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

Maria Jose Hernandez-Lopez, Joaquin Panadero, Jose Antonio Prieto, and Francisca Randez-Gil*

Department of Biotechnology, Instituto de Agroquímica y Tecnología de los Alimentos, Consejo Superior de Investigaciones Científicas, P.O. Box 73, E-46100 Burjassot, Valencia, Spain

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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* Td*CRZ1* gene, which encodes a putative zinc finger transcription factor homologue to Crz1p. Consistent with this, overexpression of Td*CRZ1* enhanced the salt tolerance of *S. cerevisiae* wild-type cells and suppressed the sensitivity phenotype of *cnb1*Δ and *crz1*Δ mutants to monovalent and divalent cations. However, *T. delbrueckii* cells lacking TdCrz1p showed phenotypes distinct from those previously observed in *S. cerevisiae crz1*Δ mutants. Quite remarkably, Td*crz1*-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 Td*ENA1* 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 rouxii*, *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 *CNA1* and *CNA2*, complexed with a regulatory subunit, the product of *CNB1*. The phosphatase activity of calcineurin is dispensable for growth under standard conditions. However, *cna1 cna2* or *cnb1* 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

* Corresponding author. Mailing address: Department of Biotechnology, Instituto de Agroquímica y Tecnología de los Alimentos, Consejo Superior de Investigaciones Científicas, P.O. Box 73, E-46100 Burjassot, Valencia, Spain. Phone: 34-963900022. Fax: 34-963636301. E-mail: randez@iata.csic.es.

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, Mn^{2+}, or Li^{+} (41, 61). Nevertheless, *crz1* and calcineurin mutant cells show opposite phenotypes under specific conditions, such as exposure to Ca^{2+} 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, particularly in highly osmotolerant species, remains unclear.

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 Génolevures sequencing project (available at http://cbi.labri.fr/Genolevures/index.php). Among these, only the *prz1*$^{+}$ and Ca*CRZ1* genes from *S. pombe* and *C. albicans*, respectively, have been studied in detail (34, 46, 51). Like Crz1p, Prz1p and CaCrz1p act downstream of calcineurin and regulate Ca^{2+} homeostasis (34, 51). However, *S. pombe prz1* 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 Td*ENA1* and Td*CRZ1* 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 *crz1*Δ 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* Td*crz1*Δ 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%$(NH_4)_2SO_4$, 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 mg/liter). 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 mg/liter of nourseothricin (clonNAT; 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 (OD_{600}) of 0.3 and spotting (3-μl) 10-fold serial dilutions onto SD or YPD agar solid medium containing NaCl, LiCl, $MnCl_2$, or $CaCl_2$. FK506 (Fujisawa GmbH) (20 mg/ml in 90% ethanol–10% Tween 20) was added at the indicated concentrations on solid and liquid media. Cyclosporine A (10 mg/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.

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
CEN.PK2-1C	*MATα ura3-52 his3-Δ1 leu2-3,112 trp1-289 MAL2-8*^{c} *SUC2*	23
YPH499	*MAT***a** *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1*	57
DD12	Same as YPH499, except *cnb1*::*hisG*	20
ASY472	Same as YPH499, except *crz1*::*loxP-kanMX-loxP*	61
ASY475	Same as DD12, except *crz1*::*loxP-kanMX-loxP*	61
ASY832	Same as YPH499, except *ura3*::*TRP1-4x CDRE-lacZ*	M. Cyert
ASY834	Same as ASY472, except *ura3*::*TRP1-4x CDRE-lacZ*	M. Cyert
ASY835	Same as ASY475, except *ura3*::*TRP1-4x CDRE-lacZ*	M. Cyert

Strain and plasmid construction. Plasmids pAMS345 (61) and pJQ10 (9), containing the *S. cerevisiae CRZ1* and *ENA1* genes, respectively, were a gift from M. Cyert and A. Rodríguez-Navarro. Plasmids pMJH1 and pMJH14, carrying DNA fragments containing the *ENA1* and *CRZ1* genes from *T. delbrueckii*, Td*ENA1* and Td*CRZ1*, 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 YEpMJH1, containing the coding region of the Td*ENA1* gene and the 5′ and 3′ noncoding regions, was moved into vector YEplac195 (26). Plasmid YEpTdCRZ1, containing the isolated Td*CRZ1* gene, was constructed by cloning a 1,980-bp ScaI/EcoRI fragment from plasmid YEpMJH14 into the vectorYEplac195 (26), previously digested with EcoRI/SmaI. The Td*CRZ1* disruption cassette containing the nourseothricin-resistance module *natMX4* (27) was constructed by restriction. First, a 3′-side fragment of Td*CRZ1* (+1322 to +1810) was obtained by PCR using two specific primers, FR142 and FR141 (Table 2), and plasmid YEpMJH14 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 pBS plasmid (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 Td*CRZ1* (−270 to +448) using oligonucleotides FR140 and FR139 (Table 2) and plasmid YEpMJH14 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 Td*CRZ1* disruption cassette, which contains the *natMX4* module flanked by 718 and 488 bp (5′ and 3′ sides, respectively), homologues to the Td*CRZ1* gene.

Correct disruption of the Td*CRZ1* 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 replaced Td*CRZ1* sequence and within the marker module (Table 2).

β-Galactosidase assay. Exponentially SD-growing cells (OD_{600}, 0.6 to 0.8) were collected, resuspended in YPD (pH 5.5) or the same medium supplemented with 0.2 M $CaCl_2$, and incubated at 30°C and 200 rpm for 45 min. Then, aliquots of the yeast suspension (15 OD_{600} units) were harvested, washed with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$), 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 2. Oligonucleotides used in this study

Primer	Sequence (5′ to 3′)	Comment(s)
FR55	ATGACCATGATTACGCCAA	Td*ENA1* and Td*CRZ1* sequencing
FR77	GTAAAACGACGGCCAGT	Td*ENA1* and Td*CRZ1* sequencing
FR94	AATCATCGGCAACCTTAG	Td*CRZ1* sequencing
FR103	GGCGACATGCTACGACTTC	Td*CRZ1* sequencing
FR136	GTGGTTAAATAGGACATCGC	Td*CRZ1* sequencing
FR90	TGCTACTGAAAAGACAAGG	Td*ENA1* sequencing
FR96	GCTTACAGGCGAGGAATT	Td*ENA1* sequencing
FR135	TGAGTCCTTGCCTATCGC	Td*ENA1* sequencing
FR110	CCTCGTTCTGCTTTGACA	Td*ENA1* sequencing
FR117	CAGGATATCAAGGGTAAGCT	Td*ENA1* sequencing
FR376	AATGGTTCAGACGTCGC	Td*ENA1* sequencing
FR377	CCAGCTGATCACTTCGG	Td*ENA1* sequencing
FR101	CCTAAAGCCCAAACTATAACA	Td*CRZ1* sequencing, verify correct targeting of the natMX4 module
FR142	CGAAGTCGACAGCTCAATCA	PCR amplification of Td*CRZ1* 3′ side
FR141	TGAATTCGGGTAAGAAAAGG	PCR amplification of Td*CRZ1* 3′ side
FR140	CATTGAGCTCCTTGGAAGG	PCR amplification of Td*CRZ1* 5′ side
FR139	ATTCGGATCCTAAGTCACTC	PCR amplification of Td*CRZ1* 5′ side
FR126	TTCAGTGCCGAAGGGACTAC	Verify correct targeting of the *natMX4* module
FR76	GTCAAGGAGGGTATTCTGG	Verify correct targeting of the *natMX4* module
FR75	AGTTAAGTGCGCAGAAAG	Verify correct targeting of the *natMX4* module
FR96	GCTTACAGGCGAGGAATT	Td*ENA1* probe for Northern blotting
FR121	GCTGCACCAACAGACAAAG	Td*ENA1* probe for Northern blotting
FR390	GGTATGTTCTAGCGCTTG	*ACT1* probe for Northern blotting
FR391	TCTGGGGCTCTGAATCTT	*ACT1* probe for Northern blotting

determined at room temperature by using the substrate ONPG (*o*-nitrophenyl-β-D-galactopyranoside) as previously described (47). One unit is defined as the amount of enzyme that is able to 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 ^{32}P-labeled probe of Td*ENA1* (+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-^{32}P]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 (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate and once with 50 ml of 0.2× 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 Td*ENA1* mRNA level for each sample analyzed was set to 100.

Sequencing and sequence analysis. DNA sequencing was performed on both strands by the dideoxy 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 (3) 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 MultAlin software (15) at INRA (http://prodes.toulouse.inra.fr/).

Nucleotide sequence accession numbers. The nucleotide sequences of Td*CRZ1* and Td*ENA1* 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-*

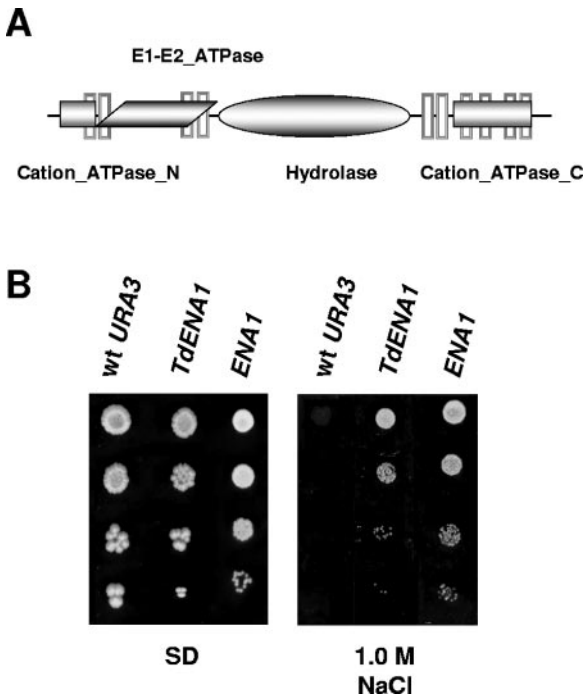

FIG. 1. Multiple copies of Td*ENA1*, encoding a P-type ATPase, enhance NaCl resistance in *S. cerevisiae*. (A) Schematic structure of TdEna1p showing the conserved domains for N-terminal cation transporting ATPase (Cation_ATPase_N), C-terminal cation transporting ATPase (Cation_ATPase_N), hydrolase, the E1-E2 ATPase-associated region, and transmembrane segments (shown as vertical boxes). (B) Mid-exponential-phase cultures of the *S. cerevisiae* strain CEN.PK2-1C transformed with plasmid YEpTdENA1 (Td*ENA1*), plasmid pJQ10 (*ENA1*), or the empty plasmid YEplac195 (wt *URA3*) were adjusted to an OD_{600} of 0.3, diluted (1 to 10^{-3}), 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.

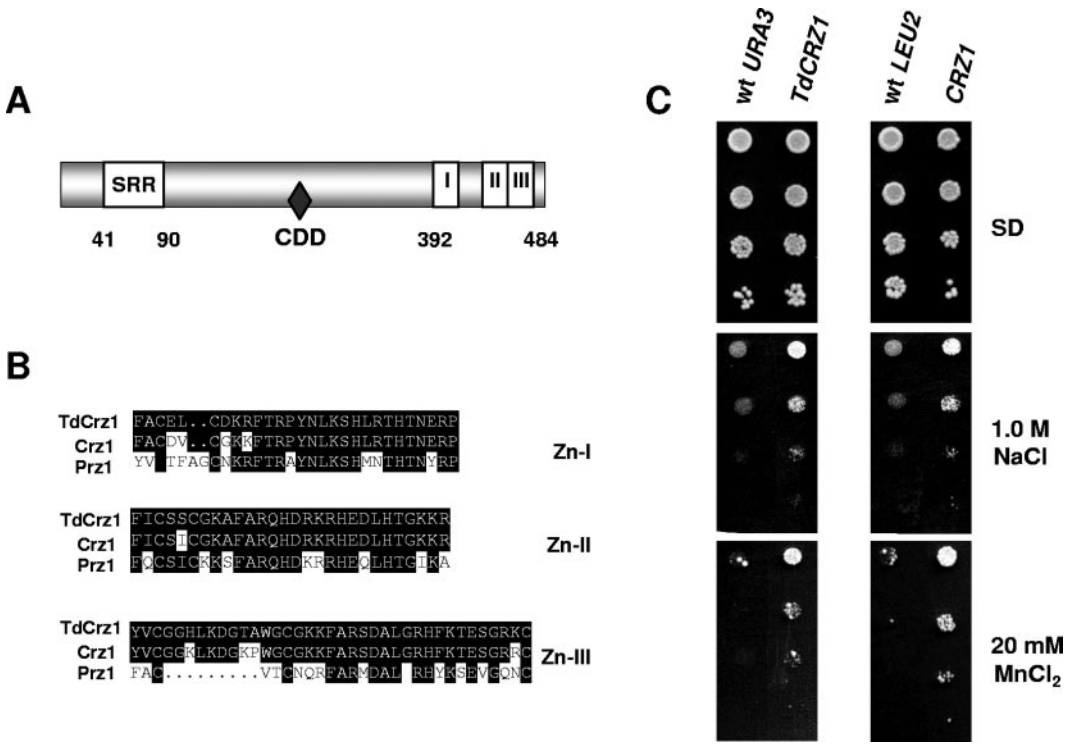

FIG. 2. Td*CRZ1* encodes the homologue to the transcriptional factor Crz1p, and its overexpression in *S. cerevisiae* confers enhanced salt tolerance. (A) Schematic representation of TdCrz1p. Denoted are the serine-rich region (SRR), the calcineurin-docking domain (CDD) and three putative C_2H_2-type zinc finger motifs at the carboxyl terminus. (B) Sequence alignment of the three zinc finger motifs from TdCrz1, 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 OD_{600} of 0.3, diluted (1 to 10^{-3}), and spotted (3 μl) onto SD plates or SD plates containing NaCl or $MnCl_2$ at the indicated concentrations. 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 α chains involved in Na^+ and K^+ 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 C_2H_2-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

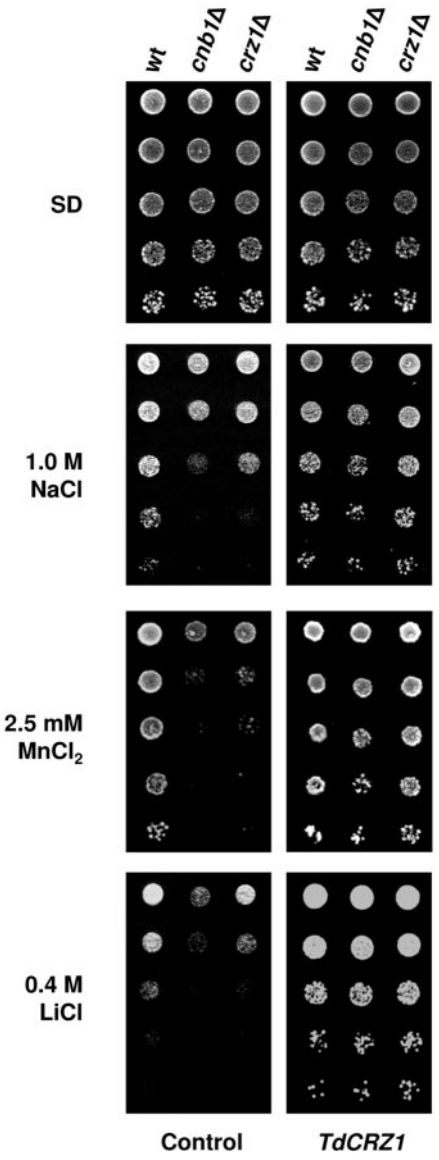

FIG. 3. TdCrz1p restores growth of *S. cerevisiae cnb1*Δ and *crz1*Δ mutants in high-salt media. Mid-exponential-phase cultures of the *S. cerevisiae* strains YPH499 (wild type [wt]), DD12 (*cnb1*Δ), and ASY472 (*crz1*Δ), transformed with plasmid YEpTdCRZ1 or YEplac195 (empty plasmid control), were examined for growth on solid SD (SD) or SD containing NaCl, $MnCl_2$, or LiCl at the indicated concentrations. SD-pregrown cells were diluted, spotted, and incubated as described in the legend to Fig. 1B. 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 of the enhanced salt resistance of the *S. cerevisiae* strain CEN.PK2-1C, we constructed plasmid YEpTd CRZ1 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 Td*CRZ1* 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 Mn^{2+} tolerance was tested. Indeed, in SD medium, the CEN.PK2-1C strain transformed with YEp TdCRZ1 grew to 20 mM $MnCl_2$, 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 Mn^{2+} ions.

Td*CRZ1* suppresses the ion sensitivity of *S. cerevisiae cnb1*Δ and *crz1*Δ mutants. Overexpression of *S. cerevisiae CRZ1* compensates for the enhanced ion sensitivity of yeast calcineurin mutants, specifically to Na^+, Li^+, and Mn^{2+} (41, 61). Therefore, we were interested in determining whether TdCrz1p could affect these calcineurin phenotypes. For this, *cnb1*Δ and *crz1*Δ mutant cells of the YPH499 strain (20, 61) were transformed with plasmid YEpTdCRZ1 and examined for growth on NaCl-, $MnCl_2$-, and LiCl-containing medium. The wild-type strain YPH499 shows higher $MnCl_2$ sensitivity than the CEN.PK2-1C 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 Mn^{2+} tolerance of a *cnb1*Δ 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 Td*CRZ1* 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 Mn^{2+} 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 $CaCl_2$ (Table 3). Consistent with previous reports (61), CDRE-driven expression was completely depen-

TABLE 3. Overexpression of Td*CRZ1* permits the calcineurin-dependent induction of a 4x CDRE-*lacZ* gene fusion in response to Ca^{2+}

Relevant genotype (+ growth condition) of *S. cerevisiae* strain used[a]	β-Galactosidase activity (U/mg of protein)[b]	
	Control	Ca^{2+}
Wild type	152.7 ± 43.2	1,336.6 ± 98.1
*crz1*Δ	0.9 ± 0.3	1.8 ± 0.4
*crz1*Δ Td*CRZ1*	57.0 ± 4.0	322.4 ± 19.8
*crz1*Δ *CRZ1*	85.0 ± 7.0	246.1 ± 23.2
*cnb1*Δ *crz1*Δ	1.7 ± 0.2	4.2 ± 1.3
*cnb1*Δ *crz1*Δ Td*CRZ1*	121.0 ± 14.2	184.6 ± 12.2
*cnb1*Δ *crz1*Δ *CRZ1*	67.8 ± 8.4	110.5 ± 17.1
*crz1*Δ Td*CRZ1* + FK506 (1 μg/ml)	52.0 ± 12.0	157.4 ± 31.0
*crz1*Δ Td*CRZ1* + FK506 (5 μg/ml)	57.0 ± 21.0	60.4 ± 18.0

[a] The *S. cerevisiae* strains used were YPH499 (wild type), ASY834 (*crz1*Δ), and ASY835 (*cnb1*Δ *crz1*Δ) transformed with plasmid YEplac195 (empty plasmid), YEpTdCRZ1 (Td*CRZ1*), or pAMS435 (*CRZ1*).

[b] Data are means ± standard errors for three independent experiments. β-Galactosidase activities are shown for the indicated strains treated with 0.2 M $CaCl_2$ in the presence or absence of FK506.

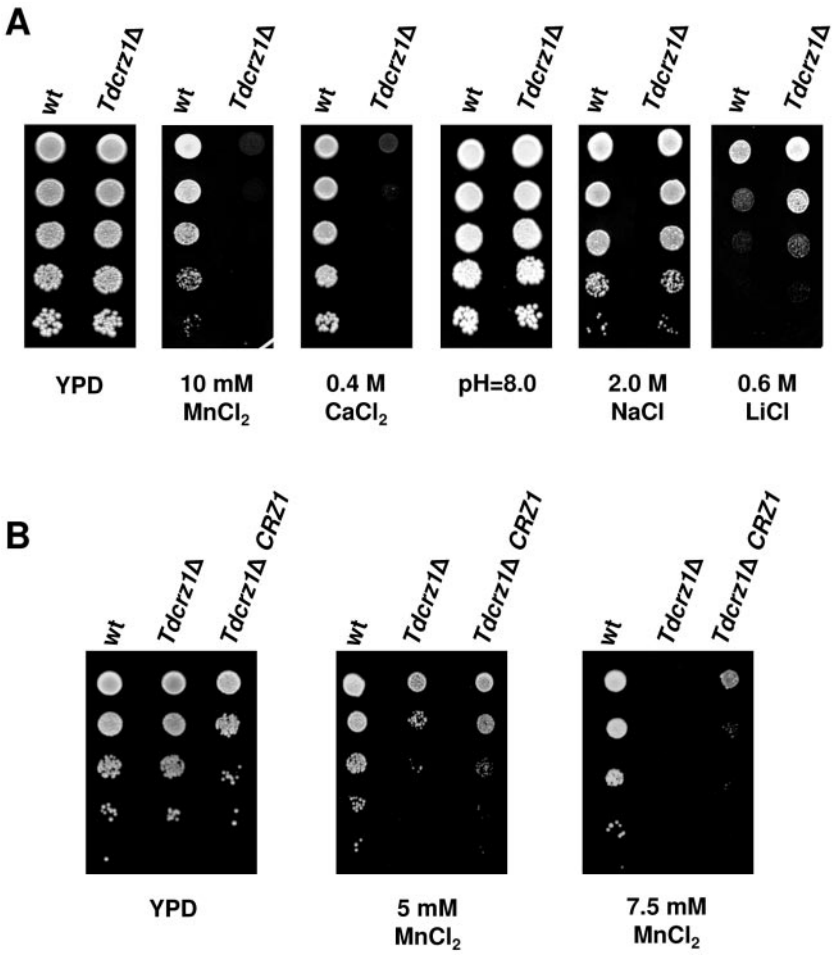

FIG. 4. Td*crz1*Δ cells exhibit specific phenotypes in response to diverse ionic stresses. (A) Exponentially growing cultures of the *T. delbrueckii* strains PYCC5321 (wild type [wt]) and MJH211 (Td*crz1*Δ) were adjusted to an OD_{600} of 0.3, diluted (1 to 10^{-4}), and spotted (3 μl) onto YPD agar medium, YPD adjusted to pH 8.0, or YPD supplemented with $MnCl_2$, $CaCl_2$, NaCl, or LiCl at the indicated concentrations. Plates were incubated at 30°C for 2 to 5 days. A representative experiment is shown. (B) Td*crz1*Δ mutant cells were transformed with plasmid pAMS354, which contains the *S. cerevisiae CRZ1* gene, and transformants (Td*crz1*Δ *CRZ1*) were examined for growth on $MnCl_2$ medium. The wild-type strain PYCC5321 (wt) and the mutant strain MJH211 (Td*crz1*Δ) were used as controls. Cells were grown in liquid YPD containing 0.2 M $CaCl_2$, diluted as described for panel A, and spotted onto YPD agar medium lacking or containing $MnCl_2$ at the indicated concentrations. In all cases, a representative experiment is shown.

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 *cnb1*Δ *crz1*Δ double mutant transformed with an empty plasmid, YEplac195 (Table 3). In contrast, overproduction of TdCrz1p in a *crz1*-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 *crz1*Δ mutant transformed with plasmid pAMS435, which contains the *S. cerevisiae CRZ1* gene (61), also showed lower induction, around threefold (Table 3). As could be expected, overexpression of Td*CRZ1* in the strain lacking both Cnb1p and Crz1p was unable to restore the CDRE-mediated expression level observed in the *crz1*Δ single mutant (Table 3). Again, similar results were obtained by overexpression of multiple copies of *CRZ1*. Hence, TdCrz1p is able to mediate CDRE-driven expression and appears to function downstream of calcineurin in *S. cerevisiae*. In order to confirm this, we analyzed the level of β-galactosidase activity in YEpTdCRZ1 transformants of the *crz1*Δ 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 *crz1*Δ cells transformed with the *S. cerevisiae CRZ1* gene (data not shown).

Td*crz1*Δ shows conserved phenotypes distinct from those of *S. cerevisiae crz1*Δ mutants. To clarify the function of Td*CRZ1* in *T. delbrueckii*, we constructed a Td*crz1*-null mutant (MJH211 strain) and analyzed cells for phenotypes previously reported for *S. cerevisiae* calcineurin and *crz1*Δ mutants (41, 44, 61, 66). On YPD plates containing 10 mM $MnCl_2$ or 0.4 M $CaCl_2$,

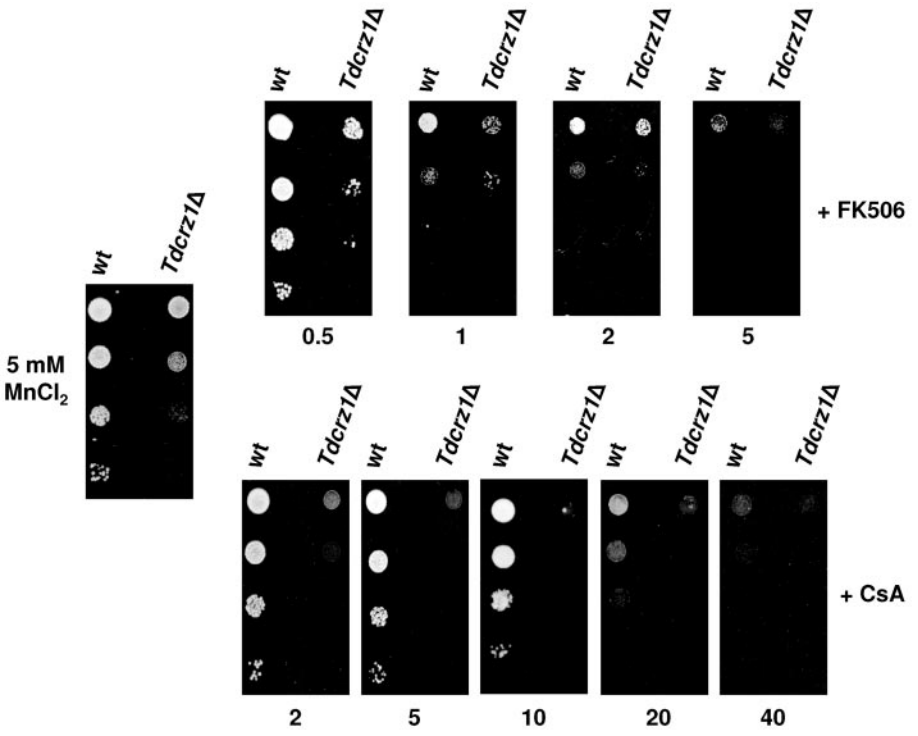

FIG. 5. The $MnCl_2$ sensitivity phenotype associated with loss of Td*CRZ1* is not suppressed by exposure to FK506 or cyclosporine A. YPD-grown cultures of the *T. delbrueckii* strains PYCC5321 (wild type [wt]) and MJH211 (Td*crz1*Δ) were adjusted to an OD_{600} of 0.3, diluted (1 to 10^{-3}), and spotted (3 μl) onto 5 mM $MnCl_2$-YPD agar medium with or without FK506 or cyclosporine A (CsA) at the indicated concentration (in micrograms per milliliter). Plates were incubated at 30°C for 2 to 5 days. A representative experiment is shown.

Td*crz1*Δ cells displayed a clear growth defect (Fig. 4A). Unlike *S. cerevisiae cnb1*Δ mutants, cells lacking TdCrz1p did not exhibit sensitivity to high pH. These phenotypes coincide completely with those reported for *crz1*Δ 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 *crz1*Δ 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*crz1*Δ mutant cells. Since the *T. delbrueckii* strains used in our work are prototrophic, pAMS435 (*CRZ1*) transformants were selected on solid YPD medium containing 0.4 M $CaCl_2$. Under these conditions, the recipient mutant strain displays residual growth (Fig. 4A). Transformants were verified by plasmid isolation and G-418 resistance prior to use (data not shown). As can be seen in Fig. 4B, the $MnCl_2$ sensitivity phenotype observed in Td*crz1*Δ mutant cells was partially suppressed by expression of multiple copies of *CRZ1*. Similar results were obtained in $CaCl_2$- or LiCl-containing medium, while no phenotype could be detected at pH 8.0 or in the presence of NaCl (data not shown). Therefore, *CRZ1* partially complements phenotypes associated with deletion of the *T. delbrueckii CRZ1* gene.

The *T. delbrueckii* calcineurin-Crz1p pathway plays no role in Na^+ tolerance. The results reported above led us to investigate whether calcineurin was involved in the phenotypes exhibited by Td*crz1*Δ 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*crz1*Δ 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 $MnCl_2$ sensitivity phenotype associated with loss of Td*CRZ1* was not suppressed in the presence of a range of cyclosporine A concentrations. Moreover, the Td*crz1*Δ 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 $MnCl_2$ in wild-type cells (Fig. 5). Similar results were obtained when phenotypes were tested in the presence of 0.5 to 5 μg/ml FK506 (Fig. 5).

The physiological role of calcineurin was also examined on $CaCl_2$ 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 $CaCl_2$. This result was surprising since *S. cerevisiae* calcineurin mutants are more Ca^{2+} tolerant than wild-type cells (66). Moreover, we found that the growth defect of the Td*crz1*Δ mutant strain was again more pronounced than that observed in the wild-type strain on Ca^{2+}/FK506-containing medium. Again, similar results were observed when cyclosporine A was used instead of FK506 (Fig. 6). Thus, our results indicate that Mn^{2+}/Ca^{2+}

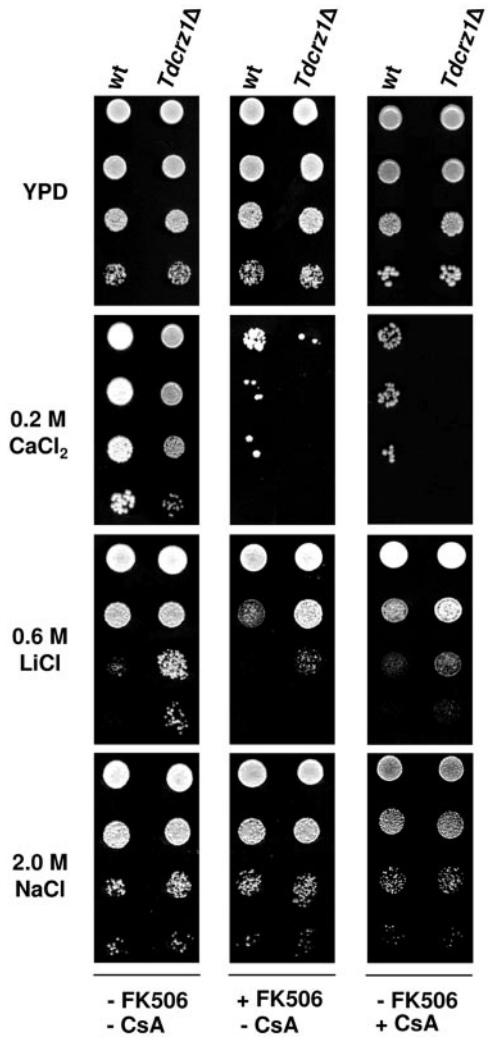

FIG. 6. The *T. delbrueckii* calcineurin-Crz1p pathway plays no role in Na^+ tolerance. Exponentially growing cultures of the *T. delbrueckii* strains PYCC5321 (wild type [wt]) and MJH211 (Td*crz1*Δ) were adjusted to an OD_{600} of 0.3, diluted (1 to 10^{-3}), and spotted (3 μl) onto YPD agar medium containing $CaCl_2$, 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.

tolerance in *T. delbrueckii* requires a functional calcineurin-Crz1p pathway. However, *T. delbrueckii* calcineurin and TdCrz1p appear to function independently.

We also characterized the properties of wild-type and Td*crz1*Δ cells exposed to NaCl or LiCl in the presence or absence of FK506 or cyclosporine A. Unlike *S. cerevisiae*, neither the lack of TdCrz1p nor exposure to the immunosuppressive agents affected the Na^+ tolerance of this organism (Fig. 6). In contrast, addition of FK506 or cyclosporine A impaired the growth of cells exposed to Li^+ (Fig. 6). Nevertheless, their presence had weak effects on Li^+ sensitivity compared to that observed for Ca^{2+} and did not affect the phenotype of Td*crz1*Δ mutant cells (Fig. 6). Hence, the *T. delbrueckii* calcineurin-Crz1p pathway plays conserved roles distinct from those reported in *S. cerevisiae* and has positive or negative effects in an ion-dependent manner.

TdCrz1p is not required for salt-induced transcriptional activation of Td*ENA1*. 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 Td*CRZ1* deletion on the levels of Td*ENA1* mRNA in cells exposed to NaCl or LiCl. Figure 7A shows a Northern blot analysis of total RNA from wild-type and Td*crz1* mutant cells probed with a 913-bp fragment of Td*ENA1*. 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. Td*ENA1* mRNA accumulation was induced rapidly after the addition of 0.4 M LiCl. Then, the Td*ENA1* 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 Td*ENA1* (Fig. 7A).

Then, we analyzed the induction profile of Td*ENA1* in the presence of FK506. As shown in Fig. 7B, addition of the immunosuppressant had no effect on the NaCl-induced expression of Td*ENA1* observed in either wild-type or Td*crz1*Δ mutant cells. In contrast, addition of FK506 affected the temporal pattern of expression of Td*ENA1* 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 Td*crz1*Δ mutant cells.

DISCUSSION

This study is the first to report the identification and characterization of a putative C_2H_2 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^{2+}/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 Td*CRZ1* in *S. cerevisiae* wild-type cells led to improved growth on media containing NaCl or $MnCl_2$. Furthermore, production of TdCrz1p suppressed the growth defect at high Na^+/Li^+ or Mn^{2+} concentrations in calcineurin and *crz1*Δ mutants and mediated the calcinuerin/Ca^{2+}-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, Td*crz1*-null phenotypes differ from those associated with Crz1p deficiency. *S. cerevisiae crz1*Δ mutants are sensitive

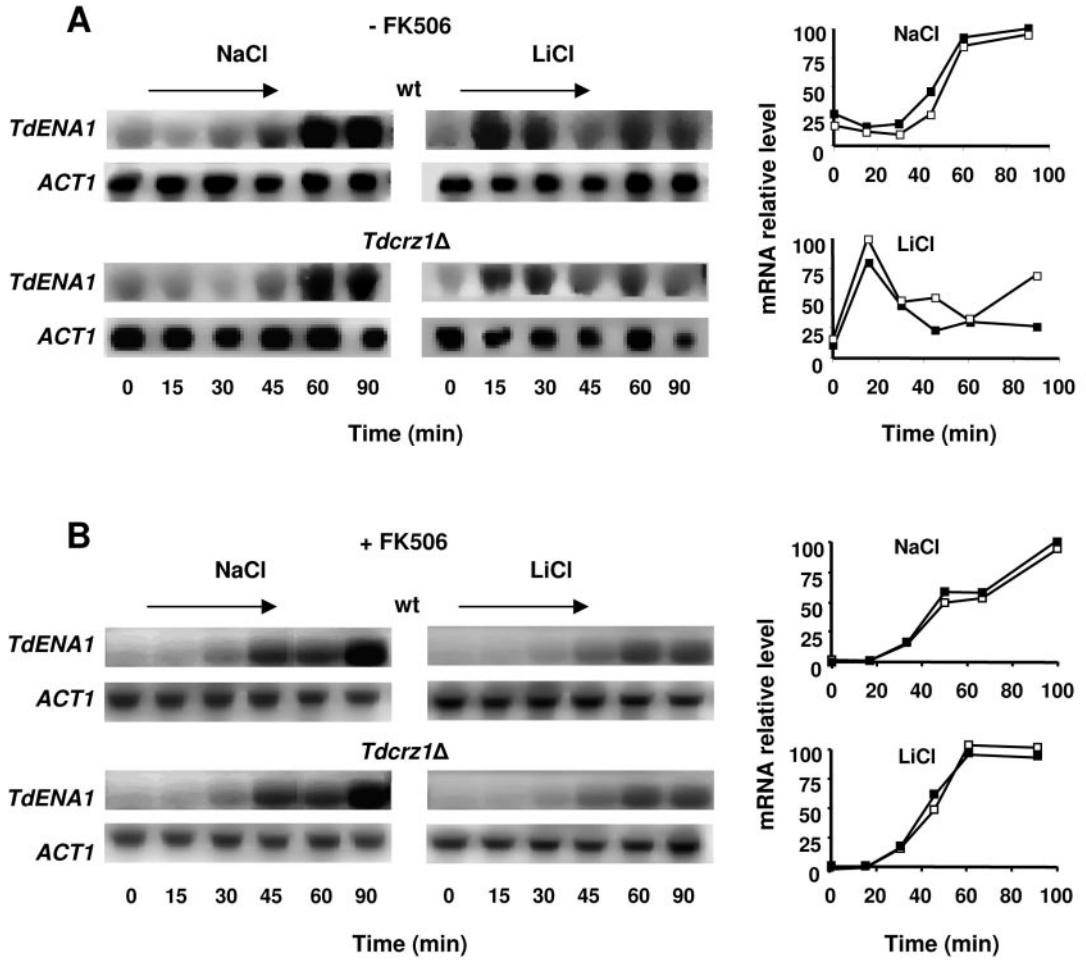

FIG. 7. Activity of TdCrz1p is not required for the salt-induced transcriptional activation of the Td*ENA1* gene. YPD-grown cells of the *T. delbrueckii* strains PYCC5321 (wild type [wt], ■) and MJH211 (Td*crz1*Δ, □) were transferred to 1.4 M NaCl-YPD or 0.5 M LiCl-YPD lacking (A) or containing (B) 5 μg/ml FK506. Samples were taken at the indicated times and analyzed by Northern blotting, as described in Materials and Methods. Filters were probed for Td*ENA1* mRNA. The graphs represent quantification of the mRNA levels of Td*ENA1* relative to those of the *S. cerevisiae ACT1* gene. Results from a representative experiment are shown.

to extracellular Ca^{2+} and Mn^{2+} and monovalent Na^+ and Li^+ cations (61). However, growth of Td*crz1*Δ 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*Δ 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 Td*crz1*Δ 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*, Td*crz1*Δ 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 Td*ENA1* 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, Td*crz1*-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 trough the same calcineurin-regulated Na^+/Li^+ ATPase, *ENA1* (40). Our results indicate that calcineurin mediates the early induction of Td*ENA1* in LiCl medium. However, the accumulation of Td*ENA1* 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 Td*crz1*Δ 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 Ca^{2+}-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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