Regulation of Salt Tolerance by *Torulaspora delbrueckii* Calcineurin Target Crz1p

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Recently, the academic interest in the yeast *Torulaspora delbrueckii* has increased notably due to its high resistance to several types of stress, including salt and osmotic imbalance. However, the molecular mechanisms underlying these unusual properties are poorly understood. In *Saccharomyces cerevisiae*, the high-salt response is mediated by calcineurin, a conserved Ca\(^{2+}\)/calmodulin-modulated protein phosphatase that regulates the transcriptional factor Crz1p. Here, we cloned the *T. delbrueckii* TdCRZ1 gene, which encodes a putative zinc finger transcription factor homologue to Crz1p. Consistent with this, overexpression of TdCRZ1 enhanced the salt tolerance of *S. cerevisiae* wild-type cells and suppressed the sensitivity phenotype of cnb\(\Delta\) and crz\(\Delta\) mutants to monovalent and divalent cations. However, *T. delbrueckii* cells lacking TdCrz1p showed phenotypes distinct from those previously observed in *S. cerevisiae* crz\(\Delta\) mutants. Quite remarkably, TdCrz1-null cells were insensitive to high Na\(^+\) and were more Li\(^+\) tolerant than wild-type cells. Clearly, TdCrz1p was not required for the salt-induced transcriptional activation of the TdENA1 gene, encoding a putative P-type ATPase homologue to the main *S. cerevisiae* Na\(^+\) pump ENA1. Furthermore, *T. delbrueckii* cells were insensitive to the immunosuppressive agents FK506 and cyclosporine A, both in the presence and in the absence of NaCl. Signaling through the calcineurin/Crz1 pathway appeared to be essential only on high-Ca\(^{2+}\)/Mn\(^{2+}\) media. Hence, *T. delbrueckii* and *S. cerevisiae* differ in the regulatory circuits and mechanisms that drive the adaptive response to salt stress.

Exposure of cells to saline stress implies both a specific cation toxicity and osmotic stress. Sodium and lithium ions are particularly toxic to the cells of most living organisms because of their ability to inhibit specific metabolic pathways. Therefore, regulation of intracellular ion content is a primary issue in the cellular reprogramming of almost all organisms subjected to salt stress (4, 58).

The high degree of evolutionary conservation of stress pathways between higher eukaryotes and *Saccharomyces cerevisiae* and the genetic advantages of budding yeast have made this organism a model system for studying stress responses (24). Yeast genes involved in salt tolerance have been identified by the ability to protect cells at increasing gene dosage or by the growth defects of yeast mutants at elevated ion concentrations (53). Thus, studies with yeast have covered basic mechanisms of ion homeostasis and have identified key genes in the maintenance of a high K\(^{+}\)/Na\(^{+}\) ratio (48, 54). Protein kinases and signaling pathways involved in salt responses have been also identified and characterized (52). Despite these advances, we are far from completely understanding the mechanisms, the nature of signaling pathways, and the functions of gene targets that allow cells to adapt to salt stress. Moreover, there is evidence that signaling pathways and stress responses have evolved in different organisms, including yeasts, in a niche-dependent manner (8, 59). It is clear, for example, that *S. cerevisiae* is not the best model of a salt-resistant microorganism. Nonconventional yeasts such as *Zygosaccharomyces rouxi*, *Debaryomyces hansenii*, and *Torulaspora delbrueckii* are by far more resistant to the combined effects of ion toxicity and osmotic stress (11, 32). Thus, the identification and characterization of the cellular mechanisms regulating salt tolerance in these non-*Saccharomyces* species are of major interest.

In *S. cerevisiae*, toxic concentrations of Na\(^+\) and Li\(^+\) promote their extrusion by induction and activation of the specific ATP-driven ion pump Ena1p (40). ENA1 expression is regulated by two different signaling pathways, the HOG (for “high osmolarity glycerol”) pathway (64), one of the five known mitogen-activated protein kinase cascades in *S. cerevisiae* (16), and the calcineurin/Crz1p pathway (19). Calcineurin is a highly conserved Ca\(^{2+}\)/calmodulin-dependent Ser/Thr protein phosphatase of type 2B (30, 49). In its native form, calcineurin is present as a heterodimer containing a catalytic subunit, encoded by the functionally redundant genes CN1A1 and CN42, complexed with a regulatory subunit, the product of *CNBI*. The phosphatase activity of calcineurin is dispensable for growth under standard conditions. However, *cnal1* *cnal2* or *cnbl1* mutants show decreased tolerance to Na\(^+\)/Li\(^+\), Mn\(^{2+}\), and OH\(^-\) ions (41, 43, 44). Calcineurin is also required for escape from cell cycle arrest after exposure to pheromone (20, 21) and plays an important role in regulating cell wall structure (22, 25).

When cells are exposed to salt stress, cytosolic Ca\(^{2+}\) levels rise, inducing its binding to calmodulin. This interaction promotes a conformational change in calmodulin, allowing it to bind and activate calcineurin (39), which in turn dephosphorylates the transcriptional factor Crz1p (41, 61). Dephosphorylation of Crz1p causes its nuclear import (62) and binding to a...
consensus DNA sequence (42), the calcineurin-dependent response element (CDRE) (61), found in the promoter of most salt-responsive genes (67). In consonance with this, cells lacking Crz1p display hypersensitivity to α-factor, Mn²⁺, or Li⁺ (41, 61). Nevertheless, crz1 and calcineurin mutant cells show opposite phenotypes under specific conditions, such as exposure to Ca²⁺ and OH⁻ ions (41, 42). This observation strongly suggests that calcineurin regulates additional yeast proteins (19). It is also possible that Crz1p might respond to signals other than those driven by calmodulin-calcineurin. Whether this signaling pathway plays a similar role in other yeasts, other than those driven by calmodulin-calcineurin, is unknown.

Recently, homologues to S. cerevisiae Crz1p have been identified in Schizosaccharomyces pombe (34) and Candida albicans (46). Proteins with some degree of similarity to Crz1p have also been found in Candida glabrata and Kluyveromyces lactis through the Genolevures sequencing project (available at http://cibi.labri.fr/Genolevures/index.php). Among these, only the prl1+ and CaCRZ1 genes from S. pombe and C. albicans, respectively, have been studied in detail (34, 46, 51). Like Crz1p, Prl1p and CaCRZ1 act downstream of calcineurin and regulate Ca²⁺ homeostasis (34, 51). However, S. pombe prl1+ and C. albicans crz1/crz1 defective strains show phenotypes distinct from those observed in S. cerevisiae crz1 mutants (34, 46). Hence, calcineurin and Crz1p homologues in fission yeast and C. albicans appear to play functional roles that are not shared by the S. cerevisiae pathway.

In this work we took advantage of the salt-sensitive phenotype of the S. cerevisiae strain CEN.PK2-1C to identify genes from T. delbrueckii that confer increased salt tolerance. Using this strategy, we cloned the TdENA1 and TdCRZ1 genes, which encode a putative Na⁺/Li⁺ P-type ATPase and a zinc finger protein homologue to S. cerevisiae Crz1p, respectively. As expected, T. delbrueckii cells lacking TdCrz1p showed some phenotypes similar to those reported for S. cerevisiae calcineurin and crzΔΔ mutant strains. However, lack of the transcriptional factor in T. delbrueckii led to enhanced resistance to Li⁺, while no growth defects were observed at high Na⁺ concentrations. Furthermore, T. delbrueckii cells did not show the same calcineurin dependency in response to saline stress as that previously reported for S. cerevisiae. These results suggest that salt stress in T. delbrueckii is regulated differently, through uncovered regulators and molecular circuits.

### MATERIALS AND METHODS

#### Strains, culture media, and general methods.

T. delbrueckii wild-type strain PYCC5321 (1) and S. cerevisiae strains (Table 1) were used throughout this work. The T. delbrueckii Tdcrz1Δ mutant strain (MJHY211) was constructed as described below. Cells were cultured at 30°C in defined medium, YPD (1% yeast extract, 2% peptone, 2% glucose) or SD (0.2% yeast nitrogen base without amino acids [Difco], 0.5% (NH₄)₂SO₄, 2% glucose), supplemented with the appropriate auxotrophic requirements (55). Escherichia coli was grown in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, 0.5% NaCl) supplemented with ampicillin (50 μg/ml). Antibiotics were filter sterilized and added to autoclaved medium. Transformation of yeasts was performed by the lithium acetate method (37). T. delbrueckii transformants containing the nourseothricin resistance module (natMX4) were grown for 4 h in YPD at 30°C before being plated on YPD agar plates containing 10 μg/ml of nourseothricin (donated by Werne Bioagents, Germany). E. coli was transformed by electroporation according to the manufacturer’s instructions (Eppendorf).

**Stress sensitivity tests.** For stress experiments, cells were grown at 30°C to mid-exponential phase, collected, and transferred to fresh medium containing the stressor to be tested at the indicated concentration. Plate phenotype experiments were made by diluting the cultures to an optical density at 600 nm (OD₆₀₀) of 0.3 and spotting (3-μl) 10-fold serial dilutions onto SD or YPD agar solid medium containing NaCl, LiCl, MnCl₂, or CaCl₂. FK506 (Fujisawa GmbH) (20 μg/ml in 90% ethanol-10% Tween 20) was added at the indicated concentrations on solid and liquid media. Cyclosporine A (10 μg/ml in ethanol) was purchased from Sigma (St. Louis, MO). Unless otherwise indicated, colony growth was inspected after 2 to 4 days of incubation at 30°C.

#### Strain and plasmid construction.

Plasmids pAM5345 (61) and pJQ10 (9), containing the S. cerevisiae CRZ1 and ENA1 genes, respectively, were a gift from M. Cyert and A. Rodriguez-Navarro. Plasmids pJH1 and pMH14, carrying DNA fragments containing the ENA1 and CRZ1 genes from T. delbrueckii, TdEna1 and TdCrz1, respectively, and flanking regions around these genes were isolated from a genomic library (31) by complementation in S. cerevisiae of the salt sensitivity phenotype of strain CEN.PK2-1C. To construct plasmid YEpTdENA1, the PstI/SpeI fragment released from plasmid YEpMH14, containing the coding region of the TdENA1 gene and the 3' and 3' noncoding regions, was moved into vector YEpLac195 (26). Plasmid YEpTdCRZ1, containing the isolated TdCRZ1 gene, was constructed by cloning a 1,980-bp SacI/EcoRI fragment from plasmid YEpMH14 into the vectorYEpLac195 (26), previously digested with EcoRI/SmaI. The TdCRZ1 disruption cassette containing the nourseothricin-resistance module natMX4 (27) was constructed by restriction. A 5'-side fragment of TdCRZ1 (+1332 to +1810) was obtained by PCR using two specific primers, FR142 and FR141 (Table 2), and plasmid YEpMH14 as a template. The PCR product was cloned into the pGEM-T Easy vector (Promega), released by restriction with SalI/EcoRI, and inserted into the pBSCRZ (Stratagene), previously digested with the same set of enzymes. The resulting plasmid, pBSCRZ, was treated with BamHI/EcoRI and used to accommodate the natMX4 module obtained from the BamHI- and EcoRI-digested plasmid pAG25 (27), creating plasmid pBSCRZ-natMX4. A PCR fragment was amplified from the 5' region of TdCRZ1 (~270 to +448) using oligonucleotides FR140 and FR139 (Table 2) and plasmid YEpMH14 as a template. The PCR product was inserted into the pGEM-T Easy vector, released with NotI and SpeI, and subcloned into plasmid pBSCRZ-natMX4, obtaining plasmid pBSCRZ-natMX4-CRZ. This was digested with EcoRV, releasing the TdCRZ1 disruption cassette, which contains the natMX4 module flanked by 718 and 488 bp (5' and 3' sides, respectively), homologues to the TdCRZ1 gene.

Correct disruption of the TdCRZ1 gene was detected by diagnostic PCR using whole yeast cells (36) from isolated colonies and a set of oligonucleotides designed to bind outside or inside of the disrupted TdCRZ1 sequence and within the marker module (Table 2).

**β-Galactosidase assay.** Exponentially SD-growing cells (OD₆₀₀, 0.6 to 0.8) were collected, resuspended in YPD (pH 5.5) or the same medium supplemented with 0.2 M CaCl₂ and incubated at 30°C and 200 rpm for 45 min. Then, aliquots of the yeast suspension (15 OD₆₀₀ units) were harvested, washed with Z buffer (60 mM Na₂HPO₄, 10 mM KCl, 1 mM MgSO₄), and centrifuged at 3,000 × g for 2 min (4°C), and the cell pellets were frozen at −20°C for further analysis. Cell extracts were prepared as previously described (14). Total protein was determined with the Bio-Rad Bradford assay kit and bovine serum albumin as the standard protein. β-Galactosidase activity was

### Table 1. S. cerevisiae strains used in this study

| Strain | Relevant genotype | Source or reference |
|--------|-------------------|---------------------|
| CEN.PK2-1C | Matα ura3-52 his3-dΔ1 leu2-3,112 | 23 |
| YPH499 | Matα ura3-52 his2-801 ade2-101 | 57 |
| DD12 | Same as YPH499, except cnb1::hisG | 20 |
| ASY742 | Same as YPH499, except crz1::loxP-KanMX::loxP | 61 |
| ASY745 | Same as DD12, except crz1::loxP-KanMX::loxP | 61 |
| ASY832 | Same as YPH499, except ura3::TRP1-4x | 6x CDRE-lacZ | M. Cyert |
| ASY834 | Same as ASY742, except ura3::TRP1-4x | 6x CDRE-lacZ | M. Cyert |
| ASY835 | Same as ASY745, except ura3::TRP1-4x | 6x CDRE-lacZ | M. Cyert |
TABLE 2. Oligonucleotides used in this study

| Primer | Sequence (5’ to 3’) | Comment(s) |
|--------|---------------------|------------|
| FR55   | ATGACCATGATTACGCGCAA|
| FR77   | GGGCAGTGCATGCTGCCCTC|
| FR94   | TGCTACTGAAAGCAAGGG|
| FR103  | GCTTACGGCCAGGAAAAATT|
| FR136  | TGAATTCGGGTAAGAAAAGG|
| FR90   | CGAAGTCGACAGCTCAATCA|
| FR86   | ATGCTTTCGACTTCGCGC|
| FR110  | CCTCTTCTCGATTTGGACA|
| FR117  | CAGGATATCAAGGGTAAGCT|
| FR376  | AATGGTTCAGACGTCGC|
| FR377  | CCAGCTGATCACTTCGG|
| FR101  | CCTAAAGCCCAAACTATAACA|
| FR142  | CGAAGTCGACAGCTCAATCA|
| FR141  | TGAATTCGGTAAAGAGGGG|
| FR140  | ATTCGGATCTCTTGGAAAGG|
| FR139  | ATTCGGATCTCTTGGAAAGG|
| FR126  | TTCAGTGCGCAAGGGGACTAC|
| FR76   | GTCAAGGAGGGTATTCTGG|
| FR75   | AGTAAAGTCGAGCAAGG|
| FR96   | GCCTACGGCCAGGAAAAATT|
| FR121  | GCTGCACCAACAGACAAAG|
| FR390  | GTATGTTCTAGGCGCTTG|
| FR391  | TCTGGGATCCTACTACCT|

-Ndetermined at room temperature by using the substrate ONPG (o-nitrophenyl-\beta-D-galactopyranoside) as previously described (47). One unit is defined as the amount of enzyme that can be convert 1 nmol of ONPG per min under the assay conditions.

Northern blotting. Total RNA from T. delbrueckii cells was prepared as described previously (55). Equal amounts of RNA (10 μg) were separated in 1% (wt/vol) agarose gels containing formaldehyde (2.5% vol/vol), transferred to a nylon membrane, and hybridized with a 32P-labeled probe of TdENA1 (+90 to +1003). A fragment of the S. cerevisiae ACT1 gene (+10 to +1066) was used as the loading control. Probes were generated by PCR and radiolabeled with the random primer Ready-to-Go kit (Amersham Biosciences, Chalfont-St. Giles, England) and [α-32P]dCTP (Amersham Biosciences). Hybridization was carried out under standard conditions (55), except for the ACT1 probe. Briefly, after hybridization overnight at 35°C, the filters were rinsed once with 50 ml of 2× SSC–0.1% sodium dodecyl sulfate at room temperature for 20 and 10 min, respectively. Filters were exposed to a high-resolution BAS-MP 2040S imaging plate (Fuji, Kyoto, Japan) for 24 h and scanned in a phosphorimager (FLA-3000; Fuji). Spot intensities were quantified with Image Gauge software, version 3.12 (Fuji). Values of spot intensity were corrected with respect to the ACT1 mRNA level and represented as the relative mRNA level. The highest relative TdENA1 mRNA level for each sample analyzed was set to 100.

Sequencing and sequence analysis. DNA sequencing was performed on both strands by the dyeideo chain termination procedure (50). Analysis of sequence data was carried out with DNAMAN sequence analysis software (Lynnon BioSoft). Similarity searches were performed using BLAST software (5) at the Munich Information Center for Protein Sequences (http://mips.gsf.de/). TdENA1p and TdCrz1p domains were searched by scanning protein sequences in the ExPASy Molecular Biology Server (http://www.expasy.ch/) from the Swiss Institute of Bioinformatics (http://www.isb-sib.ch) against the PROSITE database of protein families (56). Multiple sequence alignment was done with MultiAlin software (15) at INRA (http://prodes.toulouse.inra.fr/).

Nucleotide sequence accession numbers. The nucleotide sequences of TdCrz1 and TdENA1 have been deposited in the GenBank database (available at http://www.ncbi.nlm.nih.gov/GenBank/index.html) under accession numbers DQ097180 and DQ097181, respectively.

RESULTS

Isolation of T. delbrueckii genes that confer increased salt tolerance in S. cerevisiae. We transformed cells of the S. cer-

FIG. 1. Multiple copies of TdENA1, encoding a P-type ATPase, enhance NaCl resistance in S. cerevisiae. (A) Schematic structure of TdENA1p showing the conserved domains for N-terminal cation transporting ATPase (Cation_ATPase_N), C-terminal cation transporting ATPase (Cation_ATPase_C), and the ATPase-associating region, and transmembrane segments (shown as vertical boxes). (B) Mid-exponential-phase cultures of the S. cerevisiae strain CEN.PK2-1C transformed with plasmid YEptTdENA1 (TdENA1), plasmid pJQ10 (ENA1), or the empty plasmid YEplac195 (wt UR43). Plates were adjusted to an OD600 of 0.3, diluted (1 to 10-1), and spotted (3 μl) onto SD plates or SD plates containing NaCl at the indicated concentration. Plates were inspected after 2 to 5 days at 30°C. A representative experiment is shown.
evisiae strain CEN.PK2-1C with a high-copy-number genomic library from T. delbrueckii (31). This Saccharomyces strain is very sensitive to saline stress and therefore is a good recipient to detect genes that could confer salt tolerance. After transformation, 18 yeast colonies were isolated, purified, and confirmed on SD medium plates containing NaCl at 0.5 M, a salt concentration that inhibits the growth of the host strain. Plasmid restriction analysis established four plasmid groups that were confirmed by dot blot analysis (data not shown). Two of them were studied in detail in this work. The first, named YEpMJH1, permitted identification of a 3,273-bp open reading frame (ORF) (GenBank accession number DQ097181) that encodes a putative polypeptide closely similar to Ena proteins isolated from other yeasts, such as D. hansenii (55% identity), Schwanniomyces occidentalis (56%), Z. rouxii (67%), and S. cerevisiae (69%). These proteins belong to the large P-type ATPase family, subfamily IID, whose members perform active ion transport across biological membranes (7, 10). Consistent with this, the putative protein identified in plasmid YEpMJH1 was found to contain the typical ATPase/H9251 chains involved in Na+/H11001 and K+/H11001 transport and responsible for ATP hydrolysis (13), the E1-E2 ATPase domain characteristic of the superfamily P-type ATPases (60), and one hydrolase and 10 transmembrane domains (Fig. 1A).

In S. cerevisiae, three isoforms of Ena proteins (encoded by the ENA1, ENA2, and ENA5 genes) have been characterized. Among them, ENA1 encodes the main ATPase involved in Na+ extrusion, whose function determines tolerance to NaCl (28). Therefore, we tried to further confirm the identity of the gene contained in plasmid YEpMJH1. A PstI/SpeI restriction fragment was subcloned into the vector YEplac195 (26) and used to transform the wild-type strain CEN.PK2-1C. As expected, overexpression of Td ENA1 conferred an increased growth ability to yeast cells on NaCl medium (Fig. 1B). Moreover, the phenotype was similar to that observed in high-copy-number expression of the S. cerevisiae ENA1 gene. Thus, the ORF identified in plasmid YEpMJH1 was named Td ENA1, the ENA1 gene from T. delbrueckii.

DNA sequencing of the second plasmid analyzed in this work, YEpMJH14, revealed a 1,518-bp ORF (GenBank accession number DQ097180) that encodes a protein of 506 amino acids with overall 36% identity to S. cerevisiae Crz1p (41, 61). Accordingly, the gene was designated Td CRZ1. As shown in Fig. 2A, the gene product of Td CRZ1 contains three C2H2-type zinc finger motifs at the carboxyl terminus that are highly homologous to those of Crz1p and Prz1p (Fig. 2B). Like S. cerevisiae Crz1p and S. pombe Prz1p, the protein from T. delbrueckii displayed a serine-rich region (residues 41 to 90) (Fig. 2A) essential for protein dephosphorylation by calcineurin (62). Inspection of the protein sequence also showed the presence of a PVISVQ sequence, similar to the calcineurin-docking

FIG. 2. Td CRZ1 encodes the homologue to the transcriptional factor Crz1p, and its overexpression in S. cerevisiae confers enhanced salt tolerance. (A) Schematic representation of Td Crz1p. Denoted are the serine-rich region (SRR), the calcineurin-docking domain (CDD) and three putative C2H2-type zinc finger motifs at the carboxyl terminus. (B) Sequence alignment of the three zinc finger motifs from Td Crz1p, Crz1p, and Prz1p. Residues conserved in at least two sequences are boxed and highlighted. (C) Cells of the S. cerevisiae CEN.PK2-1C wild-type strain were transformed with plasmid YEpTdCRZ1 (Td CRZ1), plasmid pAMS354 (CRZ1), or the empty plasmids YEplac195 (wt URA3) and YEplac181 (wt LEU2). Mid-exponential-phase SD-grown cultures were adjusted to an OD900 of 0.3, diluted (1 to 103), and spotted (3 μl) onto SD plates or SD plates containing NaCl or MnCl2 at the indicated concentrations. Plates were inspected after 2 to 5 days at 30°C. A representative experiment is shown.
domains (Fig. 2A) defined in Crz1p (12) and human nuclear factors of activated T cells (NFAT) (5).

In order to confirm that the gene present in plasmid YEp MJH14 was responsible for the enhanced salt resistance of the S. cerevisiae strain CEN.PK2-1C, we constructed plasmid YEpTdCRZ1 by subcloning a 1,980-bp fragment containing the whole ORF plus 349 bp of the promoter region and 107 bp corresponding to the 3′ untranslated region into plasmid YEplac195 (26). As shown in Fig. 2C, overexpression of TdCRZ1 in the S. cerevisiae recipient strain produced a moderate increase in Na+ tolerance compared to the strain harboring an empty plasmid. These effects were more pronounced when Mn2+ tolerance was tested. Indeed, in SD medium, the CEN.PK2-1C strain transformed with YEp TdCRZ1 grew to 20 mM MnCl2, whereas the control strain showed only residual growth (Fig. 2C). Similar results were observed in transformant cells harboring plasmid pAMS345 (61), which affords high-copy-number expression of the S. cerevisiae CRZ1 gene (Fig. 2C). Hence, our results indicate that plasmid YEpMJH14 indeed contains the T. delbrueckii CRZ1 gene, the homologue to the transcriptional factor Crz1p, whose overexpression in S. cerevisiae confers enhanced tolerance to Na+ and Mn2+ ions.

TdCRZ1 suppresses the ion sensitivity of S. cerevisiae cnb1Δ and crz1Δ mutants. Overexpression of S. cerevisiae CRZ1 compensates for the enhanced ion sensitivity of yeast calcineurin mutants, specifically to Na+, Li+, and Mn2+ (41, 61). Therefore, we were interested in determining whether TdCrz1p could affect these calcineurin phenotypes. For this, cnb1Δ and crz1Δ mutant cells of the YPH499 strain (20, 61) were transformed with plasmid YEpTdCRZ1 and examined for growth on NaCl-, MnCl2-, and LiCl-containing medium. The wild-type strain YPH499 shows higher MnCl2 sensitivity than the CEN.PK-IC strain. Because of this, growth on this salt was inspected at 2.5 mM (final concentration). As shown in Fig. 3, production of the recombinant TdCrz1p increased Mn2+ tolerance of a cnb1Δ mutant to wild-type levels. Similar effects were observed in the presence of 0.4 M LiCl or 1.0 M NaCl. Moreover, overexpression of TdCRZ1 compensated for the ion sensitivity produced by the lack of Crz1p (Fig. 3). Like calcineurin mutants, crz1-null cells show retarded growth at high concentrations of Li+ /Na+ and Mn2+ cations (41, 61).

We also tested whether the production of TdCrz1p could activate the expression of a 4× CDRE::lacZ reporter, which contains four tandem copies of the 24-bp CDRE (38). Multiple copies of the CDRE increase the calcineurin-dependent transcriptional activation of the reporter gene (61). Thus, ninefold inductions were observed in wild-type cells carrying an integrated copy of the heterologous construct after 45 min of exposure to 0.2 M CaCl2 (Table 3). Consistent with previous reports (61), CDRE-driven expression was completely depen-
dent on the function of the calcineurin-Crz1p pathway. Thus, no significant β-galactosidase activity could be detected in S. cerevisiae cells lacking Crz1p or in the cnb1Δ crz1Δ double mutant transformed with an empty plasmid, YEpLac195 (Table 3). In contrast, overproduction of TdCrz1p in a crz1Δ-null background restored the CDRE-dependent transcriptional activation, although the induction level of β-galactosidase activity at high Ca^{2+} was lower, around fivefold, than that observed in the wild type. Nevertheless, the crz1Δ mutant transformed with plasmid pAMS435, which contains the S. cerevisiae CRZ1 gene, also showed lower induction, around threefold (Table 3). As expected, the addition of FK506 also impaired the β-galactosidase activity in Ca^{2+}-treated crz1Δ cells transformed with the S. cerevisiae CRZ1 gene (data not shown).

Tdcrz1Δ shows conserved phenotypes distinct from those of S. cerevisiae crz1Δ mutants. To clarify the function of TdCRZ1 in T. delbrueckii, we constructed a Tdcrz1-null mutant (MJH211 strain) and analyzed cells for phenotypes previously reported for S. cerevisiae calcineurin and crz1Δ mutants (41, 44, 61, 66). On YPD plates containing 10 mM MnCl₂ or 0.4 M CaCl₂, the level of β-galactosidase activity in YEpTdCRZ1 transformants of the crz1Δ strain treated with both Ca^{2+} and the drug FK506. The immunosuppressant FK506 is a potent inhibitor of calcineurin (6, 65) and has been used extensively for molecular studies in lower and higher eukaryotes (18, 29, 45). As shown in Table 3, addition of FK506 at 1 μg/ml decreased the Ca^{2+}-stimulated induction of β-galactosidase activity observed in the absence of the immunosuppressant, whereas at doses of 5 μg/ml, induction was eliminated altogether (Table 3). As expected, the addition of FK506 also impaired the β-galactosidase activity in Ca^{2+}-treated crz1Δ cells transformed with the S. cerevisiae CRZ1 gene (data not shown).

**FIG. 4.** Tdcrz1Δ cells exhibit specific phenotypes in response to diverse ionic stresses. (A) Exponentially growing cultures of the T. delbrueckii strains PYCC5321 (wild type [wt]) and MJH211 (Tdcrz1Δ) were adjusted to an OD₆₀₀ of 0.3, diluted (1 to 10⁻⁴), and spotted (3 μl) onto YPD agar medium. YPD adjusted to pH 8.0, or YPD supplemented with MnCl₂, CaCl₂, NaCl, or LiCl at the indicated concentrations. Plates were incubated at 30°C for 2 to 5 days. A representative experiment is shown. (B) Tdcrz1Δ mutant cells were transformed with plasmid pAMS354, which contains the S. cerevisiae CRZ1 gene, and transformants (Tdcrz1Δ CRZ1) were examined for growth on MnCl₂ medium. The wild-type strain PYCC5321 (wt) and the mutant strain MJH211 (Tdcrz1Δ) were used as controls. Cells were grown in liquid YPD containing 0.2 M CaCl₂, diluted as described for panel A, and spotted onto YPD agar medium lacking or containing MnCl₂ at the indicated concentrations. In all cases, a representative experiment is shown.
Td crz1Δ cells displayed a clear growth defect (Fig. 4A). Unlike S. cerevisiae cnb1Δ mutants, cells lacking TdCrz1p did not exhibit sensitivity to high pH. These phenotypes coincide completely with those reported for crz1Δ mutants (61). However, T. delbrueckii mutant cells were indifferent to the presence of 2.0 M NaCl and, remarkably, TdCrz1p deficiency increased Li⁺ tolerance (Fig. 4A). Both calcineurin and crz1Δ mutant cells of S. cerevisiae have been reported to be sensitive to high concentrations of monovalent Na⁺ and Li⁺ cations (41, 44, 61).

We also examined whether overexpression of the S. cerevisiae CRZ1 gene might suppress the salt-dependent phenotypes observed in Td crz1Δ mutant cells. Since the T. delbrueckii genes for the calcineurin catalytic and regulatory subunits are unknown, it is not possible to delete these genes selectively to assess their biological function. As an alternative, we analyzed the ability of FK506 and cyclosporine A to enhance the salt sensitivity of wild-type and Td crz1Δ mutant cells. Like FK506, cyclosporine A is one of the most potent, specific, and well-known inhibitors of calcineurin (49). As shown in Fig. 5, the MnCl₂ sensitivity phenotype associated with loss of Td CRZ1 was not suppressed in the presence of a range of cyclosporine A concentrations. Moreover, the Td crz1Δ mutant strain was clearly more sensitive to the immunosuppressant than was the wild-type strain. Indeed, mutant cells displayed impaired growth in response to low doses of cyclosporine A (2 μg/ml). On the contrary, much higher levels of the drug, about 20 μg/ml, were required for enhanced sensitivity to MnCl₂ in wild-type cells (Fig. 5).

The physiological role of calcineurin was also examined on CaCl₂ medium. In this case, single FK506 (1 μg/ml) and cyclosporine A (10 μg/ml) concentrations were tested. As shown in Fig. 6, addition of FK506 had dramatic inhibitory effects on the growth of wild-type cells treated with 0.2 M CaCl₂. This result was surprising since S. cerevisiae calcineurin mutants are more Ca²⁺ tolerant than wild-type cells (66). Moreover, we found that the growth defect of the Td crz1Δ mutant strain was again more pronounced than that observed in the wild-type strain on Ca²⁺/FK506-containing medium. Again, similar results were observed when cyclosporine A was used instead of FK506 (Fig. 6). Thus, our results indicate that Mn²⁺/Ca²⁺...
TdCrz1p is not required for salt-induced transcriptional activation of TdENA1. In S. cerevisiae, adaptation to salinity is primarily based on the Na⁺/Li⁺-extruding ATPase encoded by the gene ENA1. Consequently, we were interested in discovering the effects of TdCrz1Δ deletion on the levels of TdENA1 mRNA in cells exposed to NaCl or LiCl. Figure 7A shows a Northern blot analysis of total RNA from wild-type and Tdcrz1 mutant cells probed with a 913-bp fragment of TdENA1. As expected, expression of the P-type ATPase was induced in response to either Na⁺ or Li⁺, suggesting a functional role of the T. delbrueckii pump in cation homeostasis. TdENA1 mRNA accumulation was induced rapidly after the addition of 0.4 M LiCl. Then, the TdENA1 mRNA levels fell and shifted back to high at the end of the period assayed. When 1.4 M NaCl was used, the response was delayed and only one peak, at 60 min, could be detected. However, TdCrz1p was not required for the Na⁺- or Li⁺-induced expression of TdENA1 (Fig. 7A).

Then, we analyzed the induction profile of TdENA1 in the presence of FK506. As shown in Fig. 7B, addition of the immunosuppressant had no effect on the NaCl-induced expression of TdENA1 observed in either wild-type or Tdcrz1Δ mutant cells. In contrast, addition of FK506 affected the temporal pattern of expression of TdENA1 in cells exposed to Li⁺. Thus, the response was clearly delayed compared to that observed in the absence of the drug (Fig. 7A), and only a late peak of mRNA accumulation could be detected (Fig. 7B). However, similar results were found in both wild-type and Tdcrz1Δ mutant cells.

**DISCUSSION**

This study is the first to report the identification and characterization of a putative C₂H₂ zinc finger transcriptional factor, TdCrz1p, from the osmotolerant yeast T. delbrueckii; TdCrz1p is the homologue of S. cerevisiae Crz1p (41, 61), S. pombe Prz1p (34), and C. albicans Crz1p (46, 51). The budding yeast transcriptional factor Crz1p mediates the Ca⁺⁺/calcineurin-dependent induction of genes in response to salt stress and is necessary for survival under these conditions (19). Similar to its yeast homologues (12) and mammalian NFATc transcription factors (5, 63), the primary structure of TdCrz1p exhibits motifs that are characteristic of calcineurin-regulated proteins. In our study, overexpression of TdCRZ1 in S. cerevisiae wild-type cells led to improved growth on media containing NaCl or MnCl₂. Furthermore, production of TdCrz1p suppressed the growth defect at high Na⁺/Li⁺ or Mn⁺⁺ concentrations in calcineurin and crz1Δ mutants and mediated the calcineurin/Ca⁺⁺-dependent activation of a CDRE-containing reporter gene. Hence, TdCrz1p appears to be a calcineurin target and is able to compensate for the lack of a functional calcineurin-Crz1p pathway in S. cerevisiae and provide tolerance to salt stress.

These results led us to postulate that TdCrz1p and by extension calcineurin might play a role similar to that of their S. cerevisiae counterparts in regulating the salt stress response in T. delbrueckii. In contrast, however, our results demonstrated that this signaling pathway has conserved roles that are different from those described for the S. cerevisiae pathway. As shown, Tdcrz1-null phenotypes differ from those associated with Crz1p deficiency. S. cerevisiae crz1Δ mutants are sensitive to NaCl and LiCl, but have no obvious defects in growth in NaCl or LiCl (19). In contrast, TdCrz1Δ mutants are resistant to NaCl and LiCl, indicating that TdCrz1p is required for salt tolerance. The primary difference between the two organisms is that TdCrz1p is required for salt tolerance while Crz1p is not. This suggests that TdCrz1p plays a distinct role in the salt response of T. delbrueckii compared to S. cerevisiae.

It is important to note that the Na⁺/Li⁺-extruding ATPase encoded by ENA1 in S. cerevisiae is not required for salt tolerance in T. delbrueckii. Exponentially growing cultures of the T. delbrueckii strains PYCC5321 (wild type [wt]) and MJH211 (Tdcrz1Δ) were adjusted to an OD₆₀₀ of 0.3, diluted (1 to 10) onto YPD agar medium containing CaCl₂, NaCl, or LiCl at the indicated concentrations, in the presence or absence of 1 µg/ml FK506 or 10 µg/ml cyclosporine A (CsA). Plates were incubated at 30°C for 2 to 5 days. A representative experiment is shown.
to extracellular Ca\(^{2+}\) and Mn\(^{2+}\) and monovalent Na\(^{+}\) and Li\(^{+}\) cations (61). However, growth of Tdcrz1\(\Delta\) cells was diminished only upon exposure to divalent cations. In S. cerevisiae, adaptation to Ca\(^{2+}\) requires the calcineurin/Crz1p-dependent induction of genes by the vacuolar and secretory Ca\(^{2+}\) pumps Pmc1p and Pmr1p (17, 61). Mn\(^{2+}\) tolerance has also been related to the function of Pmr1p, the Golgi-localized Ca\(^{2+}\) pump (41). A similar Prz1p-dependent regulation of PMC1 expression in S. pombe has also been reported (34). Thus, a common regulatory mechanism involving Crz1p/Prz1p homologues appears to control Ca\(^{2+}\)/Mn\(^{2+}\) homeostasis in different yeasts. However, calcineurin-null cells of S. cerevisiae and S. pombe show opposite phenotypes in response to high concentrations of Ca\(^{2+}\). Whereas S. cerevisiae cnb1\(\Delta\) strains are resistant to Ca\(^{2+}\) (17), calcineurin mutants in fission yeast display decreased Ca\(^{2+}\) tolerance, a phenotype shared with prz1\(-\)null cells (34). Similarly, our results indicate that T. delbrueckii wild-type cells treated with FK506 or cyclosporine A are hypersensitive to Ca\(^{2+}\). Hence, unlike in S. cerevisiae, calcineurin activation is required for both Ca\(^{2+}\) and Mn\(^{2+}\) tolerance in T. delbrueckii. Moreover, FK506- or cyclosporine A-treated cells of the Tdcrz1\(\Delta\) strain exhibited a greater degree of sensitivity to Ca\(^{2+}\)/Mn\(^{2+}\) than did wild-type cells. Hence, TdCrz1p must carry out functions in tolerance to divalent cations that are independent of calcineurin signaling.

The differences between the S. cerevisiae and T. delbrueckii calcineurin-Crz1p pathways were further demonstrated by analysis of their respective phenotypes in response to monovalent Na\(^{+}\)/Li\(^{+}\) cations. In sharp contrast to the situation in S. cerevisiae, Tdcrz1\(\Delta\) mutants were insensitive to high external NaCl levels. Growth of wild-type cells was also unaffected by combined exposure to Na\(^{+}\)/FK506 or Na\(^{+}\)/cyclosporine A. In consonance with these phenotypes, TdCrz1p was not required to activate the NaCl-induced expression of TdENA1 either in the presence or absence of FK506. Hence, the T. delbrueckii calcineurin-Crz1p pathway has no apparent role in Na\(^{+}\) homeostasis. On the other hand, Tdcrz1\(-\)null cells are more tolerant to high levels of external Li\(^{+}\) than are wild-type cells. This fact suggests that Li\(^{+}\) and Na\(^{+}\) extrusion in T. delbrueckii is regulated, at least in part, by independent mechanisms.
Again, this is in striking contrast to the situation in *S. cerevisiae*, where both ions are extruded through the calcineurin-regulated Na⁺/Li⁺ ATPase, ENA1 (40). Our results indicate that calcineurin mediates the early induction of TdENA1 in LiCl medium. However, the accumulation of TdENA1 mRNA at the late stage after a shift to high Li⁺ was indifferent to the presence of FK506. Consistent with this, exposure of yeast cells to calcineurin inhibitors had a weak effect on Li⁺ tolerance. Interestingly, the enhanced Li⁺ tolerance observed in the Tdcrz1Δ strain indicates that TdCrz1p might function as a repressor and not as an activator of the Li⁺ extrusion system. Therefore, the calcineurin-Crz1p pathway has evolved to carry out different cellular roles in *T. delbrueckii*. Moreover, Na⁺ and Li⁺ signals appear to be transduced by unknown regulatory mechanisms and might activate distinct gene targets.

In recent years, the major biological role played by calcineurin in Ca²⁺-dependent eukaryotic signal transduction pathways has been demonstrated (49). Some of the most prominent research has been devoted to deciphering the function of the calcineurin-Crz1p pathway in the adaptation of the model yeast *S. cerevisiae* to salt stress (19, 35). However, this signaling pathway appears to have different functions in other yeasts and fungi (33, 34, 45). Moreover, stress responses and stress response mechanisms appear to have diverged among different yeasts in a niche-dependent manner. A clear example of this is the specialization of the HOG pathway toward virulence in pathogenic fungi (2, 8, 59). In conclusion, the differences in the biological functions of the calcineurin-Crz1p pathway highlighted by our results may explain the high resistance to salt stress in *T. delbrueckii* compared to *S. cerevisiae*. However, further experimentation is required to confirm this possibility and to clarify the regulatory mechanisms operating in this salt-tolerant, unconventional yeast.

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**REFERENCES**

1. Almeida, M. J., and C. Pais. 1996. Leavening ability and freeze tolerance of *Torulaspora delbrueckii*. Yeast 12:151–155.
2. Arndt, C., M. C. Cruz, M. E. Cardenas, and J. Heitman. 1999. Secretion of FK506/FK520 and ramicyn by Streptomycines inhibits the growth of competent *Saccharomyces cerevisiae* and *Cryptococcus neoformans*. Microbiology 145:1989–2000.
3. Axelsen, K. B., and M. G. Palmgren. 1998. Evolution of substrate specificities in the P-type ATPase superfamily. J. Mol. Evol. 46:84–101.
4. Blomberg, A., and L. Adler. 1992. Physiology of osmotolerance in fungi. Adv. Microb. Physiol. 33:145–212.
5. Broude, N. E., and R. Kunisawa, Y. Kottin, K. A. Moore, P. R. Young, R. A. Johnson, and G. L. P. Leavitt. 1994. The yeast FK506 gene encodes a novel membrane protein, mutations in which confer FK506 and cyclosporin A hypersensitivity and calcineurin-dependent growth. Gene 151:61–71.
6. Ciriacy, M. S. 1975. Genetics of alcohol dehydrogenase in *Saccharomyces cerevisiae*. II. Two loci controlling synthesis of the glucose-repressible ADH II. Mol. Gen. Genet. 138:157–164.
7. Cyert, M. S., and J. Thorner. 1992. Regulatory subunit (CNB1 gene product) of yeast Ca²⁺/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. Mol. Cell Biol. 12:3460–3469.
8. Cyert, M. S., R. Kunisawa, D. Kaim, and J. Thorner. 1991. Yeast has homologs (CNB1 and CNB2 gene products) of mammalian calcineurin, a calmodulin-dependent phosphoprotein phosphatase. Proc. Natl. Acad. Sci. USA 88:7376–7380.
9. Deveci, T., and J. Heitman. 2003. Calcineurin, the Ca²⁺/calmodulin-dependent protein phosphatase, is essential in yeast mutants with cell integrity defects and in mutants that lack a functional vacuolar H⁺-ATPase. J. Cell Sci. 116:413–420.
10. Hirayama, S., R. Sugiura, Y. Lu, T. Maeda, K. Kawagishi, M. Yokoyama, H. Tohda, Y. Giga-Hama, H. Shunstoh, and T. Kuno. 2003. Zinc finger protein Przl regulates Ca²⁺ but not Cl⁻ homeostasis in fission yeast. Identification
of distinct branches of calcineurin signaling pathway in fission yeast. J. Biol. Chem. 278:18078–18084.

35. Holmán, S. 2002. Osmotic stress signaling and osmoreadaption in yeasts. Microbiol. Mol. Biol. Rev. 66:380–372.

36. Huxley, C., E. D. Green, and J. Dunham. 1990. Rapid assessment of S. cerevisiae mating type by PCR. Trends Genet. 6:236.

37. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153:163–168.

38. Jiang, B., and M. S. Cyert. 1999. Identification of a novel region critical for calcineurin function in vivo and in vitro. J. Biol. Chem. 274:18543–18551.

39. Klee, C. B., and P. Cohen. 1988. The calmodulin-regulated protein phosphatase, p. 225–248. In P. Cohen and C. B. Klee (ed.), Molecular aspects of cellular regulation: calmodulin, vol. 5. Elsevier, Amsterdam, The Netherlands.

40. Marquez, J. A., and R. Serrano. 1996. Multiple transduction pathways regulate the sodium extrusion gene PMR2/ENA1 during salt stress in yeast. FEBS Lett. 382:89–92.

41. Matheos, D. P., T. J. Kingsbury, U. S. Ahsan, and K. W. Cunningham. 1997. Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in Saccharomyces cerevisiae. Genes Dev. 11:3445–3458.

42. Mendizabal, I., G. Rios, J. M. Mulet, and I. F. de Larrinoa. 1998. Yeast putative transcription factors involved in salt tolerance. FEBS Lett. 425:323–328.

43. Mendoza, I., F. Rubio, A. Rodriguez-Navarro, and J. M. Pardo. 1994. The protein phosphatase calcineurin is essential for NaCl tolerance of Saccharomyces cerevisiae. J. Biol. Chem. 269:8792–8796.

44. Nakamura, T., Y. Liu, D. Hirata, H. Namba, S. Harada, T. Hirokawa, and T. Miyakawa. 1993. Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. EMBO J. 12:4063–4071.

45. Nanthakumar, N. N., J. S. Dayton, and A. R. Means. 1996. Role of Ca2+/calmodulin binding proteins in Aspergillus nidulans cell cycle regulation. Prog. Cell Cycle Res. 2:217–228.

46. Onyewu, C., F. L. Wurmley, Jr., J. R. Perfect, and J. Heitman. 2004. The calcineurin target, Crz1, functions in azole tolerance but is not required for virulence of Candida albicans. Infect. Immun. 72:7330–7333.

47. Platt, T., B. Muller-Hill, and J. H. Miller. 1972. Assay of β-galactosidase, p. 351–355. In J. H. Miller (ed.), Experiments in molecular genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

48. Rodriguez-Navarro, A. 2000. Potassium transport in fungi and plants. Biochim. Biophys. Acta 1469:1–30.

49. Russnak, F., and P. Mertz. 2000. Calcineurin: form and function. Physiol. Rev. 80:1483–1521.

50. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.

51. Santos, M., and I. F. de Larrinoa. 2005. Functional characterization of the Candida albicans CRZ1 gene encoding a calcineurin-regulated transcription factor. Curr. Genet. 48:88–100.

52. Serrano, R., and A. Rodriguez-Navarro. 2001. Ion homeostasis during salt stress in plants. Curr. Opin. Cell Biol. 13:399–404.

53. Serrano, R., J. A. Marquez, and G. Rios. 1997. Crucial factors in salt stress tolerance, p. 147–169. In S. Hohmann and W. H. Mager (ed.), Yeast stress responses. Springer-Verlag, Heidelberg, Germany.

54. Serrano, R., J. M. Mulet, G. Rios, J. Marquez, I. de Larrinoa, M. Leube, I. Mendizabal, A. Pascual-Ahuir, M. Proff, R. Ros, and C. Montesinos. 1999. A glimpse of the mechanisms of ion homeostasis during salt stress. J. Exp. Bot. 50:1023–1036.

55. Sherman, F., G. R. Fink, and J. B. Hicks. 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

56. Sistri, C., L. Cerutti, N. Hulo, A. Gattiker, L. Falquet, M. Pagni, A. Bairako, and P. Bucher. 2002. PROSITE: a documented database using patterns and profiles as motif descriptors. Brief. Bioinformatics 3:265–274.

57. Sikorski, R. S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122:19–27.

58. Sleator, R. D., and C. Hill. 2001. Bacterial osmoreadaption: the role of osmotolys in bacterial stress and virulence. FEMS Microbiol. Rev. 26:49–71.

59. Smith, D. A., S. Nicholls, B. A. Morgan, A. J. Brown, and J. Quinn. 2004. A conserved stress-activated protein kinase regulates a core stress response in the human pathogen Candida albicans. Mol. Biol. Cell 15:4179–4190.

60. Smith, D. L., T. Tao, and M. E. Maguire. 1993. Membrane topology of a P-type ATPase. The Mg2+ transport protein of Salmonella typhimurium. J. Biol. Chem. 268:22469–22479.

61. Stathopoulos, A. M., and M. S. Cyert. 1997. Calcineurin acts through the CRZI/TCN1-encoded transcription factor to regulate gene expression in yeast. Genes Dev. 11:3432–3444.

62. Stathopoulos-Gerontides, A., J. J. Guo, and M. S. Cyert. 1999. Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation. Genes Dev. 13:798–803.

63. Sugiria, R., S. O. Sin, H. Shunstoh, and T. Kono. 2001. Molecular genetic analysis of the calcineurin signaling pathways. Cell. Mol. Life Sci. 58:278–288.

64. Westfall, P. J., D. R. Ballon, and J. Thorner. 2004. When the stress of your environment makes you go HO wild. Science 307:1511–1512.

65. Wiederrecht, G., E. Lam, S. Hung, M. Martin, and N. Sigal. 1993. The mechanism of action of FK-506 and cyclosporin A. Ann. N. Y. Acad. Sci. 696:9–19.

66. Wihee, J. L., J. Mulholland, R. Jeng, and M. S. Cyert. 1997. An essential role of the yeast pheromone-induced Ca2+ signal is to activate calcineurin. Mol. Biol. Cell 8:263–277.

67. Yoshimoto, H., K. Saltsman, A. P. Gasch, H. X. Li, N. Ogawa, D. Botstein, P. O. Brown, and M. S. Cyert. 2002. Genome-wide analysis of gene expression regulated by the calcineurin/Crz1p signaling pathway in Saccharomyces cerevisiae. J. Biol. Chem. 277:31079–31088.