to 0.3 in Tris-citrate buffer (30 mM Tris, 20 mM $MgSO_4$, 2 mM $CaCl_2$, and 2% [wt/vol] glucose brought to pH 4.5 with citric acid). At time zero, RbCl was added at the required concentration. Samples were taken periodically, filtered, and treated as for the determination of cation content. NaCl was added simultaneously with RbCl when required. In short-time experiments, uptake was linear with time and initial rates of uptake were obtained from the slope of this line.

(ii) Efflux experiments. (a) Li^+ efflux. Cells were incubated in medium with 20 μM K^+ containing 300 mM LiCl in order to load the cells with lithium. After 3 h of incubation, the cells were harvested and resuspended in medium with 20 μM or 10 mM K^+ but without Li^+. Samples taken were filtered, washed, and acid extracted, and lithium was analyzed in the extracts (23).

(b) $^{22}Na^+$ efflux. Cells were centrifuged and washed twice with cold water. The final pellet was resuspended in 5 ml of cold water with a cell concentration 40 to 50 mg (dry weight) ml^{-1} and kept on ice. Cell suspensions (400 μl) were added to the same volume of the assay buffer {5 mM MES [2-(*N*-morpholino)ethanesulfonic acid]–5 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] brought to pH 7.0 with Tris} (10) with 2% (wt/vol) glucose at 25°C. At time zero, 100 μl of ^{22}NaCl (5 μmol, 3 Bq) was added, and after 30 min 50 mM KCl was added to trigger the sodium efflux process. Samples for determination of intracellular and extracellular ^{22}Na were taken at defined time intervals. For intracellular ^{22}Na contents, 10 μl of cell suspension was filtered and washed with 10 ml of cold water and filters were transferred to gamma counter tubes containing 3 ml of water each. Simultaneously, 20 μl of cell suspension was centrifuged to measure extracellular ^{22}Na. Ten microliters of the supernatant was collected in a gamma counter tube containing 3 ml of water.

Reproducibility of results. All experiments were performed at least three times. Mean values or results of typical experiments are presented, as indicated.

Materials. Radioactive materials were from Amersham, and all other chemicals were from Merck. In all cases the water used was filtered through a Millipore Ultra Pure water system.

RESULTS

Comparison of the effects of different cations on growth of *D. hansenii* and *S. cerevisiae*. We used batch growth experiments in mineral medium in the presence of NaCl or LiCl to compare *D. hansenii* and *S. cerevisiae* (Fig. 1). *S. cerevisiae* was more affected than *D. hansenii*. A curve for the effect of KCl on *D. hansenii* is also included, showing that Na^+ and K^+ have similar effects on this yeast, as has been previously shown (16). Li^+, which has been used as an analog of Na^+ in *S. cerevisiae* in previous studies, inhibited both yeasts but was relatively more toxic for *S. cerevisiae*. In *S. cerevisiae* the ratio between the concentrations of Li^+ and Na^+ that reduced the specific growth rate by 50% was 23; this value was only 5 in *D. hansenii*, indicating that different mechanisms of toxicity are probably involved. In *D. hansenii* neither Na^+ nor K^+ disturbed growth for concentrations up to 1 M (there was even a stimulation at 0.5 M), while Li^+ was toxic at all concentrations tested. The results of a set of experiments performed at low K^+ concentrations are shown in Table 1, where the last row indicates values for standard medium, in which the concentration of K^+ is 36.7 mM. In the absence of NaCl, both yeasts were able to grow in the presence of low concentrations of K^+, although at significantly lower rates. As the concentration of Na^+ increased, *S. cerevisiae* was unable to grow at a low concentration of K^+, as previously demonstrated (4). In the case of *D. hansenii*, the presence of 1 M NaCl significantly relieved the effect of low K^+. The final biomass reached by these cultures also increased in the presence of 1 M NaCl. For example, this value was 1.67 mg ml^{-1} in cells grown with 500 μM K^+ without Na^+ and 2.02 mg ml^{-1} in cells grown with 500 μM K^+ and 1 M Na^+.

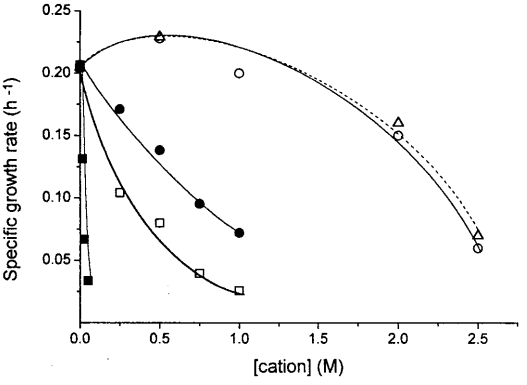


FIG. 1. Effect of different cations on the specific growth rates of *D. hansenii* (open symbols) and *S. cerevisiae* (filled symbols). Cells were inoculated in mineral medium containing the required concentration of LiCl (squares), NaCl (circles), or KCl (triangles and broken line).

Determination of the cation content of *D. hansenii*. The results in Table 1 are consistent with the hypothesis that Na^+ can substitute for K^+ in *D. hansenii*. To test this hypothesis, the cation contents of the cells were evaluated. Figure 2 summarizes the intracellular concentrations of K^+ and Na^+ for cells grown in media with different concentrations of the two cations. To calculate intracellular concentrations, the values of intracellular volume shown in the legend to Fig. 2 were used. With 1 and 2 M NaCl in the medium, the intracellular concentration of Na^+ was higher than that of K^+.

Assessment of the ability to extrude Na^+ and Li^+. One mechanism that could explain the high resistance of *D. hansenii* to NaCl could be a high ability to extrude the toxic cation. In *S. cerevisiae*, sensitivity has been associated with deletion of the *ENA* genes responsible for Na^+ extrusion (8). Two types of experiments were performed. Figure 3 shows the release of Li^+ from cells that were first loaded in 300 mM LiCl and resuspended in medium without Li^+ and with 20 μM KCl. The efflux rate constant (15 μM min^{-1}) was of the same order of magnitude as that previously reported for *S. cerevisiae* (78 μM min^{-1}) (22). The presence of a high K^+ concentration (10 mM) in the medium did not significantly change the characteristics of the efflux process (not shown). Figure 3 also compares the ability of *D. hansenii* and *S. cerevisiae* to extrude ^{22}Na. Cells were loaded with ^{22}Na in the presence of 2% (wt/vol) glucose, and the release of ^{22}Na was triggered by the addition of 50 mM KCl. The kinetics of extrusion was similar in both species.

Evaluation of the effect of Na^+ on the uptake of Rb^+. K^+ transport affects sodium tolerance (7) in *S. cerevisiae*. One hypothesis to explain the high tolerance is that a high-capacity K^+ transport system exists in *D. hansenii*. This hypothesis was weakened by the observation that when *D. hansenii* cells grown at millimolar K^+ concentrations (36.7 mM) were suspended in a 20 μM potassium medium, they lost more K^+ than did *S. cerevisiae* cells, and after 4 h the residual K^+ concentration in the medium was higher in the case of *D. hansenii* than in *S. cerevisiae*. These results suggest that the K^+ transport system of *D. hansenii* is no more efficient than that of *S. cerevisiae* (results not shown).

TABLE 1. Specific growth rates of *D. hansenii* and *S. cerevisiae* cells growing at different K^+ and Na^+ concentrations in mineral medium

$[K^+]_e$ (mM)[a]	Specific growth rate (h^{-1}) in the presence of:							
	0 M NaCl		0.6 M NaCl		1 M NaCl		2 M NaCl	
	D. hansenii	*S. cerevisiae*	*D. hansenii*	*S. cerevisiae*	*D. hansenii*	*S. cerevisiae*	*D. hansenii*	*S. cerevisiae*
0.00	0.02	0.01	0.03	—[b]	0.13	—	—	—
0.05	0.10	0.17	0.13	—	0.18	—	0.08	—
0.50	0.17	0.18	0.19	0.06	0.20	—	0.12	—
36.7	0.21	0.20	0.22	0.13	0.21	0.10	0.14	—

[a] $[K^+]_e$, external $[K^+]$.
[b] —, no growth.

In preliminary experiments we observed that in *D. hansenii*, K^+ was a competitive inhibitor of Rb^+ uptake, as in *S. cerevisiae*, indicating that Rb^+ is a good analog of K^+ for transport studies (data not shown). Analysis of the kinetic parameters of Rb^+ uptake in K^+-starved cells of *D. hansenii* and *S. cerevisiae*, pH 4.5, shows that the uptake system for Rb^+ of *S. cerevisiae* (V_{max}, 13 nmol mg [dry weight]$^{-1}$ min^{-1}; K_m, 1.1 mM) was more effective than that of *D. hansenii* (V_{max}, 4.2 nmol mg [dry weight]$^{-1}$ min^{-1}; K_m, 7.4 mM).

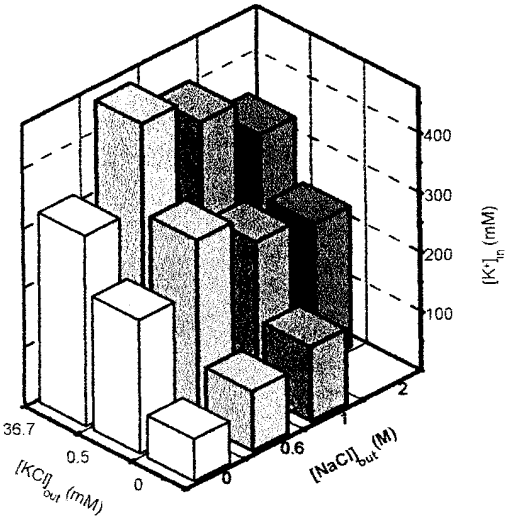
Although Na^+ is a competitive inhibitor of Rb^+ transport in *S. cerevisiae*, preliminary experiments indicated that in *D. hansenii*, Na^+ stimulated Rb^+ uptake. The presence of 50 mM NaCl in the Rb^+ uptake assay resulted in a significant increase of the affinity of the transporter for Rb^+ (the K_m for Rb^+ changed from 7.4 to 3.7 mM). We chose concentrations close to the K_m, 5 and 2 mM, to perform Rb^+ uptake assays in, respectively, *D. hansenii* and *S. cerevisiae* in the presence of different concentrations of NaCl (Table 2). The stimulation was clear in *D. hansenii*, 50 mM NaCl being more effective than 25 or 100 mM, while inhibition was observed at all concentrations tested in *S. cerevisiae*.

The situation was different when LiCl was present in the assay: in *D. hansenii*, Li^+ inhibited Rb^+ uptake (Table 2), indicating that different mechanisms are involved in the actions of Na^+ and Li^+ in *D. hansenii*.

DISCUSSION

The serendipitous observation that *D. hansenii* was able to grow in the absence of K^+ if Na^+ was present led to the research described in this report. *D. hansenii* was more tolerant than *S. cerevisiae* of Na^+ and Li^+ under all conditions tested; the toxicities of Na^+ and K^+ were similar in *D. hansenii*. This result suggests that in this yeast there is no specific toxicity associated with Na^+ other than the osmotic effect. In *S. cerevisiae* the situation is different. Wieland et al. (32) presented evidence that the toxic levels of Na^+ are much lower than those of K^+ for *S. cerevisiae*. Our observation that Li^+ was relatively more toxic to *S. cerevisiae* than to *D. hansenii* suggests that different resistance mechanisms are involved. Although it may be appropriate to consider Li^+ a transport analog of Na^+ in *S. cerevisiae* and *D. hansenii*, this analogy may not hold for toxicity. It has been recently reported that Li^+ may inhibit some steps in the metabolism of either phosphoinositide (27) or sulfate (15).

We showed that in media with low K^+ and high Na^+ concentrations (1 M), *D. hansenii* was able to grow at rates close to that obtained in standard medium and accumulated high levels of Na^+ in the cytoplasm (Table 1). This result also supports the hypothesis that Na^+ is not toxic for *D. hansenii*. The presence of 0.6 M Na^+ in the medium led to an increase in the level of intracellular K^+ (Fig. 2), which was not significantly affected by further increase in the concentration of Na^+; while the level of Na^+ increased continuously, the performance of the cells was not seriously affected. Indeed, it has been shown that in *S. cerevisiae*, Na^+ can substitute partially



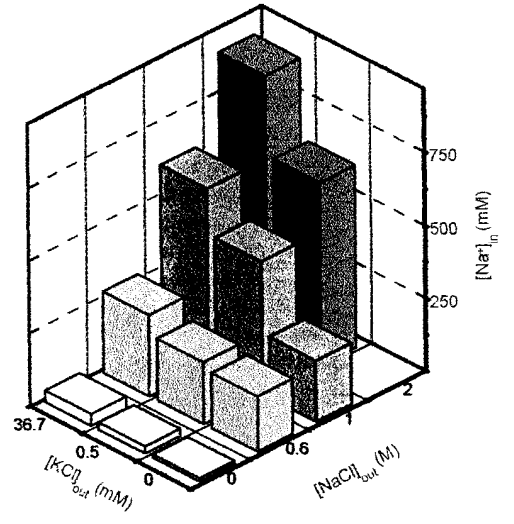


FIG. 2. K^+ and Na^+ contents of *D. hansenii* grown in media with different concentrations of KCl and NaCl. Cells were harvested by filtration at mid-exponential phase, washed, and treated with acid. K^+ and Na^+ were analyzed by atomic absorption spectrophotometry. The values of intracellular volume used to calculate K^+ and Na^+ concentrations were 1.5, 1.2, 0.95, and 1.0 μl mg (dry weight)$^{-1}$ in the presence of 0, 0.6, 1, and 2 M NaCl, respectively.

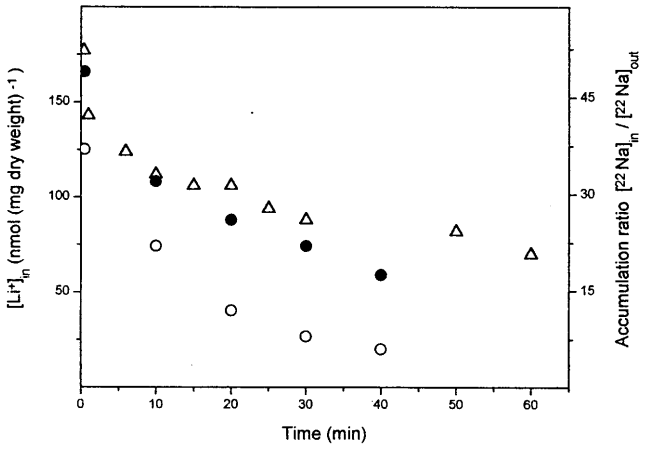


FIG. 3. Li^+ efflux in *D. hansenii* (△) and ^{22}Na efflux in *D. hansenii* (○) and *S. cerevisiae* (●). To study Li^+ efflux, cells were resuspended in a medium free of LiCl after preloading for 3 h in 300 mM LiCl. In the case of Na^+, cells were grown in mineral medium containing 300 mM NaCl, harvested, and resuspended for 30 min in buffer (MES-PIPES-Tris, pH 7.0) containing 2% (wt/vol) glucose and $^{22}NaCl$. The release of ^{22}Na was triggered by the addition of 50 mM KCl.

for K^+, but this phenomenon occurs in a narrow range of Na^+ concentrations and Na^+ becomes toxic above a certain Na^+/K^+ cellular ratio (0.7 to 1) (4).

Since *ENA* genes, responsible for Na^+ resistance in *S. cerevisiae*, code for ATPases able to extrude Na^+, we tested both yeasts for their ability to extrude Na^+. Our data suggest that the efficiency of these mechanisms was similar in both yeasts and that Na^+ extrusion alone is insufficient to explain the high Na^+ tolerance of *D. hansenii*.

One mechanism that has been proposed to explain Na^+ toxicity in *S. cerevisiae* is the competitive inhibition of K^+ uptake, leading to K^+ depletion in the cell and an increased level of sodium (7). We present two forms of evidence that the efficiency of the *D. hansenii* transporter was no better than that of the *S. cerevisiae* transporter: (i) *D. hansenii* lost more K^+ than *S. cerevisiae* when cells grown at high (millimolar) K^+ concentrations were resuspended in a low K^+ medium, and (ii) the kinetic parameters for Rb^+ uptake do not reflect a better capacity of transport in *D. hansenii* cells. We suggest that an important difference between these yeasts is the effect of Na^+ on the potassium transporter. In *D. hansenii*, Na^+ stimulated Rb^+ uptake by increasing the affinity of the transport system. For concentrations of Rb^+ close to K_m, Na^+ consistently inhibited the transport of Rb^+ in *S. cerevisiae*, while a clear stimulation was observed in *D. hansenii* (Table 2). This observation is consistent with those of Norkrans and Kylin (17) that much higher concentrations of NaCl are necessary to inhibit transport of K^+ in *D. hansenii* than in *S. cerevisiae*, results which might be explained on the basis of the activity of a putative K^+-Na^+ cotransport system, as has been recently proposed for wheat root (26). Norkrans and Kylin suggested that the higher tolerance of *D. hansenii* is a result of more efficient Na^+ extrusion and K^+ uptake. Their conclusions are based on long-term transport experiments in which the kinetics are difficult to compare. We think that their results suggest different regulation of the activities of the K^+ uptake and Na^+ extrusion transporters in *D. hansenii* and *S. cerevisiae*. We have found that although *D. hansenii* shows a Na^+ efflux process and there is a role for the uptake of potassium in salt tolerance, the main difference between both yeasts is that intracellular sodium is not toxic to *D. hansenii*.

TABLE 2. Effect of Na^+ and Li^+ on the velocity of Rb^+ uptake in *D. hansenii* and *S. cerevisiae*

Yeast	Velocity of Rb^+ uptake (nmol mg^{-1} min^{-1})[a]						
	Control[b]	[NaCl] (mM)			[LiCl] (mM)		
		25	50	100	10	25	50
D. hansenii	1.6	3.3	4.5	3.5	1.1	1.0	0.3
S. cerevisiae	8.7	7.2	4.4	2.7	—[c]	—	—

[a] The concentrations of RbCl used to study Rb^+ uptake were 5 and 2 mM in *D. hansenii* and *S. cerevisiae*, respectively.
[b] Control in the absence of Na^+ and Li^+.
[c] —, not determined.

The results obtained with Li^+ (Table 2), which inhibited Rb^+ uptake at all concentrations tested, may explain some of the differences observed in the effect on growth of Na^+ and Li^+, although, as mentioned above, a different mechanism could be involved in Li^+ toxicity.

We have identified two characteristics that may help explain why *D. hansenii* is more tolerant of Na^+ than is *S. cerevisiae*. The Na^+ content in *D. hansenii* can be quite high without significantly affecting yeast growth. The biochemical basis underlying this behavior remains to be elucidated and must be complex, since both catabolism and anabolism must occur at high salt concentrations. The activation of K^+ uptake by Na^+, at least when K^+ is present at low levels, may provide *D. hansenii* with a competitive advantage in many natural environments where K^+ is scarce and Na^+ is abundant. To date, several yeasts, including *D. hansenii*, have been considered halotolerant. Based on our present results, *D. hansenii* is probably halophilic in natural environments where the concentration of K^+ is usually much lower than that of Na^+.

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