DEPARTAMENTO DE BIOTECNOLOGÍA

TORULASPORA DELBRUECKII: APLICACIONES TECNOLÓGICAS Y AISLAMIENTO DE GENES DE RESPUESTA A ESTRÉS

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Torulaspora delbrueckii: aplicaciones tecnológicas y aislamiento de genes de respuesta a estrés

Trabajo realizado por María José Hernández López en el Instituto de Agroquímica y Tecnología de Alimentos, CSIC, para optar al grado de Doctora en Ciencias Biológicas.

Valencia, Junio 2005

Los Doctores Francisca Rández Gil y José Antonio Prieto Alamán, científicos titulares del Consejo Superior de Investigaciones Científicas en el Instituto de Agroquímica y Tecnología de los Alimentos de Valencia,

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Que Dª. María José Hernández López, licenciada en Ciencias Biológicas, ha realizado bajo su dirección el trabajo titulado "*Torulaspora delbrueckii*: aplicaciones tecnológicas y aislamiento de genes de respuesta a estrés" que presenta para optar al grado de Doctora en Ciencias Biológicas por la Universitat de València.

Valencia, 21 de Junio del 2005

Dra. Francisca Rández Gil *Dr. José Antonio Prieto Alamán*

A mis padres, a Tatín y a mi hija Carla

CONTENIDOS

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Abreviaturas

A: adenina

ATP: adenosina trifosfato

BSA: albúmina sérica bovina

CDRE: elemento de respuesta dependiente de calcineurina

C: citosina

cDNA: DNA copia de RNA

cAMP: adenosil monofosfato cíclico

CO_2: dióxido de carbono

dCTP: desoxi citosina trifosfato

DMSO: dimetil sulfóxido

DNA: ácido desoxirribonucleico

d.w.: dry weigh (peso seco)

G: guanina

h: horas

IgG: inmunoglobulina G

Kb: kilobases

MCS: multicloning sequence (sitio múltiple de clonación)

min: minutos

NFAT: nuclear factor of activated T cells (Factor Nuclear de células T Activadas)

OD_{600}: densidad óptica a 600 nm

ORF: open reading frame (pauta de lectura abierta)

rpm.: revoluciones por minuto

TBS: tampón Tris-Buffer Salino

bp: pares de bases (pb)

RNA: ácido ribonucleico

mRNA: RNA mensajero

rRNA: RNA ribosomal

spp.: especies

PYCC: Portugal Yeast Colection Cultura (colección portuguesa de cultivos de levadura)

PCR: Polimerase Chain Reaction (reacción en cadena de la polimerasa)

SD: medio sintético dextrosa

SDS-PAGE: electroforesis en gel de poliacrilamida en tampón dodecil sulfato sódico

UTR: Untranslated Region (región no traducida)

v/v: volume/volume (volumen /volumen)

w/v: weight/ volume (peso/volumen)

Introducción

Desde tiempos ancestrales, la levadura ha sido utilizada por el hombre para la transformación y elaboración de alimentos y bebidas alcohólicas. La fermentación de alimentos ha acompañado al hombre a través de la historia y ha sido considerada un arte durante muchos siglos. Ya desde el año 4.000 a.C. existe constancia de la fabricación de pan en el antiguo Egipto, donde los panaderos mezclaban cerveza con harina de trigo para la elaboración de masas. Esta práctica empezó a ser habitual en Europa a finales del siglo XVIII, aunque no fue hasta finales del XIX cuando comenzó a emerger la industria productora de cepas de levadura para panadería. Esta nueva levadura se denominó levadura prensada, debido a que se presentaba en el mercado en estado sólido y permitía la obtención de productos menos amargos y con mejor sabor.

1.- EL PROCESO DE PANIFICACIÓN

El término pan cubre una amplia variedad de productos que difieren en formulación, ingredientes o condiciones de procesamiento. En algunos de estos productos se produce una fermentación espontánea por parte de levaduras y de bacterias ácido-lácticas que están presentes de manera natural en la harina. Sin embargo, en la mayoría de ellos, la levadura se añade con el fin de obtener un producto de características constantes y con la calidad requerida por el consumidor. La principal función de la levadura es la producción de CO_2, que resulta en un incremento del volumen de la masa. La levadura también contribuye al aroma del pan debido a la formación tanto de compuestos volátiles, como de precursores de aromas durante la fermentación de la masa además de intervenir en las propiedades reológicas del pan.

El método tradicional de preparación de una masa panaria consiste en la mezcla de todos sus ingredientes, harina, agua, levadura y sal, manual o mecánicamente. El amasado permite la interacción de todos los ingredientes de la masa poniendo a disposición de la levadura una serie de nutrientes que serán metabolizados en su mayoría por un proceso de fermentación en condiciones

de microaerofília a 28 -30º C. Éste comienza con el metabolismo de los azúcares libres aportados por la harina, principalmente glucosa, fructosa, maltosa, sacarosa y una variedad de glucofructanos (Oura y col., 1979; Burrows, 1979). Una vez son consumidos éstos, la fermentación continua por el consumo de la maltosa liberada a partir del almidón, componente principal del endosperma de los granos de trigo, por parte de α y β-amilasas. La cantidad de maltosa liberada representa hasta un 3% del peso de la harina y es, por tanto, la fuente de carbono más importante para sostener la fermentación panaria (Burrows, 1979). No es de extrañar por tanto, que las cepas de levadura de panadería hayan sido seleccionadas, entre otras propiedades, en virtud de una elevada tasa de hidrólisis de este disacárido.

2.-CEPAS INDUSTRIALES DE LEVADURA DE PANADERÍA

Las levaduras comerciales de panadería son cepas de *Saccharomyces cerevisiae* que han sido seleccionadas por el hombre durante años por su buena capacidad fermentativa y optimizadas para su aplicación en panadería. Son hongos unicelulares pertenecientes a la clase *Ascomycetes*, en su mayoría homotálicas, con un alto e irregular grado de ploidía y heterocigosis y con una reducida capacidad de esporulación, originando pocas ascas con cuatro esporas, la mayoría de las cuales son inviables (Evans 1990; Codón y col., 1998). Además, presentan polimorfismos en la longitud de sus cromosomas así como reordenaciones cromosómicas con múltiples translocaciones (Codón y col., 1997). Esto hace que las cepas industriales de levadura no sean susceptibles de un análisis genético tradicional, lo que dificulta identificar genes responsables de sus propiedades fermentativas.

Las propiedades fundamentales que deben poseer las cepas industriales de panadería están relacionadas tanto con su producción industrial como con el proceso de panificación en el que van a ser empleadas. Con respecto al proceso de obtención de la levadura, ésta debe presentar un crecimiento rápido y un

elevado rendimiento de biomasa en medio de melaza, el cual es utilizado en la producción industrial de la levadura. Por otro lado, debe tener una elevada actividad fermentativa en masas panarias y su utilización debe dar como resultado la obtención de un producto estable, de sabor y olor agradables para el consumidor. El poder fermentativo de una levadura en una masa panaria depende tanto de la composición de la harina como de las características intrínsecas de la propia levadura. Entre estas últimas destaca el nivel enzimático de maltasa. Si la masa es azucarada (20-30% de sacarosa), la levadura deberá mostrar, un nivel de actividad invertasa reducido. La invertasa metaboliza la sacarosa en glucosa y fructosa. Si el nivel es alto, se produce una rápida liberación de glucosa en el medio extracelular de la levadura, con el consiguiente aumento de la presión osmótica en el exterior celular lo que impide o ralentiza el crecimiento celular y por tanto su capacidad fermentativa.

3.- ESTRÉS OSMÓTICO Y TOXICIDAD DEL SODIO

El uso industrial de la levadura implica unas condiciones muy diferentes a las de su hábitat natural por lo que deben ser capaces de adaptarse a ambientes cambiantes así como resistir distintos tipos de estrés. Como cualquier organismo vivo, *S. cerevisiae* tiene unas condiciones óptimas de crecimiento en lo que se refiere a disponibilidad de nutrientes y condiciones medioambientales. En concreto, su crecimiento es máximo cuando se encuentra en un medio rico en azúcares fácilmente fermentables, a pH en torno a 5 y a una temperatura entre 25-28º C. Sin embargo, estas condiciones son muy diferentes a las que se encuentran tanto en el proceso de obtención como durante su utilización para la elaboración de masas. En estos procesos, la levadura se encuentra sometida a diferentes fuentes de estrés que afectan tanto a la producción de biomasa como al proceso de panificación. La habilidad de la levadura de panadería *S. cerevisiae* para resistir a diferentes condiciones de estrés es clave en muchas aplicaciones de la industria panadera.

3.1. FUENTES DE ESTRÉS OSMÓTICO

3.1.1.- PRODUCCIÓN INDUSTRIAL DE LEVADURA

La producción industrial de levadura se realiza utilizando fuentes de carbono de bajo coste como son las melazas procedentes de caña de azúcar o de remolacha. Sus principales componentes son agua y carbohidratos (sacarosa, glucosa y fructosa), además de sales y otros solutos. Esto hace que durante el proceso de propagación las células de levadura se vean sometidas a un exceso de sales con la consecuente reducción de la actividad de agua. Estas condiciones de estrés osmótico son máximas cuando, una vez alcanzada la biomasa esperada, ésta se separa del medio, se lava y se procesa para obtener levadura en crema, en bloque (levadura prensada) o seca (levadura seca activa). En cualquiera de estos tres formatos se elimina un porcentaje muy elevado del agua extracelular lo cual supone un descenso drástico de la actividad de agua (Attfield, 1997).

3.1.2.- ELABORACIÓN DE MASAS DULCES

Otra situación en la que la levadura es sometida a duras condiciones de estrés, es la alta presión osmótica que presentan las masas dulces utilizadas para productos de bollería (Attfield, 1997; Randez-Gil y col., 1999). Los altos niveles de sacarosa empleados en su elaboración (de hasta un 30%), generan un ambiente de elevada presión osmótica que limita el crecimiento de las cepas de *S. cerevisiae* (Myers y Attfield, 1999) y su capacidad fermentativa. La presencia de otros ingredientes como margarina o leche en polvo supone un aumento adicional de la presión osmótica. Además, la adición de sal, con niveles de hasta un 2%, ejerce un estrés adicional debido a la toxicidad de los iones, ya que la presencia de iones Na^+ y Cl^- en el citoplasma tiene un efecto tóxico para muchos sistemas enzimáticos de la célula. (Serrano, 1996). Como consecuencia, el tiempo de fermentación se prolonga y el volumen final del

producto se reduce (Myers y Attfield, 1999). Esto obliga a los industriales a utilizar una dosis extra de levadura en las formulaciones (en torno a un 6-12%, base harina), con el consiguiente incremento del coste del producto. A su vez, esta práctica modifica las características organolépticas del producto final, lo cual genera un rechazo por parte del consumidor. Para evitar esto, una cepa de levadura industrial destinada a la elaboración de masas dulces debería exhibir tolerancia a estrés osmótico y resistencia a la toxicidad del sodio.

3.2.- RESPUESTA A ESTRÉS OSMOTICO EN *S. cerevisiae*

La respuesta a estrés osmótico y a estrés salino en *Saccharomyces cerevisiae* ha sido ampliamente caracterizada (Blomberg, 2000; Hohmann, 2002a; Hohmann, 2002b; Mendizábal y col., 1998; Rios y col., 1997; Serrano, 1996). El aumento en la osmolaridad del medio extracelular, tiene como consecuencia inmediata la pérdida de agua intracelular acompañada de una parada en el crecimiento de la célula. La pérdida de agua a través de la membrana plasmática genera una pérdida de turgencia de las células y una disminución en el tamaño de las mismas (Hohmann, 1997). La respuesta de *S. cerevisiae* frente al aumento de osmolaridad en el medio es de dos tipos, una respuesta inmediata que hace recuperar la turgencia celular impidiendo así la muerte, y una respuesta a medio-largo plazo que permite la adaptación y posterior crecimiento de la célula en el nuevo medio (Figura1).

3.2.1.-LA RESPUESTA PRIMARIA: BALANCE OSMÓTICO

La respuesta inmediata consiste en la entrada de agua extracelular, la formación de vacuolas y el bloqueo de canales de exclusión de osmolitos compatibles. Los osmolitos compatibles son componentes de bajo peso molecular, altamente solubles en agua y que pueden acumularse en el citoplasma de la célula, incluso a altas concentraciones, sin interferir con las rutas metabólicas celulares. Cuando una célula es sometida a un medio de alta

concentración osmótica, se produce una diferencia de potencial osmótico entre el interior y el exterior celular que tiene como consecuencia la salida de agua de la célula. La acumulación de osmolitos aumenta el potencial osmótico del interior celular, haciendo que se iguale con el de su exterior. Los osmolitos compatibles más utilizados son los polialcoholes, siendo el glicerol el osmolito acumulado por *S. cerevisiae* (Hohmann, 1997).

La acumulación bajo condiciones de estrés osmótico, de otros osmolitos como son eritritol, ribitol, arabinitol, xylitol, sorbitol, manitol y galactitol ha sido descrita en otras especies de levaduras y hongos por distintos autores (Blomberg y Adler, 1992). En *S. cerevisiae*, la acumulación de glicerol es controlada a dos niveles, retención y producción. La retención de glicerol forma parte de la respuesta inmediata de la célula frente a un choque osmótico. Se ha caracterizado la proteína Fps1p como una proteína canal responsable del eflujo de glicerol (Luyten y col., 1995). La presencia este canal permite a la célula controlar y ajustar la permeabilidad de la membrana al glicerol. En condiciones de estrés osmótico, el canal está cerrado y el glicerol se acumula en el interior celular. Posteriormente el canal se abre y se cierra sucesivamente, con objeto de controlar el nivel de glicerol (Tamas y col., 1999).

3.2.2. LA RESPUESTA SECUNDARIA: RUTAS DE TRANSDUCCIÓN DE SEÑAL

Dentro de los mecanismos de respuesta a medio-largo plazo, juega un papel importante la activación transcripcional de genes que habitualmente no se expresan bajo condiciones normales de crecimiento o lo hacen a niveles bajos. La modificación de la expresión génica tras un choque osmótico está mediada por diferentes mecanismos (Rep y col., 1999a; Rep y col., 1999b; Rep y col., 2000). El análisis global de la expresión génica en *S. cerevisiae* ha puesto de manifiesto la activación de entre 250-400 genes cuando las células son sometidas a diferentes condiciones de estrés osmótico y salino. La activación de

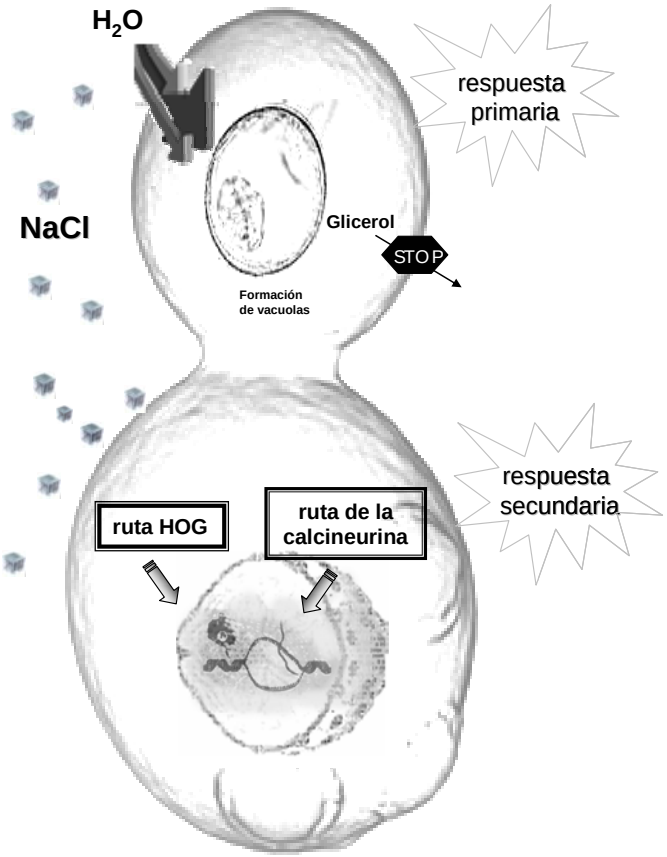


Figura 1. Representación esquemática de las respuestas primaria y secundaria de *S. cerevisiae* frente a un choque salino

estos genes tiene como consecuencia la síntesis de proteínas que cumplen una amplia variedad de funciones fisiológicas (Rep y col., 2000; Posas y col., 2000; Yale y Bohnert., 2001). En *S. cerevisiae*, esta activación transcripcional está mediada mayoritariamente a través de dos rutas de señalización celular como son la ruta HOG (por High Osmolarity Glycerol) (Hohmann, 2002a; de Nadal y col., 2002; Westfall y col., 2004; O'Rourke y col., 2002) y la ruta de la calcineurina (Rusnak y Mertz, 2000; Yoshimoto y col., 2002; Cyert, 2003).

3.2.2.1. La ruta HOG: estrés osmótico

La ruta HOG esta implicada, en mayor o menor grado, en la expresión de unos 300 genes regulados por estrés osmótico (Rep y col., 2000). Se trata de una ruta de transducción de la señal, formada por proteínas quinasas, las denominadas MAPKs (por Mitogen Activated Protein Kinase). En *S. cerevisiae* se conocen al menos cinco rutas de señalización formadas por MAPK las cuales responden a diferentes estímulos externos (revisado en Gustin y col., 1998). Las rutas de MAPK consisten en una cascada de fosforilaciones sucesivas de tres proteínas quinasas denominadas MAP quinasa quinasa quinasa (MAPKKK), MAP quinasa quinasa (MAPKK) y MAP quinasa (MAPK). Se han caracterizado dos proteínas de membrana que participan en el mecanismo de percepción por el cual la célula detecta un cambio en la osmolaridad del medio, los sensores Sln1p y Sho1p. Recientemente, se ha caracterizado un tercer sensor, Msb2p, el cual parece tener un papel redundante al de Sho1p (O´Rourke y col., 2002). El primero de los osmosensores, Sho1p, requiere de los factores Ste11p, Ste50p, Ste20p y la GTPasa Cdc42 para la transmisión de la señal bajo condiciones de estrés osmótico (Maeda y col., 1995; O´Rourke y Herskowitz, 1998; Posas y col., 1998; Raitt y col., 2000; Reiser y col., 2000). En éstas condiciones, Sho1p recluta temporalmente a Pbs2p formándose un complejo unido a la membrana con Ste11p, Ste50p, Ste20p. La formación del complejo permite la fosforilación de Pbs2p por parte de la MAPKKK, Ste11p. Esto provoca que Pbs2p se libere del complejo y fosforile a su vez a Hog1p (Reiser y col., 2000). Se considera que el otro osmosensor es la proteína Sln1p la cual forma junto con Ypd1p y Ssk1p un "sistema de dos componentes" (Maeda y col., 1994). Bajo condiciones normales de crecimiento, las tres proteínas están fosforiladas (Posas y col., 1996) y al aumentar la osmolaridad del medio se desfosforilan sucesivamente lo que provoca que Ssk1p se una a Ssk2-22p (MAPKKK) activándola y disparando así la cascada de fosforilaciónes hasta llegar a Hog1p a través de Pbs2 (MAPKK).

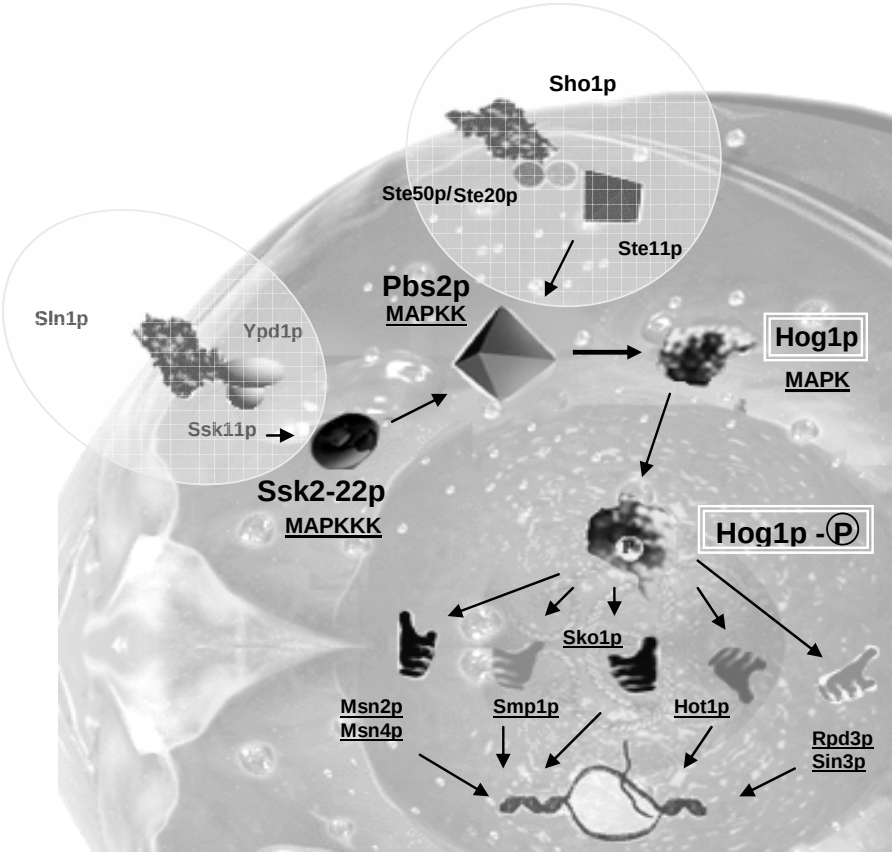


Figura 2. Representación esquemática de la ruta HOG

De este modo el sistema Sln1p-Ypd1-Ssk1p ejerce una regulación negativa sobre la ruta (Posas y Saito, 1998).

La fosforilación de Hog1p, permite su entrada transitoria al núcleo celular (Ferrigno y col., 1998; Reiser y col., 1999). Una vez dentro, Hog1p fosforila a su vez a diversos factores transcripcionales, los cuales se unen a regiones específicas de los promotores de los genes que van a activar. Msn2p/Msn4p, Msn1, Hot1 (Rep y col., 1999a), Smp1p (de Nadal y col., 2003) o Sko1 (Proft y Serrano, 1999) son algunos factores transcripcionales sobre los que actúa

Hog1p. Estos factores transcripcionales inducen la expresión de genes como *GPD1* (glicerol fosfato deshidrogenasa), *CTT1* (catalasa T citosólica) o *ENA1* (bomba de sodio y litio de la membrana plasmática) los cuales son diana de Msn2/Msn4, Hot1p y Sko1p respectivamente. Hay evidencias de que Hog1p no ejerce su acción únicamente a través de la activación de factores de transcripción mediante fosforilación sino que Hog1p también puede unirse al promotor de algunos genes facilitando la entrada de la maquinaria de transcripción (Alepuz y col., 2003; de Nadal y col., 2004; Proft y Struhl 2002).

3.2.2.2. La ruta de la calcineurina: toxicidad del sodio

La adición de NaCl en el medio de cultivo de la levadura, no sólo produce un estrés osmótico por el incremento de la diferencia del potencial osmótico de la membrana, sino que además tiene un efecto nocivo por la toxicidad de los iones. La acumulación de iones Na^+ y Cl^- en concentraciones de 50-100mM en el citoplasma, tiene efectos negativos sobre muchos sistemas enzimáticos (Serrano y col., 1997). Mientras que el potasio entra en la célula a través de canales específicos de baja afinidad, Trk1p y Trk2p (Ko y col., 1990; Ko y Gaber, 1991), el sodio carece de transportadores propios para su entrada en la célula y aparentemente entra en esta a través de los sistemas de transporte de potasio, cuya capacidad de discriminación entre ambos iones es limitada (Ramos y col., 1990). En presencia de altas concentraciones de Na^+, y a fin de evitar su entrada, el sistema de baja afinidad Trk1,2p se convierte en un sistema de alta afinidad de potasio, para aumentar la capacidad de discriminación entre ambos iones (Ramos y col., 1994). Paralelamente se induce un sistema de bombeo para expulsar el sodio intracelular. El principal sistema de detoxificación es la ATPasa de tipo P codificada por el gen *ENA1/PMR2*, la cual es capaz de bombear cationes Na^+ y Li^+ al exterior celular (Haro y col., 1991). El control de la expresión de *ENA1* está regulado por distintos mecanismos y su transcripción está, bajo condiciones normales de crecimiento, reprimida por la unión de inhibidores a su promotor.

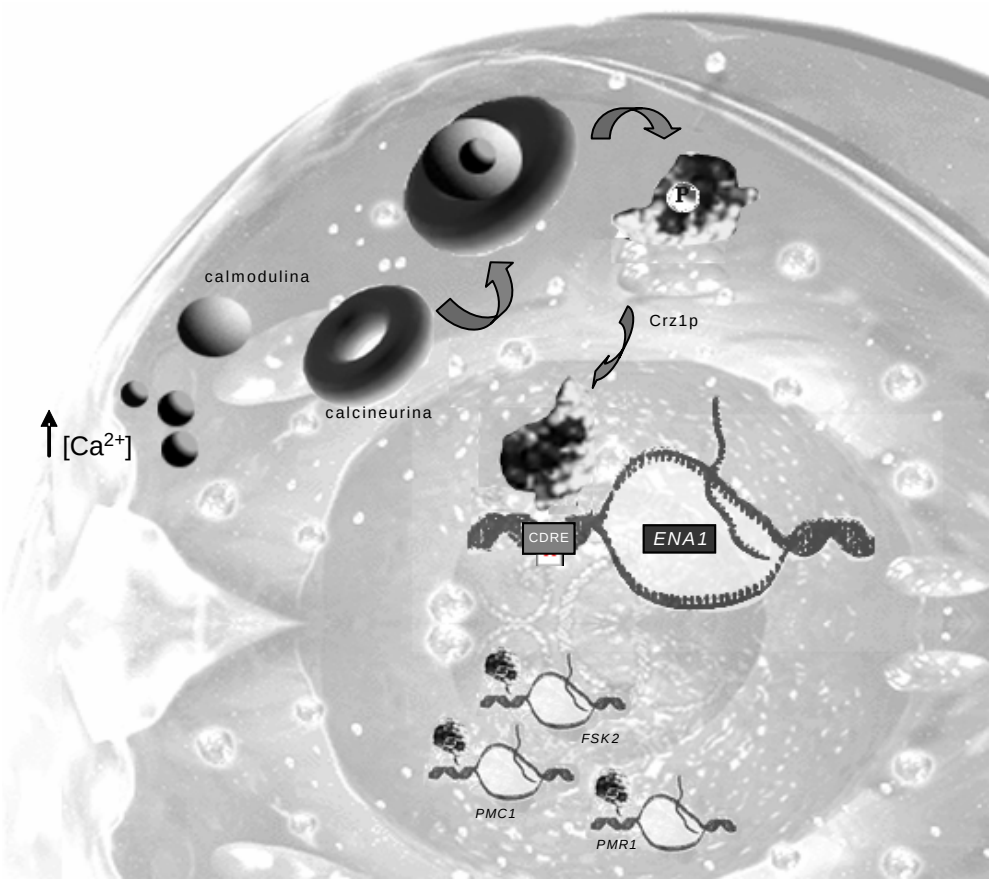


Figura 3. Representación esquemática de la ruta de la calcineurina

La expresión de *ENA1* está reprimida en presencia de glucosa en el medio. En estas condiciones los represores Mig1-2p están unidos junto con el complejo corepresor Ssn6p-Tup1p a las secuencias llamadas $URS_{MIG1\text{-}ENA1}$ del promotor de *ENA1* impidiendo su transcripción (Alepuz y col., 1997; Proft y Serrano, 1999). Este control está mediado por la proteína quinasa Snf1p y es totalmente independiente de la ruta HOG y de la ruta de la calcineurina (Alepuz y col., 1997).

Introducción

En respuesta a estrés osmótico o estrés salino moderado, la expresión de *ENA1* es activada por la ruta HOG que actúa a través del represor transcripcional Sko1p (Proft y Serrano, 1999; Marquez y col., 1998; Pascual-Ahuir y col., 2001). Este también se une, junto con el complejo corepresor Ssn6p-Tup1, a secuencias específicas del promotor de *ENA1* llamadas $URS_{CRE-ENA1}$ y en este caso es la proteína quinasa Hog1p la encargada de fosforilar a Sko1p permitiendo su liberación del promotor y por tanto la transcripción de *ENA1* (Proft y Serrano, 1999; Pascual-Ahuir y col., 2001).

Cuando las células son sometidas a un estrés salino severo, adicionalmente a la ruta HOG, se activa la ruta de la calcineurina, siendo ésta responsable de la inducción específica por sodio de *ENA1* (Marquez y Serrano, 1996; Proft y Serrano, 1999). La regulación de la transcripción de *ENA1* en condiciones de estrés salino también ha sido relacionada con la ruta TOR (de Target Of Rapamycin) a través de los factores de transcripción Gln3p y Gat1p (Crespo y col., 2001).

La calcineurina se activa como respuesta a un aumento en el contenido intracelular de Ca^{2+}, vía calmodulina (Nakamura y col., 1993; Mendoza y col., 1994), el receptor más importante de este catión. La calcineurina es una proteína fosfatasa del tipo 2B que actúa sobre residuos de serina y treonina, cuya función es dependiente de Ca^{2+} y calmodulina. La calcineurina está ampliamente conservada de mamíferos a otros eucariotas. En levadura, está implicada en distintos procesos como son la síntesis de compuestos de la pared celular (Garrett-Engele y col., 1995), el control del ciclo celular (Mizunuma y col., 1998) y es esencial para la tolerancia a estrés iónico (Rusnak y Mertz, 2000).

El análisis global de la expresión génica de células de levadura en condiciones de estrés iónico con Ca^{2+} y Na^{+}, muestra que hay 163 genes regulados por la ruta de la calcineurina (Yoshimoto y col., 2002). Los productos de estos genes

participan en una amplia variedad de procesos celulares, como el equilibrio de concentraciones de iones, la síntesis de la pared celular, el metabolismo de lípidos y esteroles o el transporte de vesículas (Yoshimoto y col., 2002). La calcineurina no es una proteína esencial en *S. cerevisiae* excepto bajo determinadas condiciones de estrés. Levaduras deficientes en calcineurina crecen pobremente en presencia de altas concentraciones de iones como Mn^{2+}, Na^{+}, Li^{+} e OH^{-} (Nakamura y col., 1993; Mendoza y col., 1994; Farcasanu y col., 1995; Pozos y col., 1996). La calcineurina se requiere para la activación transcripcional de varias bombas de iones en la célula como *ENA1*. Además controla la transcripción de *PMC1* (bomba de Ca^{2+} vacuolar) y de *PMR1* (bomba de Mn^{2+} y Ca^{2+} del aparato de Golgi). Es una proteína oligomérica formada por una subunidad catalítica A, codificada por dos genes funcionalmente redundantes (*CNA1* y *CNA2*) y una subunidad reguladora, codificada por *CNB1* (Cyert y col., 1991; Cyert y Thorner, 1992; Kuno y col., 1991; Liu y col., 1991).

La calcineurina en *S. cerevisiae*, controla también la transcripción de *FKS2*, el cual se induce por la adición de Ca^{2+} al medio siendo esta inducción dependiente de calcineurina. *FKS2* codifica para la subunidad catalítica de la beta-1,3-glucano sintasa, la cual sintetiza beta-1,3-glucano, componente principal de la pared celular de la levadura (Mazur y col., 1995). Fue en el promotor de *FKS2* donde se identificó la secuencia CDRE (calcineurin dependent response element) (Stathopoulos y Cyert, 1997). Esta secuencia consta de 24 pb y es necesaria y suficiente para la transcripción dependiente de calcineurina de *FSK2* (Stathopoulos y Cyert, 1997). Otras secuencias, conocidas como UAS_{ENA1} han sido identificadas en el promotor de *ENA1* como responsables de la inducción de la transcripción dependiente de calcineurina (Mendizábal y col., 2001). A todas estas secuencias se une un factor transcripcional conocido como Crz1p (Tcn1p). Este fue aislado e identificado por su capacidad de activar la transcripción dependiente de calcineurina de *FKS2* por su unión a la secuencia CDRE. Crz1p es sustrato de la calcineurina (Stathopoulos y Cyert, 1997). Ésta defosforila a Crz1p como respuesta al

aumento de la concentración de Ca^{2+} que se da en el citoplasma bajo distintas situaciones. Una vez desfosforilado, Crz1p cambia su localización celular pasando del citoplasma al núcleo (Statophoulos-Gerontides y col., 1999), y allí se une a los promotores de diversos genes como *ENA1*, *PMR1*, *PMC1* o *FKS2* para activar su transcripción. Estudios recientes de expresión génica muestran que Crz1p es el mayor y probablemente el único factor transcripcional dependiente de calcineurina de la levadura *in vivo* (Yoshimoto y col., 2002). También se ha descrito que Crz1p es fosforilado, y por tanto inactivado, por Hrr25p, una caseína quinasa I (Kafadar y col., 2003) además de por la PKA (de Protein Kinase A) (Kafadar y Cyert, 2004).

3.3.-ESTRATEGIAS PARA LA MEJORA DE LA OSMOTOLERANCIA EN LEVADURAS DE PANADERÍA

Numerosos esfuerzos se han dirigido a la obtención de cepas de levadura de *S. cerevisiae* con un mejor comportamiento en masas dulces. Un aspecto importante en estas masas es el nivel de actividad invertasa que presentan las cepas de panadería. Contrariamente a lo que se podría pensar, cepas con una elevada actividad invertasa (levaduras de fermentación "rápida" en masas convencionales), exhiben una producción baja de CO_2 en masas dulces. Este fenómeno se ha relacionado con una situación de bloqueo metabólico motivado por una entrada masiva de azúcares en la célula y su posterior fosforilación, lo que implica una deplección en los niveles de ATP de la célula (Thevelein y Hohmann, 1995). A esto hay que añadir el aumento de la presión osmótica que se produce como consecuencia de la hidrólisis extracelular de la sacarosa del medio, sometiendo a la levadura a un estrés osmótico. Por tanto, independientemente de los niveles de invertasa, también juega un papel importante la osmotolerancia intrínseca mostrada por distintas cepas (Oda y Ouchi, 1990). Por esta razón se han seleccionado cepas con baja actividad invertasa (cepas de fermentación "lenta") para su utilización en masas dulces (Clement y Loiez, 1983). Estas cepas son adecuadas para la elaboración de

masas con contenidos en sacarosa en torno al 8-10%. Sin embargo, para la fermentación de masas con contenidos mayores de azúcar (20-30%), donde la presión osmótica es mucho más elevada, las levaduras necesitan mostrar tolerancia a estrés osmótico.

Un contenido elevado en glicerol en la célula, está directamente relacionado con la osmotolerancia y la capacidad fermentativa en masas dulces (Myers y Attfield, 1999; Attfield y Kletsas, 2000). Se ha demostrado que células pre-estresadas antes del proceso de panificación acumulan glicerol, lo que aumenta su osmotolerancia (Hirasawa y Yokoigawa, 2001). Del mismo modo una incubación de las levaduras previa al proceso de panificación en soluciones de glicerol, también aumenta el contenido de éste y por tanto la osmotolerancia de la cepa (Myers y col., 1997). Sin embargo, estos procesos son laboriosos y a menudo costosos, por tanto son difíciles de aplicar a nivel industrial. Todo esto hace que las estrategias utilizadas se basen en la mejora genética de la propia levadura y no en su procesamiento.

Uno de los frentes abordados para conseguir aumentar la osmotolerancia de cepas industriales, se ha basado en la posible ingenierización del metabolismo del glicerol en *Saccharomyces cerevisiae*, mediante un aumento de su producción y una mejor retención del mismo. Sin embargo, estas aproximaciones han resultado infructuosas. La expresión constitutiva en ausencia de estrés de una proteína Hog1p hiperactiva, reduce la tasa de crecimiento y favorece la agregación de células y la floculación en condiciones óptimas de crecimiento (Bell y col., 2001). La activación de la ruta de MAP-quinasas, bien por la sobreexpresión de las quinasas que participan en la cascada de fosforilación o por el encendido permanente de éstas por disrupción del sensor Sln1p, resultó letal para la célula (Maeda y col., 1994).

La sobreexpresión de *GPD1* apenas aumenta el contenido de glicerol intracelular (Albertyn y col., 1994). La disrupción de *FSP1* o de éste en

combinación con la sobreexpresión *GPD1* (Luyten y col., 1995; Philips y Herskowitz, 1997; Tao y col., 1999), tiene un efecto negativo en el crecimiento de la levadura (Remize y col., 2001). Por otro lado, la expresión de una proteína Fps1p con el extremo C-terminal truncado, permite a la célula acumular más glicerol en condiciones de estrés osmótico, pero éste hecho es letal para la célula en condiciones de crecimiento isotónico (Tamas y col., 1999). Por tanto la alteración de la síntesis y/o acumulación de glicerol en levadura tiene claros efectos dañinos sobre su crecimiento. En este sentido cabe recordar que el metabolismo del glicerol juega un papel importante en varios aspectos celulares, además del de la osmoprotección.

Osmolitos compatibles diferentes al glicerol, como azucares, alcoholes o aminoácidos han sido aislados de microorganismos halotolerantes (da Costa y col., 1998). El estudio de la acumulación de estos metabolitos podría ser interesante; sin embargo hay que tener en cuenta que su acumulación puede tener un umbral máximo y que no siempre dicha acumulación se correlaciona con un aumento en la osmotolerancia (Apse y Blumwald, 2002).

La acumulación de determinadas proteínas en la membrana plasmática, protegen a esta de la desecación que tiene lugar cuando la célula está en un medio hipersalino. Una de éstas proteínas es Hsp12, cuya sobreexpresión confiere protección frente a la desecación y a estrés producido por la presencia de etanol en el medio (Sales y col., 2000). Por último, genes implicados en procesos de expulsión de Na^+ intracelular, así como de su compartimentación en vacuolas, son firmes candidatos para su manipulación genética con el fin de obtener levaduras mas osmotolerantes (Benito y col., 1997).

4.- ESTRÉS POR CONGELACIÓN

4.1 MASAS CONGELADAS

Durante las últimas décadas, la producción de masas congeladas para panadería y especialmente para bollería, ha experimentado un notable incremento. El mercado de las masas congeladas en España se inició en los años 90 principalmente en la Comunidad Valenciana y Cataluña. El desarrollo de supermercados, centros comerciales y de ocio impulsaron la creación de los denominados "puntos calientes" en los que es posible encontrar pan y bollería recién horneados. Este fenómeno ha favorecido el desarrollo de la industria de las masas congeladas. Este tipo de industria supone una modificación de los sistemas de fabricación y venta del producto. Son muchas las ventajas asociadas al uso comercial de masas congeladas. Por una parte permite una flexibilidad de horario para el panadero ya que puede almacenar el producto y hornearlo al momento. Con respecto al consumidor, le permite tener disponible a cualquier hora productos recién hechos. Sin embargo, el desarrollo de una tecnología que permita la fabricación de un producto de calidad similar al obtenido con masas frescas, se ha visto limitado por la sensibilidad al proceso de congelación-descongelación de las cepas de levadura de *S. cerevisiae* (Attfield, 1997; Randez-Gil y col., 1999) y a la reducción de su capacidad fermentativa en masas congeladas (Gelinas y col., 1993; Hatano y col., 1996) además de por la alteración física que sufre la red de gluten. Si las masas son azucaradas, el problema es todavía mayor, ya que se suma el efecto negativo que supone la adición de azucares al medio con el consiguiente aumento de la presión osmótica exterior. La resistencia a la congelación es una característica esencial de aquellas levaduras que se pretenden usar tanto en masas congeladas como en masas congeladas y además azucaradas. La pérdida de viabilidad de las células sometidas a procesos de congelación-descongelación se atribuye a la deshidratación celular provocada por un aumento del estrés

osmótico. La congelación del agua del medio da lugar a un aumento de la concentración de los electrolitos disueltos en ella antes de la congelación. (Hatano y col., 1996; Myers y Attfield, 1999). Por otro lado el almacenamiento en congelación da lugar a la formación y crecimiento de cristales de hielo, los cuales provocan daños celulares por rotura de membranas con la consecuente pérdida de la organización subcelular (Grout y col., 1990). La obtención de cepas de levadura de panadería resistentes a estrés osmótico y a estrés por congelación supondría grandes ventajas para la industria de bollería congelada ya que conllevaría un ahorro económico como consecuencia de la disminución de la dosis de levadura, así como la comodidad añadida inherente al uso de una misma cepa para distintos procesos.

4.2 ESTRATEGIAS PARA AUMENTAR LA CRIORESISTENCIA EN *S. cerevisiae*

La obtención de cepas de levadura de panadería de *S. cerevisiae* más crioresistentes se ha realizado hasta la fecha mediante selección tras ciclos sucesivos de congelación-descongelación (Oda y Tonomura, 1993; Almeida y Pais, 1996a), por hibridación (Nakagawa y Ouchi, 1994; Codón y col., 2003) o mediante mutación (Takagi y col., 1997; van Dijck y col., 2000; Teunissen y col., 2002). El estudio de las características de estas cepas ha permitido correlacionar la crioresistencia con algunos factores celulares como son la acumulación de trehalosa o la composición en ácidos grasos poliinsaturados de la membrana plasmática. Actualmente se están desarrollando distintas estrategias para construir nuevas cepas crioresistentes basadas en la disrupción o sobreexpresión de genes implicados en las rutas de síntesis y/o degradación de éstos compuestos (Kim y col., 1996; Shima y col., 1999; Izawa y col., 2004; Sakamoto y Murata 2002; Rodríguez-Vargas, 2003). La sobreexpresión en *S. cerevisiae* de aquaporinas, proteínas implicadas en la entrada y salida de agua, (Tanghe y col., 2002), así como de proteínas anticongelantes de peces también se ha utilizado como estrategia para obtener cepas de levadura más resistentes

a la congelación. (Panadero J, Rández-Gil F, Prieto JA, resultados no publicados).

5. LEVADURAS NO CONVENCIONALES. *Torulaspora delbrueckii*

La capacidad de fermentar una masa panaria no es exclusiva de *S. cerevisiae*, otras especies de levadura son capaces de producir gas y dotar de volumen a una masa panaria. Levaduras osmotolerantes pertenecientes a los géneros *Zygosaccharomyces, Pichia* o *Torulaspora* son frecuentemente encontradas en masa agrias utilizadas para la elaboración de panes tradicionales (Almeida y Pais, 1996a). La levadura *Torulaspora delbrueckii* (sin. *Saccharomyces rosei*) se encuentra frecuentemente en bebidas alcohólicas, zumos de fruta y alimentos con alto contenido en azúcares (Esteve-Zarzoso y col., 2001), así como en aislados de masas espontáneas (Almeida y Pais, 1996a). Esta especie es empleada habitualmente en países como Japón, para la fermentación panaria. Cepas de *T. delbrueckii* aisladas del suelo, grano o masas panarias han sido también seleccionadas por su elevada resistencia a estrés por congelación (Hahn y Kawai, 1990). No obstante, en la mayoría de los casos, las cepas estudiadas no combinan de forma simultánea crioresistencia y producción de CO_2. Sin embargo, dos cepas de este organismo, PYCC5321 y PYCC5323, aisladas de masas de centeno y maíz han sido caracterizadas por su elevada viabilidad y capacidad fermentativa en masas congeladas (Almeida y Pais, 1996b). Filogenéticamente *T. delbrueckii* se clasifica como un taxón diferente de *S. cerevisiae* por sus diferencias en la formación de ascas (Vaughan-Martini y Martín, 1996). Estudios de cariotipado, mediante electroforesis de campo pulsante, muestran que *T. delbrueckii* presenta 6 cromosomas de tamaños comprendidos entre 800-1600 Kb (Oda y Tonomura, 1995). De morfología oval a globular su tamaño es menor que el de *S. cerevisiae*. Pese a estas diferencias

ambas especies presentan una alta homología en la secuencia de sus genes (James y col., 1996; Oda y col., 1997; Belloch y col., 2000).

Justificación y Objetivos

Los hábitos de la población están cambiando y con ellos las exigencias del consumidor. En el campo de la panadería esto se ha traducido en la demanda de mayor variedad de productos así como de productos "listos para su uso". Esto ha supuesto que la producción de masas congeladas para panadería y sobre todo para bollería, haya experimentado un fuerte crecimiento en los últimos años. Sin embargo este desarrollo se ha visto limitado, en parte, por la inexistencia en el mercado de cepas de levadura tolerantes a la congelación y a las altas concentraciones de azúcares presentes en las masas para bollería.

La solución a este problema puede venir desde dos frentes bien diferentes. Por un lado, la construcción mediante técnicas de ingeniería genética de cepas de panadería con mayor tolerancia a congelación y estrés osmótico. Esto requiere tener un conocimiento previo de las bases moleculares que regulan ambos fenómenos, por no hablar de los problemas que implicaría en la actualidad, debido a las reticencias sociales que plantea el uso de los organismos modificados genéticamente. Pese a todos los esfuerzos dirigidos a aumentar la osmotolerancia y crioresistencia de las cepas de panadería, no existen en el mercado cepas que reúnan simultáneamente estas características y a la vez sean capaces de mantener su capacidad para producir el CO_2 requerido durante la fermentación.

La otra solución seria la utilización de levaduras con características intrínsecas de crioresistencia y osmotolerancia. Estas levaduras deberían exhibir además de una buena capacidad fermentativa, una elevada velocidad de crecimiento en medios industriales de melaza y un alto rendimiento en biomasa. Cepas de levadura de *T. delbrueckii* fueron aisladas de masas caseras de centeno y maíz (Almeida y Pais, 1996a) y han sido caracterizadas por su elevada viabilidad y capacidad fermentativa en masas congeladas (Almeida y Pais, 1996b). No obstante, no se ha determinado la tolerancia a la congelación de éstas cepas propagadas en condiciones industriales. Otros aspectos interesantes de ser esclarecidos son su osmotolerancia y comportamiento en masas azucaradas, tanto frescas como congeladas.

Justificación y Objetivos

Durante los últimos años, las técnicas de ingeniería genética han permitido la construcción de cepas de panadería de *S. cerevisiae* con nuevas características deseables en la elaboración de masas panarias y en concreto, cepas que producen proteínas o enzimas que afectan positivamente a la reología y vida media del producto final (Rández-Gil y col., 2003). Dado la posible aplicación industrial de *T. delbrueckii*, es importante conocer su capacidad de producir proteínas heterólogas. Sin embargo, el uso de técnicas de ADN-recombinante en este microorganismo es limitado, ya que se carece de las herramientas moleculares necesarias. Además, la mayoría de cepas de *T. delbrueckii* son protótrofas. En consecuencia, su transformación mediante complementación de marcadores auxótrofos, el método más común en *S. cerevisiae*, no puede ser empleado. En este escenario, la obtención de cepas auxótrofas de *T. delbrueckii* se convierte en un objetivo prioritario.

Nuestro interés por *T. delbrueckii* va más allá de su utilización como levadura de panadería en procesos industriales. Su elevada tolerancia a la congelación y el hecho de que son levaduras que se encuentran frecuentemente en alimentos y bebidas con altas concentraciones de azúcares (Esteve-Zarzoso y col., 2001), nos lleva a proponer a esta especie como modelo de estudio de tolerancia a estrés en levadura de panadería. *T. delbrueckii* y *S. cerevisiae* presentan una alta homología en la secuencia de sus genes (James y col., 1996; Oda y col., 1997; Belloch y col., 2000). Esto, y dada la disponibilidad del genoma completo de *S. cerevisiae*, nos facilita el uso en el laboratorio de *T. delbrueckii* como organismo modelo.

La identificación de genes implicados en la tolerancia a altas presiones osmóticas nos puede servir para esclarecer las bases moleculares de este fenotipo.

Con todo lo expuesto los objetivos planteados en este trabajo son:

1.- Caracterizar las cepas de *T. delbrueckii* PYCC5321 y PYCC5323 para su posible utilización industrial en la elaboración de masas dulces congeladas y estudiar sus fenotipos de crioresistencia y osmotolerancia.

2.- Desarrollar herramientas moleculares de aplicación en la levadura *Torulaspora delbrueckii.* Obtener una librería genómica de *T. delbrueckii* que nos permita aislar y caracterizar genes que codifican para marcadores auxotróficos y construir cepas de esta levadura interrumpidas para estos marcadores de selección.

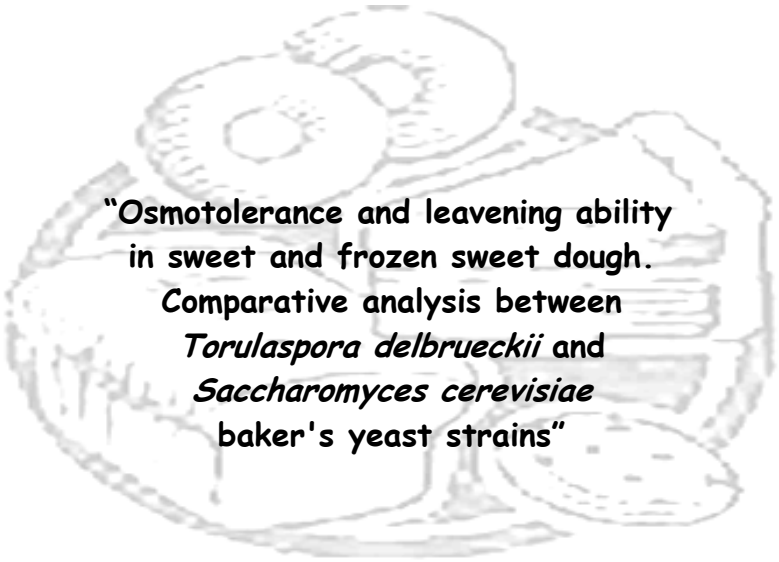
3.- Estudiar el posible empleo de *T. delbrueckii* para la expresión de genes heterólogos.

4.-Aislamiento y caracterización del *HOG1* de *T. delbrueckii* PYCC5321 y estudio de su implicación en la respuesta a estrés osmótico en esta levadura.

5.-Aislamiento, secuenciación y caracterización de genes de *T. delbrueckii* PYCC5321 que confieren una mayor osmotolerancia a cepas de *S. cerevisiae.*

Capítulo 1

"Caracterización tecnológica de cepas de *Torulaspora delbrueckii* para su uso en la elaboración de masas dulces congeladas"



"Osmotolerance and leavening ability in sweet and frozen sweet dough. Comparative analysis between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* baker's yeast strains"

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Osmotolerance and leavening ability in sweet and frozen sweet dough. Comparative analysis between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* baker's yeast strains

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Abstract

The response of *Saccharomyces cerevisiae* and freeze-tolerant *Torulaspora delbrueckii* strains to osmotic stress and their CO_2 production capacity in sweet and frozen-sweet dough has been examined. *T. delbrueckii* strains, IGC5321 and IGC5323 showed higher leavening ability than *Saccharomyces*, specially after exposure to hyperosmotic stress of bread dough containing 20% sucrose and 2% salt added. In addition, *Torulaspora* and especially *T. delbrueckii* IGC5321 exhibited no loss of CO_2 production capacity during freeze-thaw stress. Overall, these results appeared to indicate that *Torulaspora* cells are more tolerant than *Saccharomyces* to osmotic stress of bread dough. This trait correlated with a low invertase activity, a slow rate of trehalose mobilisation and the ability to respond rapidly to osmotic stress. Growth behaviour on high osmotic synthetic media was also examined. Cells of the IGC5321 strain showed intrinsic osmotolerance and ion toxicity resistance. However, *T. delbrueckii* IGC5323 exhibited a clear phenotype of osmosensitivity. Hence, this characteristic may not be essential or the only determinant for leavening ability in salted high-sugar dough.

Introduction

The ability of the baker's yeast *Saccharomyces cerevisiae* to cope with different stress conditions is a key factor in many commercial applications. One of the most extreme challenges for yeast is the high osmotic pressure encountered in sweet dough (Attfield 1997; Randez-Gil et al. 1999). These bakery products contain up to 30% added sucrose or glucose/fructose syrup as a sweetener agent, often combined with fat, powder milk and salt (Sultan 1990), which reduces the water activity of the dough.

Exposure of yeast to hyperosmotic stress results in rapid cell dehydration that limits growth and gas-production capacity (Blomberg and Adler 1992; Hohmann 1997; Varela and Mager 1996). As a consequence, the proofing time increases and the final volume of the baked product is reduced (Myers et al. 1997). This situation gets worse in frozen-sweet dough, because the water activity is reduced by the freezing and thawing processes. In addition, freezing and frozen storage of dough has a negative influence on baking performance due to freeze cell injury (Gélinas et al. 1993; Hatano et al. 1996; Neyreneuf and Van Der Plat 1991). To overcome these problems, it is common for bakers to add extra yeast to sweet and frozen-sweet dough. However, this practice increases process cost, and affects negatively the taste and texture of the product. Consequently, the development of yeast strains with better leavening capability for sweet and frozen-sweet dough production has a great economic interest.

An important trait that influences the baking performance of yeast in sweet dough is the level of invertase activity (Evans 1990). This enzyme catalyses the hydrolysis of sucrose into glucose and

fructose, increasing osmotic pressure. Therefore, baker's yeast strains with low invertase activity have been selected for sweet dough applications. These strains are suitable to elaborate dough containing low concentrations of sucrose (< 8–10%). However, these strains do not show intrinsic osmotolerance and at higher sugar contents the osmotic pressure inhibits their activity (Myers et al. 1997).

On the other hand, the addition of salt (around 2%, flour basis) to sweet dough may also cause further effects on baking performance which are different from those of osmotic stress. Indeed, accumulation of sodium and chloride in the cytosol of yeast cells has harmful effects for most of the enzyme systems (Serrano 1996). These toxic effects are observed at intracellular concentrations of Na^+ and Cl^- of 50–100 mM, which is much lower than the levels required for ionic strength effects (Serrano et al. 1997). Such ionic contents are easily achieved because the low water activity in sweet dough enhances the effective concentration of both ions. Consequently an industrial baker's yeast, which is well adapted to sweet dough processes, should be osmotolerant and salt toxicity resistant. However, in spite of the efforts, none of the baker's yeast strains reported so far combines the desirable technological properties.

Dough leavening abilities have been reported for yeasts other than *S. cerevisiae*. Yeasts such as *Issatchenkia orientalis*, *Pichia membranaefaciens* and *Torulaspora delbrueckii* were the most abundant non-*Saccharomyces* species present in home-made corn and rye bread dough (Almeida and Pais 1996a). Freeze-tolerant strains of *T. delbrueckii* (Almeida and Pais 1996b; Hahn and Kawai 1990; Sasaki and Oshima 1987), *T. pretoriensis* (Oda and Tonomura 1993) and *Kluyveromyces thermotolerans* (Hino et al. 1987) have also been isolated and characterised as suitable for frozen dough. However, most of the selected freeze-tolerant strains have not been evaluated extensively for other properties of interest in baking, or do not fulfil the most important requisites on commercial baker's yeast.

In this work, we have investigated the response of two freeze-tolerant *T. delbrueckii* strains to salt and osmotic stress and their leavening ability in sweet and frozen-sweet dough in comparison with commercial baker's yeast strains of *Saccharomyces*. In addition, we have analysed the effect of various cellular factors on stress behaviour and fermentation characteristics of these strains.

Materials and methods

Strains and culture media

T. delbrueckii yeast strains IGC5321 and IGC5323 (Almeida and Pais 1996b) were used throughout this work. The commercial baker's yeast named Cinta Roja (Burns Philp) and Plus Vital (Lesaffre) were used as controls. Biomass from yeast was prepared by culturing cells in a liquid molasses medium according to an industrial recipe: 116.6 g of beet molasses (49% sucrose), 1.5 g of ammonium sulphate, 0.15 g of orthophosphoric acid, and 20 g of biotin per litre; adjusted to a final pH 5.0. Cultures were incubated and cells collected as described previously (Randez-Gil et al. 1995). The yeast cake was kept at 4°C for later use. Storage time was no longer than 24 h. Yeast cells were also cultured in defined media, YNB (0.67% yeast nitrogen base without amino acids [DIFCO] plus 2% glucose) or YPD (1% yeast extract, 2% peptone, 2% glucose). Cells were grown routinely in 250 ml or 1000 ml Erlenmeyer flasks at 30 °C on an orbital shaker (200 rpm).

For stress experiments on solid media, yeast cells were grown to exponential phase (OD_{600} of 0.3–0.5) in YPD, harvested, diluted, and plated on YPD agar containing NaCl (1.4 M), KCl (1.4 M) or LiCl (0.4 M). Yeast growth was also assayed on YP agar medium containing 20% sucrose (YPS) in the absence of NaCl or in the presence of 0.7 M NaCl. Plates were incubated at 30 °C for 2–4 days.

Enzyme determinations

Maltase activity was determined in crude extracts as described by Okada and Halvorson (1964) using p-nitrophenyl-α-D-glucopyranoside (pNPG) as substrate. To prepare yeast extracts, cells were resuspended in 0.3 ml of cold homogenisation buffer (0.1 M potassium phosphate buffer, pH 6.5) and transferred into a tube containing 1.0 g of glass beads (acid washed, 0.4-mm diameter). The mixture was vortexed for three periods of 1 min, centrifuged at 12,000 × g (4 °C) for 10 min, and the clear supernatant was used for further analysis. Total protein was determined by the method of Bradford (1976) using the commercial BioRad protein assay kit and rabbit IgG as standard. One unit (U) of specific activity is defined as the amount of enzyme that produces 1 nmol of pNPG/min/mg of protein under the assay conditions.

Invertase was assayed as described by Niederacher and Entian (1987), and is expressed as nmols of glucose released from sucrose/min/mg of protein.

Glycerol and trehalose determination

To determine glycerol content under salt-stress conditions, cells were grown in YNB medium (DO_{600} of 1.0) and then transferred to 1.4 M NaCl containing medium. At different times, cells were collected by filtration and washed twice with 5 ml of chilled 1.4 M NaCl. The filter was transferred quickly to a cold tube containing 1 ml of distilled water, and the yeast suspension was boiled for 10 min, cooled on ice, and centrifuged at 15,300 × g for 10 min (4 °C). Finally, the supernatant was collected and used for further analysis. Glycerol was determined colorimetrically with a commercial kit (Boehringer), following the manufacturer's instructions.

For the trehalose assay, cells were harvested, washed three times in cold TBS buffer, and suspended in 0.5 M Tris-HCl (pH 7.5) buffer. The yeast suspension was boiled for 10 min, cooled on ice, centrifuged at 15,300 × *g* for 10 min (4 °C), and the supernatant collected for further assay. Trehalose was determined colorimetrically by measuring the glucose generated by hydrolysis with commercial trehalase. Briefly, the reaction mixture contained 5 μl of trehalase (1.4 units per mg of protein, SIGMA) and 50 μl of crude extract, in 200 μl of 25 mM sodium acetate buffer, pH 5.5. The mixture was incubated at 37 °C overnight, afterwards 100 μl were withdrawn for glucose determination.

Mobilisation of trehalose in stationary-phase molasses-grown yeast (OD_{600} of 24–26) was induced by transfer of cells to YPD medium. Cultures were incubated by shaking (200 rpm) at 30 °C. Samples were taken and analysed for trehalose content at 25 and 180 min after the transfer.

Table 1. Growth rate (μ) and final biomass concentration of different baker's yeast grown in molasses

Strain	Growth rate* (h^{-1})	Final biomass* (g/l)
T. delbrueckii IGC5321	0.32 ± 0.01	23.5 ± 2.8
T. delbrueckii IGC5323	0.29 ± 0.01	25.1 ± 1.5
Plus Vital	0.41 ± 0.01	27.9 ± 2.3
Cinta Roja	0.38 ± 0.01	24.1 ± 2.1

*Values are given as means and standard deviations of three independent experiments.

Dough preparation and gas production measurements

A commercial wheat bread flour (moisture, 16%; W, 113.5 × 10^{-4} J [Alveograph]; P/L, 0.26 [Alveograph]) was used throughout this work. Bread dough was prepared using the formula: flour (100%), yeast (2.8%), salt (2%) and tap water (47.4%). Sweet dough contained sucrose also, 8% or 20% (flour basis). Ingredients were mixed for 8 min in a MAHOT LABO 25 mixer (VMI France), and the resulting dough was divided into 315-g pieces. One piece as a control dough was analysed immediately for gassing power. The rest of the pieces were moulded by hand to 0.5-cm thickness, placed in aluminium salves, and wrapped in plastic bags. Dough was quickly frozen at −80 °C for 1 h and stored at −20 °C for 3 h (0' time) or up to 60 days. After each storage stage, the frozen dough was left to thaw at 30 °C for 30 min before gassing power was measured. CO_2 production was recorded in the Chopin rheofermentometer (Chopin, France) at 28.5 °C for 3 h. The increase of specific loaf volume during fermentation was measured in a calibrated vessel (Afora) using 100 g-pieces of hand-balled bread dough. Technological parameters were assayed in duplicate for each yeast tested. Yeast biomass for each experiment was obtained from different propagation batches.

Results and discussion

Dough-leavening activity and growth characteristics

The most important requisites in the commercial production of baker's yeast are rapid growth and high biomass yield (Randez-Gil et al. 1999). Therefore, we analysed these characteristics in two *T. delbrueckii* strains, previously isolated from home-made bread dough (Almeida and Pais 1996a), and reported as freeze-tolerant (Almeida and Pais 1996b). We used molasses as culture medium, the most common substrate in the baker's yeast industry, and two commercial strains from *S. cerevisiae*, Cinta Roja and Plus Vital, as controls. Culture of *T. delbrueckii* on molasses medium showed lower growth rate values than those observed for *Saccharomyces* strains, although the final biomass did not differ among the four strains assayed (Table 1).

We determined the gas-production capacity of the yeast strains in lean and sweet-dough. Table 2 shows that CO_2 production in lean dough, was slightly higher in *T. delbrueckii* strains than in Cinta Roja, a baker's yeast commercialised for unsugared bread dough applications. As expected, the differences were more pronounced when the dough-leavening activity of the *Torulaspora* cells was compared with that observed for the Plus Vital strain (Table 2). This strain shows a poor ability to ferment maltose, but performs properly in low-sucrose bread dough. In consonance with this, the CO_2 production in bread-dough elaborated with this strain increased by the addition of 8% sucrose, whereas that of the Cinta Roja strain diminished (Table 2). The best results were again for both *Torulaspora* strains, and especially for IGC5321. Furthermore, yeast exposure to hyperosmotic stress of bread dough containing 20% sucrose resulted in a dramatic drop of the fermentative capacity of the commercial strains (Table 2). Under the same conditions, the *T. delbrueckii* strains displayed a decrease of CO_2 production of about 30% relative to that found in lean-dough. We further compare these data with those of 20%-sucrose dough elaborated with commercial compressed yeast biomass. Growth under industrial dynamic fed-batch conditions provide broad stress tolerance and high fermentative capacity. Despite this, CO_2 production for the Cinta Roja strain was still lower, 196 ± 15 ml per g of cells (d.w.), than observed for batch-grown *Torulaspora* cells (Table 2), and only dough yeasted with Plus Vital rendered values in a similar range, 658 ± 42. Therefore, both *Torulaspora* strains are suitable for the production of both lean and sweet-dough and display higher leavening ability than commercial strains of *Saccharomyces*, when grown under these laboratory conditions.

Table 2. Gas production capacity of different baker's yeast strains in lean and sweet dough

Strain	ml of CO_2/g of cells (d.w.)[a]		
	Lean dough	Sweet dough	
		8% sucrose	20% sucrose
T. delbrueckii IGC5321	811 ± 24	840 ± 25	529 ± 25
T. delbrueckii IGC5323	780 ± 19	765 ± 15	525 ± 32
Plus Vital	585 ± 29	699 ± 14	158 ± 20
Cinta Roja	719 ± 12	529 ± 42	n.d.[b]

[a] CO_2 production in lean and sweet dough was recorded in the rheofermentometer. Bread dough containing the yeast strains under study was prepared as described in the Materials and Methods section. Values are given as means and standard deviations of three independent experiments. [b] n.d. non detected.

Maltase and invertase activities

The yeast-gassing rate depends on the dough formulation, on specific fermentation parameters and especially on intrinsic characteristics of baker's yeast. Therefore, we tried to analyse the implications of several cellular factors in the baking performance of the baker's yeast. One important trait that determines the fermentation activity of *S. cerevisiae* in lean and low-sucrose bread dough is the level of maltase and invertase activity. In sweet bread dough, it is believed that the level of invertase correlates inversely with CO_2 production (Myers et al. 1997). A good dough-leavening activity in lean dough has been correlated with a high potential maltose-fermentation rate (Beudeker et al. 1990; Oliver 1991). As shown in Figure 1A, *T. delbrueckii* IGC5321 exhibited a low maltase activity (< 100 U mg^{-1} of protein), whereas the commercial control strain employed in lean dough production, Cinta Roja, showed the highest maltose-degradation rate (> 300 U mg^{-1} of protein) (Figure 1A). These results did not correlate with the fermentative activities in lean dough shown above (see Table 2). Therefore, a correlation between maltase activity and CO_2 production in lean dough appears to be unclear; at least for the baker's yeast strains analysed. This was not the case for the invertase activity (Figure 1B). Indeed, *T. delbrueckii* strains gave a similar ranking of invertase levels to that found for Pus Vital. In contrast, Cinta Roja, which showed an impaired leavening ability in sugared bread dough (Table 2), displayed the highest invertase activity (Figure 1B).

Salt addition. Effect on leavening ability

The data shown above indicated an unexpected poor behaviour of strain Plus Vital in high-sugar bread dough. Since these experiments were performed with bread dough elaborated with the addition of salt, we investigated in greater depth the effects of this compound in yeast gassing capacity. We measured the increase of specific loaf volume in 20% sucrose bread dough with or without salt added for the Plus Vital and *T. delbrueckii* strains. This test allows a fermentation capacity assay for longer periods than those recorded (3h) in the rheofermentometer. As shown in Figure 2A, all strains analysed showed a similar leavening ability in high-sugar dough lacking salt. As expected, the rate of loaf volume increase was clearly reduced,

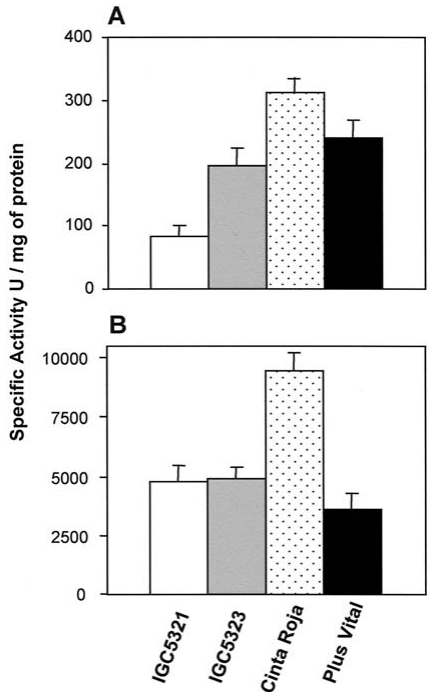

Figure 1. Maltase and invertase levels in different baker's yeast strains. Samples from *T. delbrueckii* IGC5321 and IGC5323, Plus Vital and Cinta Roja cells cultured in molasses medium (OD_{600} of 24–26) were processed for maltase (A) and invertase (B) activity analysis. Preparation of crude extracts and biochemical assays were performed as described in the Materials and Methods section. Values are given as means and standard deviations of three independent experiments.

when 2% salt was added (Figure 2B). However, this effect was more pronounced for dough yeasted with the *T. delbrueckii* IG5323, and specially with the Plus Vital strain, which presented a longer lag phase and a reduced maximal loaf volume as compared with the *T. delbrueckii* IGC5321 strain (Figure 2B).

Growth under osmotic and salt stress conditions

We further examined the effects of osmotic and salt stress on different synthetic media. Sodium chloride generates two different stress conditions to *S. cerevisiae* cells: osmotic stress and sodium toxicity (Serrano et al. 1997). Thus, NaCl is much more toxic than equivalent concentrations of KCl. This is also the case for the ion lithium, its threshold for toxicity is even lower (Dichtl et al. 1997). As shown in Figure 2C, growth of Plus Vital cells on YPD medium was impaired in the presence of high osmotic concentrations of KCl (1.4 M) and almost abolished at 0.4 M LiCl or 1.4 M NaCl. Similar results were observed in YP media containing 20% sucrose plus 0.7 M NaCl (Figure 2C). *T. delbrueckii* IGC5323 showed a clear phenotype of osmosensitivity. Indeed, this strain failed to grow in all media tested. However, cells of *T. delbrueckii* IGC5321 continued growing in high-osmotic environments, even when exposed to ion toxicity from NaCl or LiCl (Figure 2C). Hence, the *T. delbrueckii* IGC5321 strain shows intrinsic osmotolerance and ion toxicity resistance. Nevertheless, the results with the IGC5323 strain suggest that this characteristic may not be the essential or the only determinant for leavening ability in salted high-sugar dough.

Glycerol production

A general osmoregulatory mechanism in different micro-organisms is the synthesis and accumulation of one or more compatible solutes (Blomberg and Adler 1992). Between them, polyols are widely distributed in fungi, being glycerol and arabinitol, the most common polyols for non-tolerant and osmotolerant yeasts, respectively (Blomberg and Adler 1992). Preliminary analysis by gas chromatography and high performance anion-exchange of the pattern of polyol production by *T. delbrueckii* strains, indicated that glycerol was the main osmoregulator in salt-stressed cells. Smaller amounts of arabinitol, galactitol and methyl-galactictol were also found, but no additional polyol was detected (data not shown). According to this, we analysed more deeply the intracellular levels of glycerol after a shift to NaCl-containing medium.

Cells of *Saccharomyces* displayed a relatively high glycerol content, around 4 mg per g of cells, under non-stress conditions (Figure 3). This should be attributed to the fact that glycerol production in *S. cerevisiae* has a role in redox control during fermentative growth (Ansell et al. 1997). On the contrary, *Torulaspora* cells showed low glycerol levels before osmotic shock, suggesting that polyol production in this organism could have no additional functions to those of osmotic adjustment. A similar situation has been previously proposed for the osmotolerant yeast *Zygosaccharomyces rouxii* (Shen et al. 1999).

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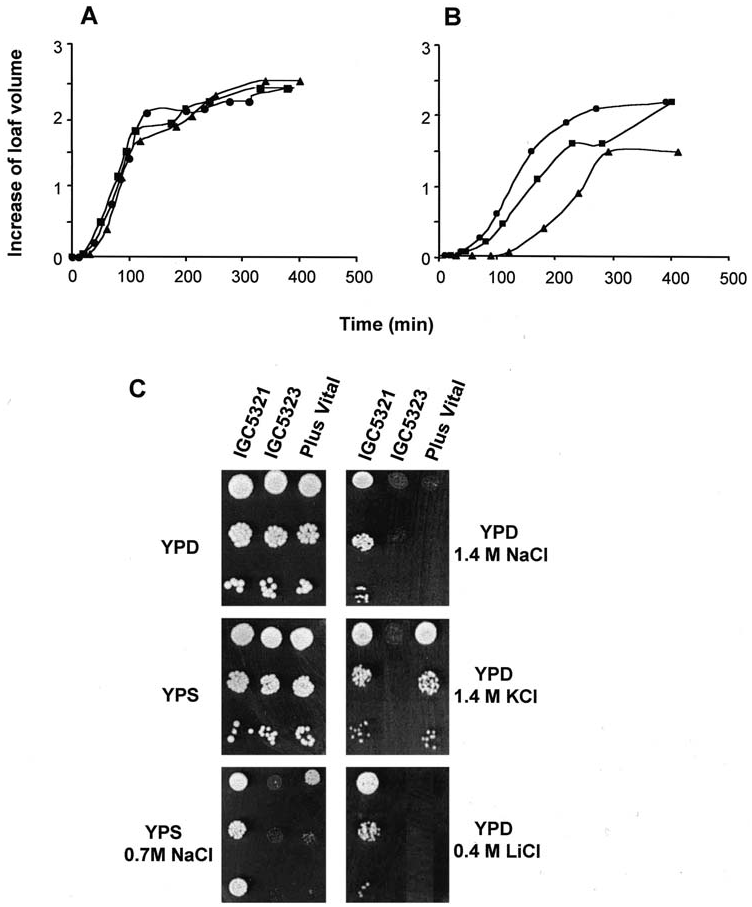

Figure 2. Effects of osmotic and salt stress on dough leavening ability and growth behaviour of baker's yeast strains. Increase of specific loaf volume in 20% sucrose bread dough without (A) or with (B) 2% salt added. Symbols for the *T. delbrueckii* IGC5321 (circles), IGC5323 (squares) and Plus Vital (triangles) strains. Bread dough was prepared as described in the Materials and Methods section. Values are given as means of three independent experiments. Growth on different synthetic media (C). Cells grown to exponential phase (OD_{600} of 0.3–0.5) in YPD medium were spotted in a 1:10 dilution series onto YPS (20% sucrose) or YPD containing NaCl, KCl or LiCl. Plates were incubated at 30 °C for 2–4 days.

Despite of these initial differences, the intracellular glycerol content in *Torulaspora* cells exhibited a sharp increase upon a shift to high-osmotic medium reaching values higher than those observed for the *Saccharomyces* strains after 3 h (Figure 3). Thus, the estimated rate of glycerol production during the first three hours of osmotic shock, a time that fits well with the period of CO_2 measurements in high-sugar dough (Table 2 and Figure 2B), was clearly higher for *Torulaspora* strains, 60–80 against 20–30 g glycerol per min and g of cells (d.w.) for *Saccharomyces*. These results suggest that *Torulaspora* responds more rapidly than *Saccharomyces* to high osmotic pressures. The kinetics of glycerol production at

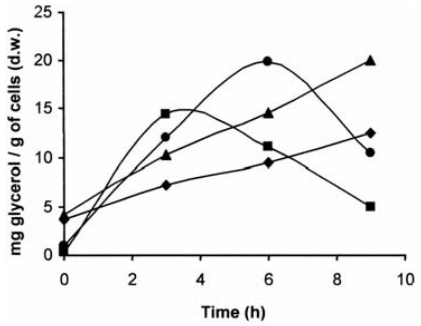

Figure 3. Intracellular glycerol content in yeast strains during culture in salt-stress medium. Yeast cells from *T. delbrueckii* IGC5321 (circles), IGC5323 (squares) – and *S. cerevisiae*, Plus Vital (triangles) and Cinta Roja (diamonds) – were cultured in YNB medium (OD_{600} of 1.0) and then transferred to 1.4 M NaCl-containing medium. Samples were taken at the indicated times and analysed for glycerol content in cells. Biochemical assays were performed as described in the Materials and Methods section. A representative experiment is shown. Independent experiments revealed identical kinetics of metabolic values.

longer times also suggest that *Torulaspora* cells were conditioned earlier than *Saccharomyces* to tolerate high NaCl concentrations. Indeed, the maximum glycerol level in cells of *T. delbrueckii* IGC5323 and IGC5321 was observed just after 3 and 6 h of osmotic stress, respectively, and later, the amount of intracellular glycerol started to decrease. Conversely, hyperaccumulation of glycerol was maintained longer in strains of *Saccharomyces*. Thus, the maximum glycerol content, 25–35 mg per g of cells, was only reached after 20–24 h of salt stress (data not shown). As a consequence, the total production of glycerol was considerably greater in *Saccharomyces* cells. This was clearly demonstrated by analysis of the glycerol accumulated in the supernatant of long-term cultures. After 120 hours of exposure to salt-stress, the levels of glycerol in the culture supernatant ranged from 80 to 90 mg per g of cells (d.w.) for *Torulaspora* strains, whereas values of 280 and 550 were detected for Plus Vital and Cinta Roja. Overall, these results indicated that during water stress, baker's yeast strains of *S. cerevisiae* deviated an increased amount of energy into maintenance glycerol production. This could be an additional factor determining the differences in osmotolerance between *Saccharomyces* and *Torulaspora*. Thus, it has been proposed that the high osmotolerance of yeast species, like *Debaryomyces hansenii*, might be a mere consequence of a reduced catabolic energy expenditures during growth in salt medium (Gustafsson and Larsson 1990). It has been reported also that *Z. rouxii* achieves glycerol accumulation by increasing the proportion retained within the cell and not by inducing glycerol production (Van Zyl and Prior 1990).

Freezing and frozen storage resistance

Frozen resistance is an essential property of a yeast strain if it is to be suitable for use in frozen and frozen-sweet dough. Loss of leavening activity of yeast cells upon freezing and thawing has been mainly attributed to desiccation and electrolyte release giving to hyperosmotic stress (Hatano et al. 1996; Myers and Attfield 1999). In addition, frozen storage allows ice crystals to be grown, which results in cellular damage by fracture of the cell membrane and destruction of sub-cellular organisation (Grout et al. 1990). Therefore, we analysed both effects by measuring the CO_2 production capacity of the selected yeast strains after freezing at − 20 °C for 3 h (0' time in the graphic), or during frozen dough storage. Experiments testing the effect of freezing and thawing of dough showed a marked decrease of fermentative capacity (around 40%), of either lean or sweet-dough elaborated with the commercial strains (Figure 4, see 0' time). This effect was less pronounced for dough made with *T. delbrueckii* strain IGC5321, whose leavening ability appeared to be unaltered. Similar behaviour was observed for the IGC5323 strain (data not shown). These results were in good concordance with the apparent tolerance of *Torulaspora* strains to osmotic stress of high-sugar dough (Table 2 and Figure 2B).

As expected, effects of frozen storage were more pronounced in sweet dough, although similar for the strains tested (Figure 4B). Only in lean dough, *Torulaspora* cells appeared to have a better performance than *Saccharomyces*. Therefore, our results confirm previous reports about the freeze-tolerant phenotype of the *Torulaspora* strains IGC5321 and IGC5323 (Almeida and Pais 1996b) and extend this property to frozen-sweet dough. This phenotype appears to correlate mainly with the ability of these strains to prevent damage by osmotic effects during freezing and thawing of bread dough.

Trehalose content and mobilisation

The results reported above suggested that the freeze-

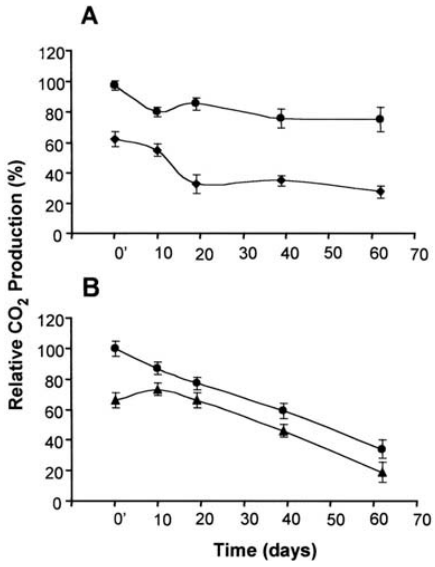

Figure 4. Effect of freezing and frozen storage on CO_2 production by baker's yeast strains. Lean- (A) and 8% sucrose sweet-dough (B) made with *T. delbrueckii* IGC5321 (circles), Cinta Roja (diamonds) or Plus Vital (triangles) was frozen at − 80 ℃ for 1 h and stored at − 20 ℃ for 3 h (0') or up to 60 days. After each storage stage the CO_2 production level was tested in the rheofermentometer. Results are expressed as the percentage of CO_2 production relative to unfrozen control dough. Values are given as means and standard deviations of three independent experiments.

tolerant phenotype of *T. delbrueckii* could be mainly correlated with their ability to cope with water stress. We also showed in this work, that *Torulaspora* cells displayed a higher rate of glycerol production than *Saccharomyces*. However, yeast cells have not enough time at freezing rates applied in frozen dough preparation to produce significant levels of glycerol (Myers and Attfield 1999). Therefore, we focused our attention on another compatible osmolyte, trehalose, which is a widely established determinant in stress tolerance of *Saccharomyces* (Singer and Lindquist 1998), including osmotic shock (Hounsa et al. 1998; Sano et al. 1999). In baker's yeast strains, trehalose is believed to be particularly critical for the preservation of yeast viability in frozen dough (Hino et al. 1990; Meric et al. 1995; Shima et al. 1999). Because of this, culture conditions for yeast propagation have been optimised to promote hyperaccumulation of trehalose (Beudeker et al. 1990). However, mixing of yeast cells with nutrients during dough preparation triggers rapid mobilisation of trehalose by hydrolysis and loss of performance in frozen dough (Shima et al. 1999; Van Dijck et al. 1995). According to this, we determined the level of trehalose in stationary-phase baker's yeast cells grown in molasses and the effects on the trehalose content after a cell shift to a glucose-containing medium. As depicted in Figure 5, trehalose levels of 2.5 to 4.5% of the dry weight, were found for the baker's yeast under study, with the highest differences among *Torulaspora* strains IGC5321 and IGC5323. These values were consistent with previously reported data for cells of *S. cerevisiae* grown in batch culture (Van Dijck et al. 1995). As expected, the amount of trehalose accumulated began to decrease rapidly when cells were transferred to a YPD medium. However, the rate of trehalose mobilisation differed clearly between *Saccharomyces* and *Torulaspora* strains. In Cinta Roja and Plus Vital cells, the trehalose level dropped to less than 0.6% after 25 min, whereas no residual trehalose was detected after 3 h of the transfer (Figure 5). On the contrary, the content of trehalose accumulated in cells of *T. delbrueckii* remained quite high after the onset of fermentation. Moreover, significant amounts of the disaccharide, more than 1% of the dry weight, were still detected for both strains of *Torulaspora* after 3 h of a shift to YPD (Figure 5). These results are in good agreement with the previous report of Almeida and Pais (1996b) showing that strains IGC5321 and IGC5323 are not

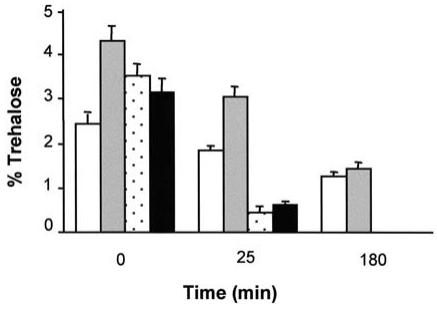

Figure 5. Trehalose content and mobilisation after the transfer to YPD medium of stationary-phase molasses-grown yeast cells. Samples of the *T. delbrueckii* IGC5321 (white bars) *T. delbrueckii* IGC5323 (grey bars), Plus Vital (dotted bars) and Cinta Roja (black bars), were taken at the indicated times and analysed for trehalose content as described in the Materials and Methods section. Values are given as means and standard deviations of three independent experiments.

affected by fermentation before freezing. Yokoigawa et al. (1995) have also shown that freeze tolerant *T. delbrueckii* strains exhibit lower trehalase activity than sensitive strains. Therefore, our results suggest that the stability of yeast cells of *T. delbrueckii* after the initial freezing and thawing of bread dough can be correlated with a slow rate of trehalose mobilisation during fermentation.

Conclusions

The results shown in this work further confirm that *T. delbrueckii* strains IGC5321 and IGC5323 fulfil the most important requisites of modern baking, rapid growth and high biomass coupled with a high-dough leavening activity in lean and frozen dough. Moreover, our results extend these properties to sweet and frozen sweet dough. These traits respond to the demands of baker's yeast producers and consumers of strains with improved osmotolerance and cryoresistance. Moreover, the application of these strains would have additional economic advantages since a single strain can be used for most or all baking applications with the highest performance, including those containing salt. Additional work is needed to confirm the results reported here at the industrial level, in particular in cells grown under dynamic fed-batch conditions, which determine the fermentative power and the stress behaviour of *S. cerevisiae* in downstream and baking applications. Preliminary experiments in our laboratory indicate that the use of *Torulaspora* strains do not modify bread quality parameters, like crumb texture, colour or flavour. These studies should be extended to other commercially important characteristics in order to ensure the acceptance of baker's yeast strains of *Torulaspora* by producers and consumers.

But the importance of *T. delbrueckii* strains goes beyond this. As we show in this work, the fermentative potential of this yeast in sweet and frozen dough correlates with low invertase activity, a slow rate of trehalose mobilisation and the ability to adapt rapidly to high osmotic pressures environments. Moreover, strain IGC5321 shows a clear phenotype of osmotolerance and Na^+ toxicity resistance in synthetic media. Therefore, these strains could be a good model to identify co-ordinately expressed genes under those physiological conditions encountered in bread dough and to understand how the regulation of stress pathways can be applied to the improvement of industrial yeast strains.

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References

Almeida M.J. and Pais C.S. 1996a. Characterization of the yeast population from traditional corn and rye bread doughs. Lett. Appl. Microbiol. 23: 154–158.

Almeida M.J. and Pais C.S. 1996b. Leavening ability and freeze tolerance of yeasts isolated from traditional corn and rye bread doughs. Appl. Environ. Microbiol. 62: 4401–4404.

Ansell R., Granath K., Hohmann S., Thevelein J.M. and Adler L. 1997. The two isoenzymes for yeast NAD-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. EMBO J. 16: 2179–2187.

Attfield P.V. 1997. Stress tolerance: The key to effective strains of industrial baker's yeast. Nature Biotechnol. 15: 1351–1357.

Beudeker R.F., van Dam H.W., van der Plaat J.B. and Vellenga K. 1990. Developments in baker's yeast production. In: Verachtert H. and De Mot R. (eds), Yeast Biotechnology and Biocatalysis. Marcel Dekker, New York, pp. 103–146.

Blomberg A. and Adler L. 1992. Physiology of osmotolerance in fungi. Adv. Microb. Phys. 33: 145–212.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Dichtl B., Stevens A. and Tollervey D. 1997. Lithium toxicity in yeast is due to inhibition of RNA processing enzymes. EMBO J. 16: 7184–7195.

Evans I.H. 1990. Yeast strains for baking: recent developments. In: Spencer J.F.T. and Spencer D.M. (eds), Yeast Technology. Springer-Verlag, Berlin, pp. 13–54.

Gélinas P., Lagimonière M. and Dubord C. 1993. Baker's yeast sampling and frozen dough stability. Cereal Chem. 70: 219–225.

Grout B., Morris J. and McLellan M. 1990. Cryopreservation and the maintenance of cell lines. Trends Biotechnol. 8: 293–297.

Gustafsson L. and Larsson C. 1990. Energy budgeting in studying the effect of environmental factors on the energy metabolism of yeasts. Thermochimica Acta 172: 95–104.

Hahn Y.-S. and Kawai H. 1990. Isolation and characterization of freeze-tolerant yeasts from nature available for the frozen-dough method. Agric. Biol. Chem. 54: 829–831.

Hatano S., Udou M., Koga N., Honjoh K. and Miyamoto T. 1996. Impairment of the glycolytic system and actin in baker's yeast during frozen storage. Biosci. Biotechnol. Biochem. 60: 61–64.

Hino A., Takano H. and Tanaka Y. 1987. New freeze-tolerant yeast for frozen dough preparations. Cereal Chem. 64: 269–275.

Hino A., Mihara K., Nakashima K. and Takano H. 1990. Trehalose levels and survival ratio of freeze-tolerant versus freeze-sensitive yeasts. Appl. Environ. Microbiol. 56: 1386–1391.

Hohmann S. 1997. Shaping up: the response of yeast to osmotic stress. In: Hohmann S. and Mager W.H. (eds), Yeast Stress Responses. Springer, New York, pp. 101–146.

Hounsa C.-G., Brandt E.V., Thevelein J., Hohmann S. and Prior B.A. 1998. Role of trehalose in survival of *Saccharomyces cerevisiae* under osmotic stress. Microbiology 144: 671–680.

Meric L., Lambert-Guilois S., Neyreneuf O. and Richard-Molard D. 1995. Cryoresistance of baker's yeast *Saccharomyces cerevisiae* in frozen dough: contribution of cellular trehalase. Cereal Chem. 72: 609–615.

Myers D.K. and Attfield P.V. 1999. Intracellular concentration of exogenous glycerol in *Saccharomyces cerevisiae* provides for improved leavening of frozen sweet doughs. Food Microbiol. 16: 45–51.

Myers D.K., Lawlor D.T.M. and Attfield P.V. 1997. Influence of invertase activity and glycerol synthesis and retention on fermentation of media with high sugar concentration by *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 63: 145–150.

Neyreneuf O. and Van Der Plat J.B. 1991. Preparation of frozen French bread dough with improved stability. Cereal Chem. 68: 60–66.

Niederacher D. and Entian K.-D. 1987. Isolation and characterization of the regulatory *HEX2* gene necessary for glucose repression in yeast. Mol. Gen. Genet. 206: 505–509.

Oda Y. and Tonomura K. 1993. Selection of a novel baking strain from the *Torulaspora* yeasts. Biosci. Biotech. Biochem. 57: 1320–1322.

Oliver S. 1991. Classical Yeast Technology. In: Tuite M.F. and Oliver S.G. (eds), Saccharomyces. Biotechnology handbooks Vol. 4. Plenum Press, London, pp. 213–248.

Okada H. and Halvorson H.O. 1964. Uptake of alpha-thioethylglucopyranoside by *Saccharomyces cerevisiae*. 1. The genetic control of facilitated diffusion and active transport. Biochim. Biophys. Acta 82: 538–542.

Randez-Gil F., Prieto J.A., Murcia A. and Sanz P. 1995. Construction of baker's yeast strains that secrete *Aspergillus oryzae* alpha-amylase and their use in bread making. J. Cereal Sci. 21: 185–193.

Randez-Gil F., Sanz P. and Prieto J.A. 1999. Engineering baker's yeast: room for improvement. Trends Biotechnol. 17: 237–244.

Sano F., Asakawa N., Inoue Y. and Sakurai M. 1999. A dual role for intracellular trehalose in the resistance of yeast cells to water stress. Cryobiology 39: 80–87.

Sasaki T. and Oshima Y. 1987. Induction and characterization of artificial diploids from the haploid yeast *Torulaspora delbrueckii*. Appl. Environ. Microbiol. 53: 1504–1511.

Shen B., Hohmann S., Jensen R.G. and Bohnert H.J. 1999. Roles of sugar alcohols in osmotic stress adaptation. Replacement of glycerol by mannitol and sorbitol in yeast. Plant Physiol. 121: 45–52.

Serrano R. 1996. Salt tolerance in plants and microorganisms: toxicity targets and defense responses. Int. Rev. Cytol. 165: 1–52.

Serrano R., Márquez J.A. and Ríos G. 1997. Crucial factors in salt stress tolerance. In: Hohmann S. and Mager W.H. (eds), Yeast Stress Responses. Springer, New York, pp. 147–169.

Shima J., Hino A., Yamada-Iyo C., Suzuki Y., Nakajima R., Watanabe H. et al. 1999. Stress tolerance in doughs of *Saccharomyces cerevisiae* trehalase mutants derived from commercial baker's yeast. Appl. Environ. Microbiol. 65: 2841–2846.

Singer M.A. and Lindquist S. 1998. Thermotolerance in *Saccharomyces cerevisiae*: the Yin and Yang of trehalose. Trends Biotechnol. 16: 460–468.

Sultan W.J. 1990. Sweet yeast dough products. In: Practical baking. 5th edn. Van Nostrand Reinhold, New York, pp. 217–327.

Varela J.C.S. and Mager W.H. 1996. Response of *Saccharomyces cerevisiae* to changes in external osmolarity. Microbiology 142: 721–731.

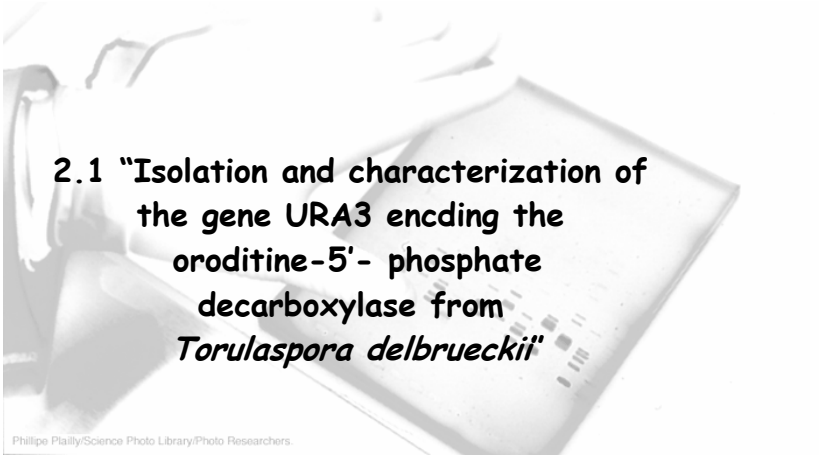
Van Dijck P., Colavizza D., Smet P. and Thevelein J.M. 1995. Differential importance of trehalose in stress resistance in fermenting and nonfermenting *Saccharomyces cerevisiae* cells. Appl. Environ. Microbiol. 61: 109–115.

Van Zyl P.J. and Prior B.A. 1990. Water relations of polyol accumulation by *Zygosaccharomyces rouxii* in continuous culture. Appl. Microbiol. Biotechnol. 33: 12–17.

Yokoigawa K., Murakami Y. and Kawai H. 1995. Trehalase activity and trehalose content in a freeze-tolerant yeast, *Torulaspora delbrueckii*, and its freeze-sensitive mutant. Biosci. Biotech. Biochem. 59: 2143–2145.

Capítulo 2

"Obtención de herramientas moleculares para el estudio de *Torulaspora delbrueckii*"



2.1 "Isolation and characterization of the gene URA3 encding the oroditine-5'- phosphate decarboxylase from *Torulaspora delbrueckii*"

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Yeast Sequencing Report

Isolation and characterization of the gene *URA3* encoding the orotidine-5′-phosphate decarboxylase from *Torulaspora delbrueckii*

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Abstract

A DNA fragment containing the *URA3* gene from *Torulaspora delbrueckii* was isolated by complementation cloning in *Saccharomyces cerevisiae*. DNA sequence analysis revealed the presence of an ORF of 795 bp, encoding a 264 amino acid protein, which shares a high similarity to the *Saccharomycetaceae* Ura3 proteins. Furthermore, the cloned ORF fully complemented the *ura3* mutation of *S. cerevisiae*, confirming that it encodes for the TdUra3 protein. The GeneBank Accession No. for *TdURA3* is AF518402. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: ***Torulaspora delbrueckii*; *URA3*; orotidine-5′-phosphate decarboxylase; OMPdecase**

Introduction

Applicability to bread-making by the frozen-dough method is now a desirable property for a baker's yeast strain (for review, see Randez-Gil *et al*., 1999). For this purpose, yeast cells are required to survive freezing and then, when warmed, to produce CO_2 quickly to make dough rise. However, none of the commercial strains of *Saccharomyces cerevisiae* available so far combines freeze–thaw resistance and high leavening ability. Consequently, a variety of non-conventional yeasts isolated from expontaneous bread-dough have been explored for such traits (Hino *et al*., 1987; Oda and Tonomura, 1993; Almeida and Pais, 1996a). Between them, the yeast *Torulaspora delbrueckii* (Sasaki and Oshima, 1987; Hahn and Kawai, 1990) and in particular strain IGC5321 (Almeida and Pais, 1996a) has been recognized as an attractive alternative for this purpose (Almeida and Pais, 1996b). Moreover, this strain has shown to exhibit osmotolerance and Na^+ toxicity resistance (Hernandez-Lopez MJ, Prieto JA, Randez-Gil F, unpublished), two important properties for sweet dough preparations (Randez-Gil *et al*., 1999).

The potential biotechnological applications of *T. delbrueckii* strains in bread-making make this yeast an interesting host for expressing foreign proteins, such as enzymes, widely used in the industrial process. The possibilities of this approach have been extensively demonstrated for baker's yeast strains of *S. cerevisiae* (Randez-Gil *et al*., 1999). Furthermore, this yeast could be used as a model to improve our knowledge about osmotolerance and cryoresistance in baker's yeast.

The genetic engineering of industrial *T. delbrueckii* strains is limited by the lack of auxotrophic markers. This implies that manipulation in these strains should involve the use almost exclusively of dominant drug resistance markers. However, the use of this selection system is hampered by legal regulations concerning the commercial exploitation of genetically modified organisms (Linko *et al*., 1997). In this study, we show the isolation, cloning and preliminary characterization of the *T. delbrueckii URA3* gene. To our knowledge, this is the first report describing the

complete sequence of a gene encoding an essential biosynthetic enzyme of this freeze- and osmotolerant yeast. These results should allow the construction of molecular tools for the manipulation of this organism in fundamental and applied research.

Materials and methods

Strains and culture media

T. delbrueckii IGC5321 (Almeida and Pais, 1996a) and *S. cerevisiae* CEN.PK2-1A (*MAT***a** *ura3-52 his3-*Δ1 *leu2-3,112 trp1-289 SUC3*) yeast strains were used throughout this work. Unless indicated, the yeast cells were cultured at 30 °C in defined media, YPD (1% yeast extract, 2% peptone, 2% glucose) or SD [0.67% yeast nitrogen base without amino acids (DIFCO) plus 2% glucose] supplemented with the appropriate auxotrophic requirements. *Escherichia coli* DH5α host strain was grown in Luria–Bertani (LB) medium (1% peptone, 0.5% yeast extract, 0.5% NaCl) supplemented with ampicillin (50 mg/l). Yeast cells were transformed by the lithium acetate method (Ito *et al*., 1983). *E. coli* was transformed by electroporation following standard protocols (Dower, 1990).

Genomic DNA library and plasmids

The *T. delbrueckii* IGC5321 genomic library was constructed by partial digestion of genomic DNA with *Sau*3AI and later ligation of DNA fragments (4–10 Kb) into the *Bam*HI-digested vector YEplac181 (Gietz and Sugino, 1988). Amplification was carried out in the *E. coli* DH5α strain. Plasmid YEpTdURA-1, carrying the original insert containing the *URA3* gene from *T. delbrueckii* (*TdURA3*), was isolated from the genomic library by complementation screening (see Results and discussion). A PCR reaction to amplify the *TdURA3* gene flanked by DNA sequences of the *TdURA3* promoter and terminator was performed using the primers PR85 and PR86 (Table 1) and plasmid YEpTdURA-1 as template. The fragment was digested with *Eco*RI–*Hin*dIII and inserted into the YEplac181 plasmid cleaved with the same set of enzymes, resulting in plasmid YEpTdURA-2.

DNA manipulations and sequencing

Standard DNA manipulations were carried out as described by Sambrook *et al*. (1989). Genomic DNA from *T. delbrueckii* cells was prepared as described by Sherman *et al*. (1986). DNA sequencing was performed in both strands by the dideoxy-chain termination procedure (Sanger *et al*., 1977). Analysis of sequence data was carried out using DNAMAN sequence analysis software (Lynnon BioSoft). Similarity searches were performed at the Swiss Institute of Bioinformatics using BLAST software (Altschul *et al*., 1997). Search of Ura3p domains was carried out by scanning the sequence against protein profile databases, PROSITE and Pfam. Multiple sequence alignment was done using the ClustalW program (Thompson *et al*., 1994). For phylogenetic inference, we used the neighbour-joining method (Saitou and Nei, 1987). The neighbour-joining tree was performed using the computer program MEGA, version 1.0 (Kumar *et al*., 1993). The sequence obtained for the *TdURA3* gene has been submitted to GeneBank under Accession No. AF518402.

Table 1. Oligonucleotides used in this study

Primer	Sequence 5′ to 3′	Comments
PR10	CCGACATTATAGTACTGAAGC	Sequencing
PR11	GTGAATACACCCAAGGTACTG	Sequencing
PR16	GCAGGCAGAAAATCAAGC	Sequencing
PR17	TGCGCAATTCTGTAGACA	Sequencing
PR18	TCACTGGGTTGAAACAAG	Sequencing
PR19	TCTCTGAGGTATGAACAG	Sequencing
PR85	GCAAGCTTAAGGTCAAACAA	YEpTdURA-2 plasmid construction
PR86	GCTGGAATTCATATCTCTG	YEpTdURA-2 plasmid construction

Results and discussion

Cloning of the *TdURA3* gene

The *T. delbrueckii URA3* gene was isolated from a genomic library constructed into the vector YEplac181 (*LEU2*) by complementation screening in *S. cerevisiae* ura3-52 strain. Out of about 20 000 Leu^+ transformants, only six were able to grow in the absence of leucine and uracil. Plasmid DNA was rescued from all of them, amplified in *E. coli* and tested again by retransformation in *S. cerevisiae*. The Ura^+ prototrophic phenotype was confirmed for one plasmid, termed YEpTdURA3-1, which was selected for further characterization.

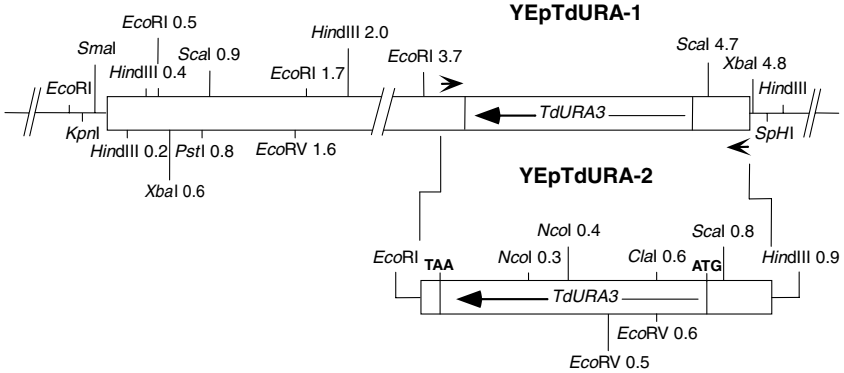

Figure 1. Schematic representation and restriction map of the DNA contained in plasmids YEpTdURA-1 and YEpTdURA-2. The arrows outside the drawing indicate the positions of the primers used to amplify by PCR the *TdURA3* ORF subcloned in plasmid YEpTdURA-2. Numbers indicate distances from the restriction site at the left of each drawing. Only relevant restriction sites are indicated. Fragment sizes are not to scale

Restriction analysis revealed that this contained a DNA insert of approximately 4.7 kb (Figure 1) that hybridized by Southern blot with a probe of the *S. cerevisiae URA3* gene (data not shown). DNA sequence analysis of the left side of the insert revealed the presence of an open reading frame (ORF) of 795 bp showing significant similarity to other reported *URA3* genes. Furthermore, a PCR fragment containing the whole ORF was subcloned and the resulting plasmid, YEpTdURA3-2 (Figure 1), introduced into *S. cerevisiae*. As a result, YEpTdURA3-2 complemented the Ura$^-$ phenotype of the CEN.PK2-1A strain, confirming that the ORF encodes for the *TdURA3* gene.

Characterization of the *TdURA3* gene

Nucleotides and deduced amino acid sequences of the *TdURA3* gene, including 109 pb upstream from the ATG codon and 266 pb of the 3′-UTR, are shown in Figure 2. No evident consensus sequence known to be transcriptional start or termination (Guo and Sherman, 1995) was found, although some in-frame stop codons are present after the TAA codon at 793. Out of this, the only relevant feature from the flanking sequences of *TdURA3* was the presence of A at positions −1 and −3 with respect to the initiation codon. This suggests a high expression of the *TdURA3* gene. Indeed, this characteristic is common among highly expressed genes from *S. cerevisiae* and in general of eukaryotic genes (Kozak, 1987). In agreement with this, a codon bias index of 0.397 was calculated for the *TdURA3* gene, a similar value to that found for the *S. cerevisiae* gene (0.388).

The deduced 264 amino acid protein of *TdURA3* showed the typical structure of a member of the orotidine-5′-phosphate decarboxylase family (OMPdecase), with four strongly conserved domains, 1–4 (see Figure 2), and a lysine residue in the region 2 (position 93) as the active site (Kimsey and Kaiser, 1992).

As expected, comparison of the TdUra3p sequence with those of other previously reported members of this family revealed a high similarity to the *Saccharomycetaceae* Ura3 proteins, with overall identity values ranking from 84% (*Kluyveromyces lactis*) to 75% (*Pachysolen tannophilus*). 83% identity is found among the *T. delbrueckii* and *S. cerevisiae* proteins. Nevertheless, similarity was also high to proteins from less related yeast species, like *Candida utilis* (78% overall identity), confirming that all yeast OMPdecases, except that from *Schizosaccharomyces pombe* (54% vs. the TdUra3p) are closely related (Yang *et al*., 1994).

The similarity of the TdUra3p to other Ura3 proteins was further revealed by analysis of the phylogenetic relationship between yeast Ura3p homologues (Figure 3). The dendrogram constructed by the neighbour-joining method (Saitou and Nei, 1987) from the amino acid sequences of 11

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-109   TCTAAAGGTCAAACAAGAACTTTATTTTCTCCAAGTCATCGCTAGACAAAAAGTTTCATAAAGAAAAGCAGGCAGAAAATCAAGCTATATATTTCGCTTC

-9     AGTACTATAATGTCGGTTGCTACTTATCAAGAGAGGGCTGCAAAGCATCCAAGTCCTGTGGCTTCAAAACTTTTGAATTTGATGCATGAAAAGAAGACCA
              M  S  V  A  T  Y  Q  E  R  A  A  K  H  P  S  P  V  A  S  K  L  L  N  L  M  H  E  K  K  T  N    31

92     ATCTATGTGCTTCATTAGATGTGAATACCACCGAAGAATTGCTTAAATTAGTCGATACTCTAGGTCCTTACATCTGTTTGTTGAAAACACACATCGATAT
         L  C  A  S  L  D  V  N  T  T  E  E  L  L  K  L  V  D  T  L  G  P  Y  I  C  L  L  K  T  H  I  D  I    64

192    CATTTCTGATTTTTCCATTGATGGTACTGTGAAGCCTTTGAAAGAATTGGCCAAAAAGCACAACTTTATGATCTTTGAGGACAGGAAATTCGCCGATATC
         I  S  D  F  S  I  D  G  T  V  K  P  L  K  E  L  A  K  K  H  N  F  M  I  F  E  D  R  K  F  A  D  I    97

292    GGTAACACTGTCAAGCTACAGTATTCTTCAGGTGTCTACAGAATTGCCGAGTGGGCTGATATTACAAACGCCCATGGTGTCACTGGTGCTGGGATCGTCA
         G  N  T  V  K  L  Q  Y  S  S  G  V  Y  R  I  A  E  W  A  D  I  T  N  A  H  G  V  T  G  A  G  I  V  T   131

392    CTGGGTTGAAACAAGCGGCTCAAGAGACTACAAACGAACCAAGAGGTCTATTAATGTTGGCTGAACTATCTTCTAAAGGTTCTTTGGCCCATGGTGAATA
         G  L  K  Q  A  A  Q  E  T  T  N  E  P  R  G  L  L  M  L  A  E  L  S  S  K  G  S  L  A  H  G  E  Y   164

492    CACCCAAGGTACTGTCGAAATTGCTAAGACAGACAAAGATTTCGTCATTGGATTCATTGCCCAAAGAGACATGGGAGGAAGAGAAGAAGGATATGACTGG
         T  Q  G  T  V  E  I  A  K  T  D  K  D  F  V  I  G  F  I  A  Q  R  D  M  G  G  R  E  E  G  Y  D  W   197

592    CTAATCATGACACCAGGTGTTGGCCTAGACGACAAGGGCGATGCGCTGGGTCAACAGTACAGAACTGTCGATGAAGTTGTCTCTACGGGGTCAGACATCA
         L  I  M  T  P  G  V  G  L  D  D  K  G  D  A  L  G  Q  Q  Y  R  T  V  D  E  V  V  S  T  G  S  D  I  I   231

692    TCATTGTCGGCAGAGGACTTTTCGCCAAGGGAAGAGATCCAAGAGTAGAAGGCGAACGTTACCGTAAAGCCGGCTGGGAAGCATACGAGAAGAGATGCCA
         I  V  G  R  G  L  F  A  K  G  R  D  D  P  R  V  E  G  E  R  Y  R  K  A  G  W  E  A  Y  E  K  R  C  Q   264

792    GTAAGATGTAGCTTCTTTATACTGTTCATACCTCAGAGATATAAATTCCATTACCCGGCGGATTGCTCTTTATTTGAATGAAAAATTTCAAGTAGCACAA
                *
892    CTTAACATAGTATTTCAACGATTGATTGCCAGTAAAGGAGCCTTTCAATAACTTACTAATCAACTTATCAACTCACAATGGGATGCACTAAACAAGTTTG

992    AGGAACAGCAAAGAATTCCAAAAATTGGGTGGGACAAAAAACAAAATGAAAGGATTCATGCCGTCCGGTT
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Figure 2. DNA and deduced protein sequences corresponding to the *TdURA3* gene. The characteristic two As immediately in front of the initial ATG codon are indicated in bold. The four conserved domains defined for all the members of the OMPdecase protein family (Kimsey and Kaiser, 1992), 1 (position 45–65), 2 (position 74–101), 3 (position 196–204) and 4 (position 227–238) are underlined. The lysine residue (K93) is essential for the catalytic function. The stop codon is marked by *

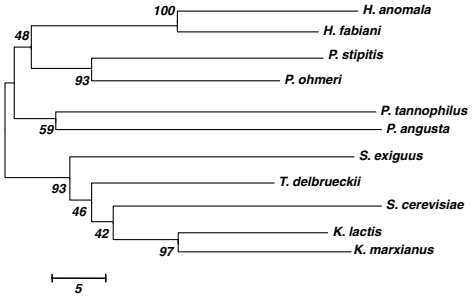

Figure 3. Tree representation of the relationship between TdUra3p and 10 yeast Ura3p homologues, constructed by the neighbour-joining method. The numbers on the nodes correspond to the percentages with which a cluster appears in a bootstrap test based on 1000 pseudoreplicates

Ura3p from *Saccharomycetaceae* spp. indicated that *T. delbrueckii* is grouped in a taxon independent from that of *S. cerevisiae*, although they are genealogically closely related, as has been previously reported by sequence comparisons of conserved rRNA genes (Belloch *et al*., 2000), 18S–28S rRNA spacer regions (James *et al*., 1996; Oda *et al*., 1997) and electrophoretic karyotyping (Oda and Tonomura, 1995). Furthermore, the two *Kluyveromyces* spp. analysed were clearly resolved and appeared as a sister group of the *S. cerevisiae* group. This proximity has been also inferred from the phylogenetic analysis of the genus *Kluyveromyces*, based on the sequence analysis of mitochondrial *COII* and nuclear 26S rRNA genes (Belloch *et al*., 2000).

The data presented here demonstrated that the *URA3* genes in *T. delbrueckii* and *S. cerevisiae* are closely related and functionally interchangeable. The *URA3* gene has been widely used as auxotrophic marker for transformation by plasmids and for gene disruptions in *S. cerevisiae*. Therefore, the availability of the complete sequence of *TdURA3* opens the possibility for the development of basic studies in this freeze- and osmotolerant organism. Moreover, this information can be used to design *URA3*-targeted cassettes for the integration of exogenous genes in the yeast genome.

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References

Altschul SF, Madden TL, Schäffer AA, *et al*. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Almeida MJ, Pais CS. 1996a. Characterization of the yeast population from traditional corn and rye bread doughs. *Lett Appl Microbiol* **23**: 154–158.

Almeida MJ, Pais CS. 1996b. Leavening ability and freeze tolerance of yeasts isolated from traditional corn and rye bread doughs. *Appl Environ Microbiol* **62**: 4401–4404.

Belloch C, Querol A, García MD, Barrio E. 2000. Phylogeny of the genus *Kluyveromyces* inferred from the mitochondrial cytochrome *c* oxidase II gene. *Int J Syst Evol Microbiol* **50**: 405–416.

Dower WJ. 1990. Electroporation of bacteria: a general approach to genetic transformation. In *Genetic Engineering Principles and Methods*, vol 12, Plenum: New York; 275–296.

Gietz RD, Sugino A. 1988. New yeast–*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**: 527–534.

Guo Z, Sherman F. 1995. 3′-End-forming signals of yeast mRNA. *Mol Cell Biol* **15**: 5983–5990.

Hahn Y-S, Kawai H. 1990. Isolation and characterization of freeze-tolerant yeasts from nature available for the frozen-dough method. *Agric Biol Chem* **54**: 829–831.

Hino A, Takano H, Tanaka Y. 1987. New freeze-tolerant yeast for frozen dough preparations. *Cereal Chem* **64**: 269–275.

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

James SA, Collins MD, Roberts IN. 1996. Use of an rRNA internal transcribed spacer region to distinguish phylogenetically related species of the genera *Zygosaccharomyces* and *Torulaspora*. *Int J Syst Bacteriol* **46**: 189–194.

Kimsey HH, Kaiser D. 1992. The orotidine-5′-monophosphate decarboxylase gene of *Myxococcus xanthus*. Comparison to the OMP decarboxylase gene family. *J Biol Chem* **267**: 819–824.

Kozak M. 1987. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acid Res* **15**: 8125–8148.

Kumar S, Tamura K, Nei M. 1993. *MEGA: Molecular Evolutionary Genetics Analysis*, vol 1.0. Pennsylvania State University: University Park, PA.

Linko Y-Y, Javanainen P, Linko S. 1997. Biotechnology of breadbaking. *Trends Food Sci Technol* **8**: 339–344.

Oda Y, Tonomura K. 1993. Selection of a novel baking strain from the *Torulaspora* yeasts. *Biosci Biotech Biochem* **57**: 1320–1322.

Oda Y, Tonomura K. 1995. Electrophoretic karyotyping of the yeast genus *Torulaspora*. *Lett Appl Microbiol* **21**: 190–193.

Oda Y, Yabuki M, Tonomura K, Fukunaga M. 1997. A phylogenetic analysis of *Saccharomyces* species by the sequence of 18S–28S rRNA spacer regions. *Yeast* **13**: 1243–1250.

Randez-Gil F, Sanz P, Prieto JA. 1999. Engineering baker's yeast: room for improvement. *Trends Biotechnol* **17**: 237–244.

Saitou N, Nei M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press: New York.

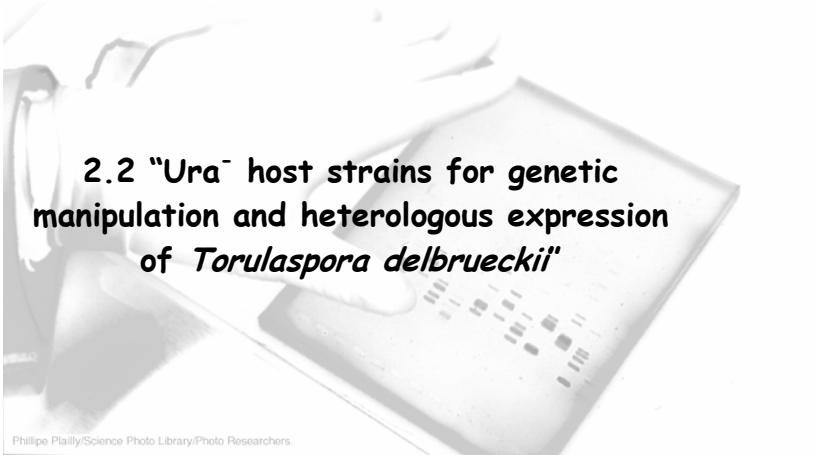
Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463–5467.

Sasaki T, Oshima Y. 1987. Induction and characterization of artificial diploids from the haploid yeast *Torulaspora delbrueckii*. *Appl Environ Microbiol* **53**: 1504–1511.

Sherman F, Fink GR, Hicks JB. 1986. *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press: New York.

Thompson JD, Higgins DG, Gibson TJ. 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680.

Yang VW, Marks JA, Davis BP, Jeffries TW. 1994. High-efficiency transformation of *Pichia stipitis* based on its *URA3* gene and a homologous autonomous replication sequence, *ARS2*. *Appl Environ Microbiol* **60**: 4245–4254.



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Ura⁻ host strains for genetic manipulation and heterologous expression of *Torulaspora delbrueckii*

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Abstract

Recently, the industrial and academic interest in the yeast *Torulaspora delbrueckii* has increased notably due to its high resistance to several stresses. This characteristic has made of this organism a very attractive model to study the molecular basis of the stress response in yeast. However, very little is known about the physiology and genetics of this yeast, and the tools for its manipulation have not been developed. Here, we have generated Ura⁻ strains of the baker's yeast *T. delbrueckii* IGC5323 by either 5-FOA-aided selection or transformation with a PCR-based disruption cassette, natMX4, which confers nourseothricin resistance. Furthermore, the mutant and disruptant strains were used as recipient of a plasmid containing the *xlnB* cDNA from *Aspergillus nidulans*. Our results indicate that *Torulaspora* transformants produce active recombinant protein at a similar level to that found for *Saccharomyces*.

Keywords: *Torulaspora delbrueckii*; Baker's yeast; *URA3*; natMX4; 5-FOA; Heterologous expression

1. Introduction

The yeast *Torulaspora delbrueckii* is frequently found in alcoholic beverages, fruit juices and high-sugar-containing foods (Esteve-Zarzoso et al., 2001), often being considered as a spoilage yeast (Schuller et al., 2001). Phylogenetically, *T. delbrueckii* is grouped in an independent taxon to that of *Saccharomyces cerevisiae*, although they are genetically closely related, as it has been previously reported by sequence comparisons (James et al., 1996; Oda et al., 1997; Belloch et al., 2000). Unlike *Saccharomyces*, *T. delbrueckii* strains exhibit an exceptional resistance to different stress conditions, which made of this yeast a potential model to explore and understand the mechanisms underlying stress resistance in yeast.

From a practical point of view, the ability of *Torulaspora* cells to cope with several stresses has led to their utilisation in some biotechnological processes. In the wine-making industry, *T. delbrueckii* strains are utilised in re-fermentation steps due to its high ethanol tolerance and its ability to ferment at low temperatures. *T. delbrueckii* has been also isolated as one of the most abundant non-Saccharomyces species found in home-made corn and rye bread dough (Hahn and Kawai, 1990; Almeida and Pais, 1996a) and two

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strains, IGC5321 and IGC5323, have been characterised as freeze–thaw tolerant (Almeida and Pais, 1996b). These strains were further examined by our group in order to establish its fermentative potential in sweet and frozen sweet dough. Leavening activity of *T. delbrueckii* IGC5321 and IGC5323 upon freeze–thaw appeared to be unaltered in comparison with the decrease of fermentative capacity (40%) in *S. cerevisiae* baker's yeast (Hernandez-Lopez et al., 2003). This result could be explained, at least in part, by the slow rate of trehalose mobilisation found in these strains and their ability to respond rapidly to osmotic stress. Yokoigawa et al. (1995) have also shown that freeze tolerant *T. delbrueckii* strains exhibit lower trehalase activity than sensitive strains. As expected, effects of frozen storage were deleterious for all the strain tested, being less pronounced in *T. delbrueckii* (Hernandez-Lopez et al., 2003). In addition, *T. delbrueckii* strains showed Na^+ toxicity resistance and a good leavening activity in all the formulations tested, lean, regular and sweet dough (Randez-Gil et al., 2002). Thus, the application of these strains in bread making would have additional advantages, since a single strain can be used for most or all baking applications with the highest performance.

During last years, the genetic engineering techniques have led to the possibility of constructing new strains of baker's yeast of *S. cerevisiae* with improved traits or able to produce proteins that can modify bread flavour, dough rheology or shelf-life (for a review, see Randez-Gil et al., 1999). Various laboratories have also reported the obtention of recombinant wine yeasts that express heterologous enzymes with positive effects on the colour and aroma of the wines obtained (for a review, see Querol and Ramón, 1996).

Like *Saccharomyces*, the biotechnological applications of *Torulaspora* strains render them an interesting host to express foreign proteins that can improve the specific industrial processes in which they are involved. However, the use of the recombinant DNA technology in this organism is limited by the lack of genetics and molecular tools. Moreover, most of the *T. delbrueckii* strains are prototrophic. Therefore, selection of transformants by complementation of auxotrophic mutations, the most common approach in *S. cerevisiae*, cannot be employed.

Recently, we have shown the isolation, cloning and preliminary characterisation of *T. delbrueckii URA3* gene, *TdURA3* (Hernandez-Lopez et al., 2002). This gene encodes for the orotidine-5′ -phosphate decarboxylase, an essential enzyme in the biosynthesis pathway of uracil, which has been widely used as selection marker in yeast. Here, we describe the construction of Ura^- *T. delbrueckii* strains and its application as potential host for the production of heterologous enzymes. These mutant strains should allow to extend our knowledge on the physiology and molecular biology of *T. delbrueckii*.

2. Materials and methods

2.1. Strains and culture media

T. delbrueckii IGC5323 (Almeida and Pais, 1996b) and *S. cerevisiae* HS13 (*ura3*, Lesaffre International, France) yeast strains were used in transformation experiments. *T. delbrueckii* IGC5323 derivatives FRY111 (*ura3*) and FRY112 (*ura3::*natMX4) were constructed during this work, as described below. Unless indicated, yeast cells were cultured at 30 °C in defined media, YPD (1% yeast extract, 2% peptone, 2% glucose) or YNB (0.17% yeast nitrogen base without amino acids [DIFCO] plus 0.5% ammonium sulphate and 2% glucose) supplemented with the appropriate auxotrophic requirements (Sherman et al., 1986). Yeast transformants, containing the nourseothricin resistance module (natMX4), were selected on YPD-agar plates supplemented with 50 μg/ml of nourseothricin (clonNAT, WERNER Bioagents, Germany). *ura3* mutants were selected by platting an unmutagenized population of cells on YNB-agar plates supplemented with uracil (10 mg/l), proline (1 mg/ml) and 5-fluoroorotic acid, 5-FOA (1 mg/ml) (McCusker and Davis, 1991). Production of recombinant xylanase was detected in solid medium by the formation of haloes in YNB-plates containing the coloured xylane substrate azo-xylan (birchwood, 0.1%). For the assays in liquid medium, yeast cells were cultivated in Sugar Nutrient medium, SN (0.25% yeast extract, 1.5% glucose, 4.5% maltose, 0.2% magnesium sulphate, 0.08% potassium chloride, 0.47% ammonium phosphate, 4 ppm thiamine, 4 ppm pyridoxine, 40 ppm nicotinic acid, 0.15 M citrate buffer pH = 5.5). *Escherichia coli* DH5α host strain was grown in Luria Bertani (LB) medium (1% pep-

tone, 0.5% yeast extract, 0.5% NaCl) supplemented with ampicillin (50 mg/l). Antibiotics, amino acids and 5-FOA were filter-sterilised and added to autoclaved medium.

2.2. Plasmids and disruption cassette

Construction of the *TdURA3* disruption cassette containing the nourseothricin-resistance natMX4 module was performed by PCR using the pAG25 plasmid (Goldstein and McCusker, 1999) as template and two specific synthetic oligonucleotides, PR12 and PR13 (Table 1), which contain 18 and 19 bases, respectively, homologous to the pFA6-natMX4 MCS (Goldstein and McCusker, 1999), flanked by short homology regions to the *TdURA3* gene (Hernandez-Lopez et al., 2002). Because of the high GC content of the nourseothricin acetyltransferase-encoding gene *nat1* from *Streptomyces noursei* (Krugel et al., 1993), the PCR-reaction mixture contained 5% DMSO, as previously described (Goldstein and McCusker, 1999). The YEplac195 vector (Gietz and Sugino, 1988) carrying the *URA3* gene of *S. cerevisiae* was used to complement the Ura$^-$ phenotype in the FRY111 and FRY112 strains of *T. delbrueckii*. The plasmid YEp195ACT-X24 used to obtain xylanase-producing yeast cells was generated by digestion with *Sac*I/*Hin*dIII of the plasmid YEpACT-X24 (Monfort et al., 1997), and later ligation of the released fragment, which contains the *xlnB* cDNA from *A. nidulans* (Perez-Gonzalez et al., 1996) under the control of the promoter of the *ACT1* gene from *Saccharomyces* into the YEplac195 plasmid (Gietz and Sugino, 1988).

2.3. Transformation

Yeast transformation was carried out as described by Ito et al. (1983), using 1 μg of plasmid. In order to obtain disrupted transformants, we used linear DNA fragments (1–5 μg) generated by PCR. Cultures transformed with the natMX4 cassette were grown for 4 h in YPD at 30 °C before platting on selective medium. *E. coli* was transformed by electroporation, as previously described (Dower, 1990).

2.4. DNA manipulations

Standard DNA manipulations were done, as described by Sambrook et al. (1989). Genomic DNA from *T. delbrueckii* cells was prepared, as described by Sherman et al. (1986). Verification of correct *URA3* replacement in *T. delbrueckii* was carried out by PCR and Southern blot analysis. A PCR-amplified fragment (primers PR85 and PR86, Table 1) containing the whole sequence of *TdURA3* was used to probe the corresponding genomic DNA. Probes were labelled with the DIG-High Prime Kit (Boehringer Mannheim) following the manufacturer's instructions. DIG-labelled DNA was detected using anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) and the chemiluminescent substrate CPD-*Star* (Roche).

2.5. Enzyme determinations

Xylanase activity was measured in the culture supernatant of SN-grown cells according to the meth-

Table 1
Oligonucleotides used in this study

Primer	Sequence 5′ to 3′	Comments
PR12	TGTCGGTTGCTACTTATCAAGAGAGG GCTGCAAAGCATCCAAGTCCGTAC GCTGCAGGTCGAC	Disruption cassette construction
PR13	ATCTTACTGGCATCTCTTCTCGTATG CTTCCCAGCCGGCTTTATCGATGAA TTCGAGCTCG	Disruption cassette construction
PR16	GCAGGCAGAAAATCAAGC	Verify correct targeting of the natMX4 module
PR19	TCTCTGAGGTATGAACAG	Verify correct targeting of the natMX4 module
PR75	AGTTAAGTGCGCAGAAAG	Verify correct targeting of the natMX4 module
PR85	GCAAGCTTAAGGTCAAACAA	Probe, Southern
PR86	GCTGGAATTCATATCTCTG	Probe, Southern
PR198	CACGTCAAGACTGTCAAG	Verify correct targeting of the natMX4 module

odology described by Bailey et al. (1992), except that oat spelt xylan was used as substrate at pH 5.5. One unit of activity is defined as the amount of enzyme that is able to release 1 μmol of xylose per min at 50 °C under the assay conditions.

3. Results and discussion

3.1. Obtention of T. delbrueckii spontaneous URA3 mutants

URA3-based selectable marker systems have specific advantages. Thus, it is possible to easily select for Ura$^-$ strains on media containing the drug 5-FOA, 5-fluoroorotic acid (McCusker and Davis, 1991). This compound is metabolised by the *URA3* or *URA5* gene products to a harmful toxic in a variety of eukaryotic and procaryotic cells (Boeke et al., 1984). 5-FOA-aided selection allows also the repeated use of *URA3*-based disruption vectors, after the "pop-out" or in vivo excision (Alani et al., 1987). Finally, the 5-FOA-based selection system of Ura$^-$ strains is particularly useful because the resulting strain is considered as a food-safe-grade.

Previous papers have shown that uracil-auxotrophic mutants derived from wild-type strains of *T. pretoriensis* (Oda and Tonomura, 1995) and *T. delbrueckii* (Watanabe et al., 1996) can be selected using the 5-FOA method. Therefore, we tested this selection system in cells of the freeze- and osmotolerant strain IGC5323. This strain has been proposed as a potential model organism for basic and applied research on stress (Hernandez-Lopez et al., 2002). Moreover, its use on frozen-high-sugar-dough bread making has been recently patented (Randez-Gil et al., 2002). Accordingly, Ura$^-$ mutants were selected by spreading an unmutagenized population of IGC5323 cells on 5-FOA-containing medium and later replica plating onto the same medium. All of the 5-FOA-resistant colonies analysed were unable to grow in uracil-lacking media. Twenty of these Ura$^-$ mutants were further tested by their defectiveness in the *URA3* gene. A recent report has demonstrated that the *URA3* genes in *T. delbrueckii* and *S. cerevisiae* are closely related and functionally interchangeable (Hernandez-Lopez et al., 2002). In consonance with this, five of the Ura$^-$ mutants were transformed to Ura$^+$ with the YEplac195 plasmid (Gietz and Sugino, 1988), which contains the *URA3* gene from *S. cerevisiae*. A positive mutant referred as FRY111 strain was further examined by Southern blot.

As it is depicted in Fig. 1, hybridisation using a *TdURA3* fragment as a probe showed the presence in the wild-type IGC5323 of three bands, that were not

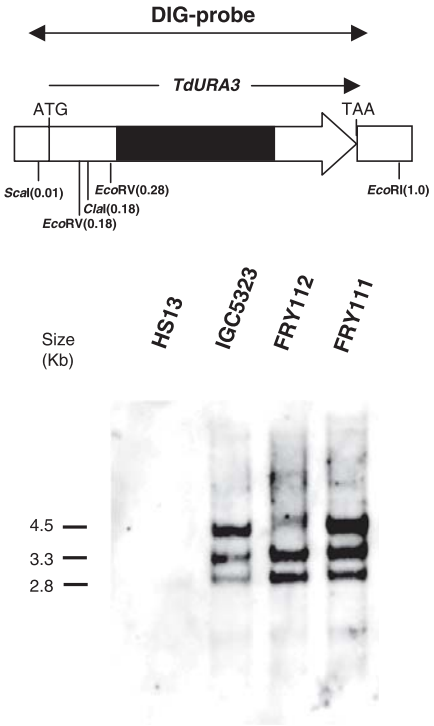


Fig. 1. Southern blot analysis of Ura$^-$ strains. Genomic DNA from strains IGC5323, FRY111 (*ura3*) and FRY112 (*ura3*::natMX4) of *T. delbrueckii* and HS13 of *S. cerevisiae* were digested with *Eco*RI and the released fragments were separated by electrophoresis in 1% agarose, transferred to Nylon membrane and hybridised with a *TdURA3* PCR-generated probe labelled with digoxigenin (DIG). A schematic representation of the wild-type *URA3* locus is shown. The black box indicates the fragment, which is replaced by the natMX4 module in the FRY112 strain. The numbers in parenthesis indicate distances (in Kb) from the restriction site to the ATG. Only the main restriction sites are shown. The sequences flanking the *TdURA3* gene, except 109 and 266 bp at the 5′- and 3′-side (Hernandez-Lopez et al., 2002), are unknown.

present in the negative control, a baker's yeast strain of *S. cerevisiae*. Moreover, the FRY111 strain displayed the same profile of bands that the IGC5323 strain, indicating that the Ura$^-$ phenotype was not the consequence of a genomic rearrangement at the *URA3* locus. Similar results were obtained by PCR (data not shown).

3.2. Construction of a URA3-targeted disruption cassette

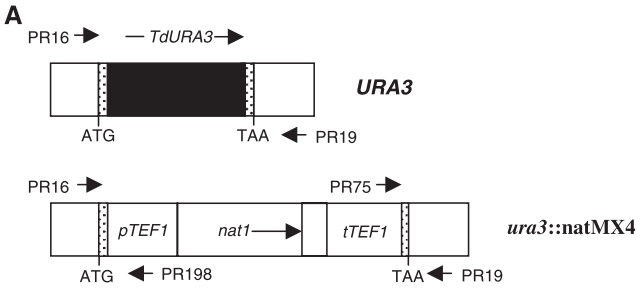
The development of molecular and genetic tools is essential to gain knowledge on the basis of the high stress resistance of *T. delbrueckii* strains. Phenotypes observed with directed gene alterations, a common practice in many organisms, have proven to be an important source of information for functional analyses. In *S. cerevisiae*, one-step gene disruptions based in the use of heterologous G418 resistance modules, kanMX3 and kanMX4 (Wach et al., 1994), have been successfully employed for whole genome analysis http://sequence-www.stanford.edu/group/yeast_deletion_project). Recently, a new dominant disruption cassette containing the nourseothricin (clonNAT) resistance cassette natMX4 has been shown to be phenotypically neutral and suited for creating mutated *S. cerevisiae* strains (Goldstein and McCusker, 1999).

According to this, we tested the power of natMX4-based modules as a tool for gene disruptions in *T. delbrueckii*. For this, we constructed a PCR-generated fragment containing the natMX4 module flanked by 45 and 42 bp homologous to the 5′-and 3′-site of *TdURA3*, positions +2/+46 and +798/+757 from the start codon, respectively. This linear fragment was used (2 μg) to transform the industrial *T. delbrueckii* IGC5323, and transformants were selected on YPD-clonNAT (50 μg/ml) plates. At this concentration, the growth of the parental strain was prevented (data not shown). Transformation efficiency was lower (2-5 transformants per μg of DNA) than previously reported for laboratory (Wach et al., 1994; Goldstein and McCusker, 1999) or industrial (Puig et al., 1998) strains of *S. cerevisiae* transformed with PCR-targeting disruption cassettes. Like in this yeast, a background of abortive transformants was observed, and in consequence, the clonNAT-resistance phenotype of the colonies was confirmed by streaking out and regrowth on clonNAT-containing plates.

Detection of the correct disruption of *TdURA3* was done by either Southern bot analysis or diagnostic PCR. As it can be seen in Fig. 1, the integration appeared to occur in the *URA3* locus, since in the resulting strain, referred as FRY112 (*ura3::*natMX4), the more intense band observed in the wild-type sample was lost. Since this strain was Ura$^-$, the presence of two weak extra bands corresponding with lower molecular size fragments in both wild-type and mutant cells (Fig. 1) should be associated to unspecific hybridisation of the probe. Indeed, this was confirmed by PCR, using primers designed to bind outside of the replaced *URA3* sequence (Primer PR16 and PR19, Table 1) and within the marker module (PR75 and PR198, Table 1). A schematic representation of this type of analysis is depicted in Fig. 2. As it is shown, the disruptant strain showed the pattern of fragments expected for the presence of a *ura3::*natMX4 allele, whereas the wild-type band was only visible for the IGC5323 strain. Overall, these results appear to indicate that *URA3* is present in a single copy in the genome of this industrial strain. In consonance with this, analysis by flow cytometry indicated that *T. delbrueckii* IGC5323 displays approximately haploid DNA content (data not shown).

3.3. Expression of heterologous genes in URA$^-$ T. delbrueckii strains

An important point in the potential biotechnological application of *Torulaspora* is the possibility of its use as a recipient of foreign genes. Therefore, we tested the utility of *T. delbrueckii* IGC5323-derivative Ura$^-$ strains, FRY111 and FRY112, by transforming cells with the YEp195ACT-X24 plasmid (*URA3*) which contains the *xlnB* cDNA under the control of the promoter of the *ACT1* gene from *Saccharomyces*. The *xlnB* cDNA encodes for the xylanase X24 from *Aspergillus nidulans* (Perez-Gonzalez et al., 1996), an enzyme which shows positive effects on bread quality parameters (Monfort et al., 1996, 1997). As a control strain, we used the *S. cerevisiae* baker's yeast strain HS13 (*ura3*). Transformants, selected by its ability to grow in the absence of uracil and to produce haloes of hydrolysis on xylane-plates, were further analysed in liquid medium. As it is shown in Fig. 3, transformants cells of the FRY112 strain were able to express and export active recombinant xylanase to the culture



B

lane	strain	primers	PCR products (expected size, bp)
1	wt	PR16-PR19	871
2	FRY112	PR16-PR19	1471
3	wt	PR16-PR198	-
4	FRY112	PR16-PR198	219
5	wt	PR19-PR75	-
6	FRY112	PR19-PR75	194

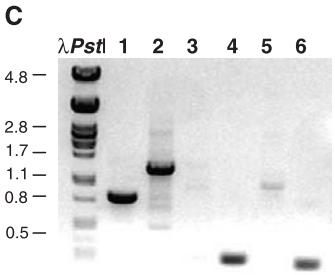


Fig. 2. PCR verification of *URA3* disruption. (A) Schematic diagram of strategy for diagnostic PCR of *URA3* loci, wild-type (*URA3*) and mutant (*ura3::*natMX4) where a gene fragment (black box) has been replaced by the natMX4 module. Fragment sizes are not to scale. (B) Combination of primers used to detect the correct integration of the disruption cassette and expected size for the PCR products. (C) Analysis of cells of the wild-type (wt) and FRY112 strain. PCR-amplified fragments were separated by electrophoresis in 1% agarose and visualised with ethidium bromide.

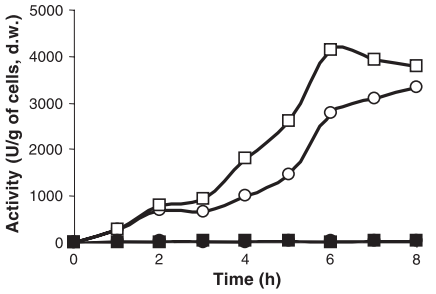


Fig. 3. Production of xylanase by transformants of *T. delbrueckii* FRY112 (○) and *S. cerevisiae* HS13 (□). Cells were grown on molasses plates, collected and resuspended in SN medium [7.5 mg of cells (d.w.) per ml]. At different times, the levels of xylanase were measured in the culture supernatant as described in the Materials and methods section. Cells harbouring YEp195ACT-X24 (open symbols) or YEplac195 (closed symbols).

supernatant. The enzyme production rate was lower for *Torulaspora* than for *Saccharomyces*, although at the end of the period analysed (8 h), the level of extracellular activity was similar for both strains, around 4000 U/g of cells (d.w.). Similar results were observed for transformants of the mutant FRY111 strain (data not shown).

4. Concluding remarks

The aim of this work was to obtain genetic and molecular tools for industrial *T. delbrueckii* strains and to demonstrate the possibilities of its use as host of heterologous genes. As described in this report, classical and modern techniques, widely used to obtain auxotrophic mutants of *S. cerevisiae*, can be successfully employed in *Torulaspora* strains. A PCR-target-

ing disruption cassette allowed the knock-out of *TdURA3*. Since, several dominant drug resistance markers, each with a unique resistance phenotype are now available (Goldstein and McCusker, 1999), this result opens the possibility to construct multiple mutations in *Torulaspora* strains and to conduct functional studies in this putative model organism. Of special interest was to know that Ura$^-$ mutants of baker's yeast of *Torulaspora* could be useful for the generation of recombinant strains. At this respect, it would be important to investigate if the auxotrophic marker modify the baking traits of the parental strain and to construct food-safe-grade cassettes for the stable integration of foreign genes according to current regulations.

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References

Alani, E., Cao, L., Kleckner, N., 1987. A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. Genetics 116, 541–545.

Almeida, M.J., Pais, C.S., 1996a. Characterization of the yeast population from traditional corn and rye bread doughs. Lett. Appl. Microbiol. 23, 154–158.

Almeida, M.J., Pais, C.S., 1996b. Leavening ability and freeze tolerance of yeasts isolated from traditional corn and rye bread doughs. Appl. Environ. Microbiol. 62, 4401–4404.

Bailey, M., Biely, P., Poutanen, K., 1992. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23, 257–270.

Belloch, C., Querol, A., García, M.D., Barrio, E., 2000. Phylogeny of the genus *Kluyveromyces* inferred from the mitochondrial cytochrome-c oxidase II gene. Int. J. Syst. Evol. Microbiol. 50, 405–416.

Boeke, J.D., LaCroute, F., Fink, G.R., 1984. A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. Mol. Gen. Genet. 197, 345–346.

Dower, W.J., 1990. Electroporation of bacteria: a general approach to genetic transformation. In: Setlow, J.K., Hollaender, A. (Eds.), Genetic Engineering Principles and Methods, vol. 12. Plenum, New York, pp. 275–296.

Esteve-Zarzoso, B., Gostincar, A., Bobet, R., Uruburu, F., Querol, A., 2001. Selection and molecular characterization of wine yeasts isolated from the "El Penedes" area. Food Microbiol. 17, 553–562.

Gietz, R.D., Sugino, A., 1988. New yeast—*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74, 527–534.

Goldstein, A.L., McCusker, J.H., 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast 15, 1541–1553.

Hahn, Y.-S., Kawai, H., 1990. Isolation and characterization of freeze-tolerant yeasts from nature available for the frozen-dough method. Agric. Biol. Chem. 54, 829–831.

Hernandez-Lopez, M.J., Prieto, J.A., Randez-Gil, F., 2002. Isolation and characterisation of the gene *URA3* encoding the orotidine-5′-phosphate decarboxylase from *Torulaspora delbrueckii*. Yeast 19, 1431–1435.

Hernandez-Lopez, M.J., Prieto, J.A., Randez-Gil, F., 2003. Osmotolerance and leavening ability in sweet and frozen sweet dough. Comparative analysis between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* baker's yeast strains. Antonie van Leeuwenhoek (in press).

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

James, S.A., Collins, M.D., Roberts, I.N., 1996. Use of an rRNA internal transcribed spacer region to distinguish phylogenetically related species of the genera *Zygosaccharomyces* and *Torulaspora*. Int. J. Syst. Bacteriol. 46, 189–194.

Krugel, H., Fiedler, G., Smith, C., Baumberg, S., 1993. Sequence and transcriptional analysis of the nourseothricin acetyltransferase-encoding gene nat1 from *Streptomyces noursei*. Gene 127, 127–131.

McCusker, J.H., Davis, R.W., 1991. The use of proline as a nitrogen source causes hypersensitivity to, and allows more economical use of 5FOA in *Saccharomyces cerevisiae*. Yeast 7, 607–608.

Monfort, A., Blasco, A., Prieto, J.A., Sanz, P., 1996. Combined expression of *Aspergillus nidulans* endoxylanase X24 and *Aspergillus oryzae* α-amylase in industrial baker's yeasts and their use in bread making. Appl. Environ. Microbiol. 62, 3712–3715.

Monfort, A., Blasco, A., Prieto, J.A., Sanz, P., 1997. Construction of baker's yeast strains that secrete different xylanolytic enzymes and their use in bread making. J. Cereal Sci. 26, 195–199.

Oda, Y., Tonomura, K., 1995. Molecular genetic properties of the yeast *Torulaspora pretoriensis*: characterization of chromosomal DNA and genetic transformation by *Saccharomyces cerevisiae*-based plasmids. Curr. Genet. 27, 131–134.

Oda, Y., Yabuki, M., Tonomura, K., Fukunaga, M., 1997. A phylogenetic analysis of *Saccharomyces species* by the sequence of 18S-28S rRNA spacer regions. Yeast 13, 1243–1250.

Perez-Gonzalez, J.A., De Graaff, L.H., Visser, J., Ramon, D., 1996. Molecular cloning and expression in *Saccharomyces cerevisiae* of two *Aspergillus nidulans* xylanase genes. Appl. Environ. Microbiol. 62, 2179–2182.

Puig, S., Ramón, D., Perez-Ortin, J.E., 1998. Optimized method to obtain stable food-grade recombinant wine yeast strains. J. Agric. Food Chem. 46, 1689–1693.

Querol, A., Ramón, D., 1996. The application of molecular techniques in wine microbiology. Trends Food Sci. Technol. 7, 73–78.

Randez-Gil, F., Sanz, P., Prieto, J.A., 1999. Engineering baker's yeast: room for improvement. Trends Biotechnol. 17, 237–244.

Randez-Gil, M.F., Prieto Alamán, J.A., Hernandez-Lopez, M.J., 2002. Utilization of *Torulaspora delbrueckii* strains in the production of sweet dough. PCT ES02/00431.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sherman, F., Fink, G.R., Hicks, J.B., 1986. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schuller, D., Corte-Real, M., Leao, C., 2001. A differential medium for the enumeration of the spoilage yeast *Zygosaccharomyces bailii* in wine. J. Food Prot. 63, 1570–1575.

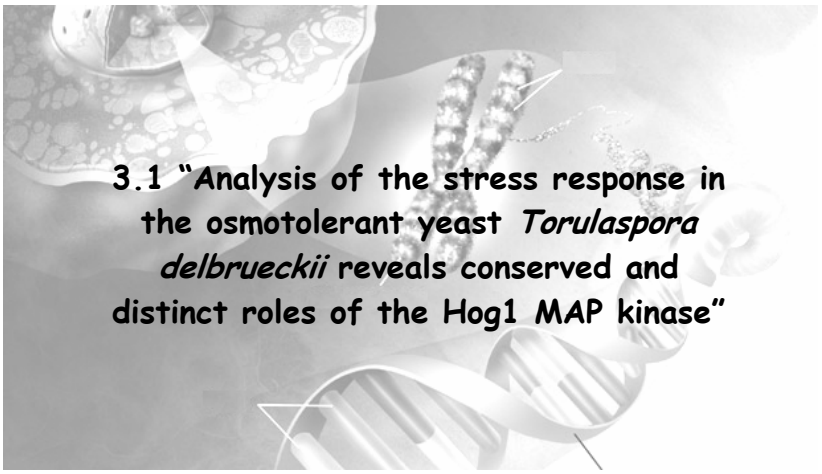
Wach, A., Brachat, A., Pohlmann, R., Philippsen, P., 1994. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. Yeast 10, 1793–1808.

Watanabe, Y., Imai, K., Oishi, H., Tamai, Y., 1996. Disruption of phospholipase B gene, *PLB1*, increases the survival of baker's yeast *Torulaspora delbrueckii*. FEMS Microbiol. Lett. 145, 415–420.

Yokoigawa, K., Murakami, Y., Kawai, H., 1995. Trehalase activity and trehalose content in a freeze-tolerant yeast *Torulaspora delbrueckii*, and its freeze-sensitive mutant. Biosci. Biotechnol. Biochem. 59, 2143–2145.

Capítulo 3

" *Torulaspora delbrueckii*, aislamiento de genes y caracterización de rutas de respuesta a estrés"



3.1 "Analysis of the stress response in the osmotolerant yeast *Torulaspora delbrueckii* reveals conserved and distinct roles of the Hog1 MAP kinase"

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Analysis of the stress response in the osmotolerant yeast *Torulaspora delbrueckii* reveals conserved and distinct roles of the Hog1 MAP kinase

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Summary

T. delbrueckii **has emerged during evolution as one of the most osmotolerant yeasts. However, the molecular mechanisms underlying this unusual stress resistance are poorly understood. In this study, we have characterised the functional role of the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway in mediating the osmotic and other stress responses in *T. delbrueckii*. We show that *T. delbrueckii* Hog1p homologous, TdHog1, is phosphorylated after transfer of cells to NaCl- or sorbitol-containing medium. However, TdHog1p plays a minor role in tolerance to moderate osmotic stress conditions, a trait mainly related with the osmotic balance. In consonance with this, lack of TdHog1p only produced a weak defect in the timing of the osmostress-induced glycerol and *GPD1* mRNA overaccumulation. *Tdhog1*Δ mutants also failed to display aberrant morphology changes in response to osmotic stress. Furthermore, our data indicate that the *T. delbrueckii* HOG pathway has evolved to respond to specific environmental conditions and to play a pivotal role in the stress-cross protection mechanism.**

Introduction

The ability of *Saccharomyces cerevisiae* to withstand increasing osmotic pressures is critical for its survival in natural habitats and an essential trait in many biotechnological processes. Variation in ambient osmolarity is a constant phenomenon occurring in almost all steps from yeast biomass production to downstream applications, in which cells have to grow and perform optimal fermentation under hostile conditions (Randez-Gil *et al.*,

2003). However, the success of this industrial microorganism is based upon its capacity to transform rapidly carbohydrates into ethanol and CO_2, and not on its unusual resistance to environmental stresses. Yeasts other than *S. cerevisiae,* like *Zygosaccharomyces rouxii*, *Debaryomyces hansenii* or *Torulaspora delbrueckii*, display much higher resistance to adverse conditions, and in particular to osmotic stress. This characteristic made of these yeasts a potential model to explore and understand the mechanisms underlying osmotic tolerance in eukaryotic cells. However, little is know about the molecular basis of this phenotype and, in most cases, the characterisation of signalling pathways, transcription factors and gene targets in these non-*Saccharomyces* species remains elusive.

Stress responses have been extensively investigated in *S. cerevisiae*. Yeast cells exposed to an osmostressing environment show a particular transcription profile. Thus, over 250-400 genes covering a wide variety of physiological functions, are up-regulated after different conditions of osmotic shock (Gasch *et al.*, 2000; Rep *et al.*, 2000; Causton *et al.*, 2001). Stimulated expression of these genes appears to be mainly dependent on three well characterised molecular signalling pathways, the cAMP-activated PKA (for Protein Kinase A) pathway (Thevelein and de Winde, 1999), the HOG (for High Osmolarity Glycerol) pathway (Westfall *et al.*, 2004), one of the five MAP (for Mitogen Activated Protein) kinase (MAPK) pathways known in *S. cerevisiae* (Gustin *et al.*, 1998) and the calcineurin/Crz1p pathway, which is specifically required for adaptation to high salt (Rusnak and Mertz, 2000).

The HOG pathway consists of two discrete signalling branches composed by a putative osmosensor coupled to a MAPKs cascade, any of which can lead to the phosphorylation and activation of the core MAPK Hog1p, the ortholog to mammalian p38 and fission yeast Sty1p SAPK (for Stress Activated Protein Kinase) (Gacto *et al.*, 2003). Osmostress-induced phosphorylation of Hog1p triggers its nuclear accumulation (Ferrigno *et al.*, 1998; Reiser *et al.*, 1999) and the later induction of many osmostress-responsive genes (Rep *et al.*, 2000; O'Rourke and Herskowitz, 2004), between them *GPD1* (Rep *et al.*, 1999), the main gene for the production of the compatible osmolyte glycerol in *S. cerevisiae* (Albertyn *et al.*, 1994). Although the correlation between Hog1p activation, enhanced glycerol production and osmotic resistance is well established in *S. cerevisiae*, it is unclear if the MAPK pathway plays a similar role in other yeasts, particularly in highly osmotolerant species.

MAP kinases homologous to *S. cerevisiae* Hog1p, have been identified in different yeast species, like *S. pombe* (Millar *et al.*, 1995), *Candida albicans* (San Jose *et al.*, 1996), *Z. rouxii* (Iwaki *et al.*, 1999) and *D. hansenii* (Bansal and Mondal, 2000), and multicellular fungi, between others *Aspergillus nidulans* (Han and Prade, 2002), *Neurospora crassa* (Zhang *et al.*, 2002) and the human pathogen *Crytoccoccus neoformans* (Bahn *et al.*, 2005). Although only a few of them have been studied in

detail, it appears that Hog1p orthologs share conserved and distinct roles in response to a variety of environmental cues. Whereas inactivation of the MAPK in *S. cerevisiae* has dramatic effects on growth under hyperosmotic conditions, *hog1* null mutants from *Z. rouxii* could grow as well as the parental strain in the presence of 2 M NaCl (Iwaki *et al.*, 1999). Disruption of *HOG1*-homologus MAPKs from *C. neoformans* (Bahn *et al.*, 2005) or *A. nidulans* (Kawasaki *et al.*, 2002) also results in weak or no growth defects, respectively, in the presence of high osmolyte concentrations. Moreover, the HOG-pathway appears to have evolved in some species to respond to additional extracellular stimuli and execute different cellular roles in a niche-depending manner. Examples include cell-to-cell signalling and virulence in *C. albicans* (Alonso-Monge *et al.*, 1999; Smith *et al.*, 2004) or *C. neoformans* (Bahn *et al.*, 2005), fungicide resistance in *N. crassa* (Zhang *et al.*, 2002) or methylglyoxal tolerance in *S. cerevisiae* (Aguilera *et al.*, 2005). If this niche-specific evolution also apply to highly osmotolerant species and its significance in the adaptation of eukaryotic cells to high osmotic environments remains unclear.

In this study, we have isolated the Hog1p homologous of the yeast *T. delbrueckii*, a facultative fermentative specie characterised by its exceptional resistance to osmotic stress (Kurtzman, 1998; Hernandez-López *et al.*, 2003). Our aim in this work was to investigate the functional role of the HOG pathway in the stress response of *T. delbrueckii* and determine if this signalling route has evolved to allow this yeast to proliferate in high osmolarity environments. Surprisingly, we found that glycerol accumulation, the key feature for osmotic tolerance, is mainly controlled by regulatory mechanisms other than the HOG pathway. On the contrary, the *T. delbrueckii* HOG pathway has undergone a functional specialization, being used as central module of the stress cross-protection mechanism in this yeast.

Results

Cloning and molecular characterisation of T. delbrueckii HOG1 *gene*

A *T. delbrueckii* genomic library (Hernandez-Lopez *et al.* 2002) was screened via complementation of a *S. cerevisiae hog1*Δ mutant. From more than 1,000 transformants, only five were able to restore growth of the osmosensitive strain on 0.5 M NaCl. Plasmidic DNAs were rescue from all of them, amplified in *E. coli*, and compared on the basis of their restriction map, which revealed three different patterns (data not shown). Analysis by Southern blot of these plasmids allowed identifying one, pMJH28, which hybridised under heterologous conditions with a probe of the *S. cerevisiae HOG1* gene (data not shown). DNA sequencing of the fragment revealed the presence of a 1,284-bp-length uninterrupted open reading frame encoding a putative 427-amino acids protein, TdHog1p. This peptide showed 94 and 88% overall identity with *Z. rouxii* ZrHog2 and ZrHog1 proteins, respectively, 88% with *S. cerevisiae* Hog1p and 85% with *D. hansenii* DHog1p. TdHog1p also displayed a high degree of identity with other members of the Hog1/Spc/p38 MAPK

subfamily, like *N. crassa* Os-2 (83%), *C. albicans* CaHog1p (78%), *A. nidulans* HogA (76%), *C. neoformans* Hog1p (76%) and mammalian p38 (47%).

Protein domain analysis using the PROSITE software at the Expert Protein Analysis System server (available at http://www.expasy.org), showed that TdHog1p contains a typical catalytic protein kinase domain (positions 23-302), also present in other Hog1p and p38 MAPK homologues. Within this region we identified a protein kinase ATP-binding region (positions 29-53) and a consensus Ser/Thr protein kinase active site (positions 140-152). In addition, the *T. delbrueckii* Hog1 protein contains a TGY dual phosphorylation signature (amino acids 174-176), characteristic of hyperosmolarity-activated MAP kinases (Cano and Mahadevan, 1995), a CD (for Common Docking) domain (Tanoue *et al.*, 2000) and an alanine rich region at the C-terminus, positions 302 to 316 and 364 to 383, respectively, also found in *S. cerevisiae* Hog1p.

To further confirm the identity of the *TdHOG1* gene product, we cloned the entire open reading frame (GenBank accession number DQ020519), including 132 bp upstream from the ATG and 125 bp of the 3'-UTR, into the yeast multicopy expression vector YEplac195, and the resulting construct YEpTdHOG1, was transformed into *S. cerevisiae* W303-1A *hog1*Δ strain and examined for salt tolerance. Mutant cells transformed with the empty plasmid were used as control. As it is shown in Figure 1A, overexpression of *TdHOG1* complemented the osmosensitivity phenotype of the *S. cerevisiae hog1*Δ mutant strain on 1 M NaCl. We also analysed if the replacement of the Hog1p MAPK by its counterpart TdHog1p could improve the intrinsic salt-sensitivity of *S. cerevisiae*. Since the *HOG1* gene is present in single copy in the *S. cerevisiae* genome, cells of the *hog1*Δ mutant strain were transformed with centromeric plasmids, YCpHOG1 and YCpTdHOG1, carrying the genes from *S. cerevisiae* and *T. delbrueckii*, respectively, and transformants were tested under severe salt-stress conditions. On SD liquid medium supplemented with 1.5 M NaCl, either YCpHOG1 or YCpTdHOG1 transformants showed a lag phase of about 15 h. After this time, growth was resumed in both cases, displaying almost parallel growth curves and reaching by 60 h a similar OD_{600} (Fig. 1B). Hence, structural and functional evidences indicate that TdHog1p is a member of the Hog1/p38-MAPK protein family able to complement the osmosensitive phenotype of a *hog1*Δ mutant. However, low-copy number expression of *TdHOG1* in *S. cerevisiae* does not confer enhanced salt-tolerance.

TdHog1p is phosphorylated upon osmotic stress

We examined the effect of a shift to NaCl-containing medium on the phosphorylation state of TdHog1p in cells of the wild-type strain PYCC5321. Fig. 2A shows the results of a Western blot analysis of protein extracts analyzed with anti-phospho p38 antibody, which recognizes the dual-phosphorylated form of several p38/Hog1p-homologues, and polyclonal anti-Hog1p, an antibody raised against the carboxy terminus of *S. cerevisiae* Hog1p, as a loading control. TdHog1p was

clearly activated when YPD-grown cells (DO_{600} = 1.0) were subjected to mild NaCl-stress conditions (0.5 M). The kinetics of phosphorylation was similar to that previously reported for *S. cerevisiae* cells exposed to similar NaCl concentrations (Van Wuytswinkel *et al.*, 2000). Indeed, the phosphorylation level reached a maximum within 1-5 min of exposure to 0.5 M NaCl and decreased markedly after 10 min (Fig. 2A). As expected, TdHog1p was also sensitive to the presence of external sorbitol (Fig. 2B). Thus, both NaCl and sorbitol trigger the activation of the HOG pathway.

The phosphorylation pattern of TdHog1p was also analysed in samples of the T. delbrueckii PYCC5323 wild-type strain. Both PYCC5321 and PYCC5323 were isolated in a broad survey of bread dough microbiota (Almeida and Pais, 1996). Remarkably, cells of this strain were unable to induce the overphosphorylation of TdHog1p in response to NaCl exposure (Fig. 2A). Similar results were obtained when the activation was tested using sorbitol (data not shown). This defect is likely caused by a missfunction of any HOG-pathway element upstream the MAPK, since TdHog1 proteins from both

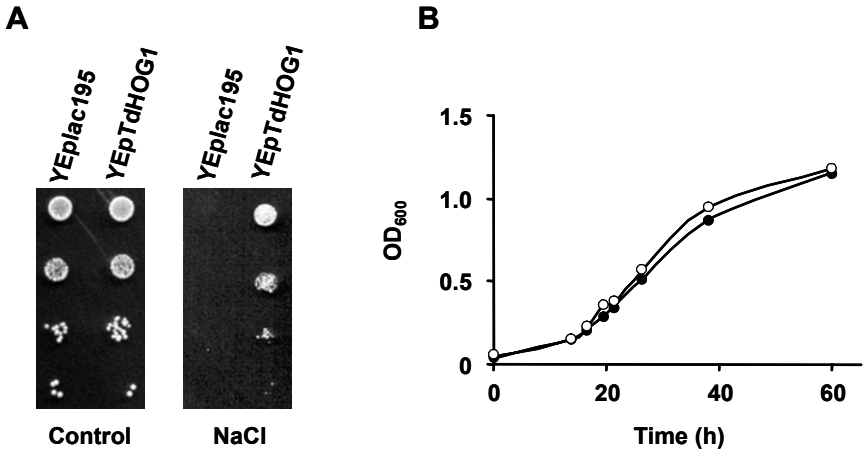

Fig. 1. Expression of *TdHOG1* complements the osmosensitive phenotype of a *hog1*Δ mutant, but does not confer enhanced salt-tolerance in *S. cerevisiae*. A: Mid-exponential grown cultures of W303-1A *hog1*Δ mutant cells transformed with the empty plasmid YEplac195 or YEpTdHOG1 were adjusted to OD_{600} = 0.3, diluted ($1\text{-}10^{-3}$) and spotted (3 μl) onto SD plates lacking (control) or containing 1 M NaCl (final concentration). Plates were inspected after 2-4 days at 30ºC. **B:** YCpHOG1 (○) and YCpTdHOG1 (●) transformants of the *S. cerevisiae* *hog1*Δ mutant strain were grown on SD liquid medium, collected, transferred (initial OD_{600} = 0.05) to the same medium containing 1.5 M NaCl, and the OD_{600} was measured at regular intervals. A representative experiment is shown. Independent experiments revealed identical kinetics of growth.

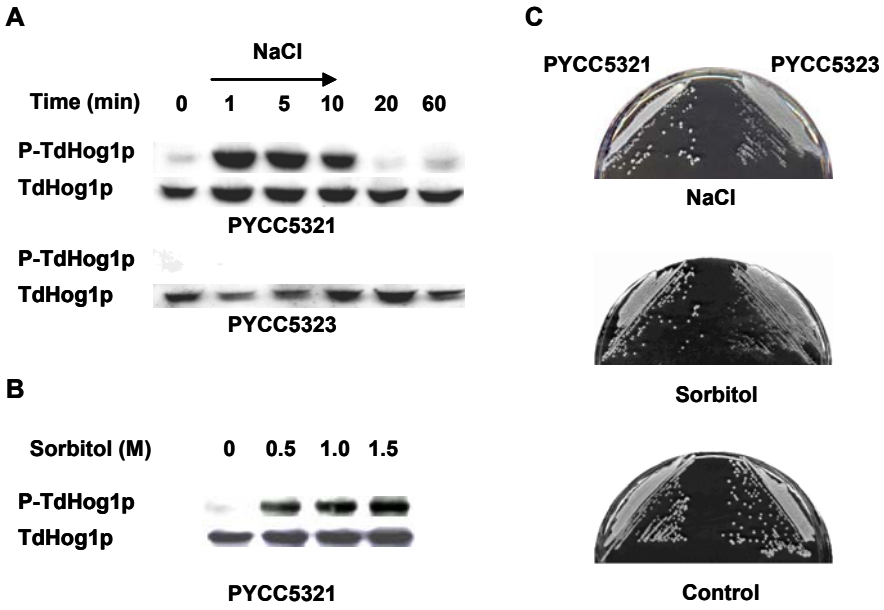

Fig. 2. TdHog1p phosphorylation and growth of *T. delbrueckii* strains under osmotic stress conditions. A: cells of the *T. delbrueckii* PYCC5321 and PYCC5323 wild-type strains were grown in YPD at 30ºC until early-exponential phase (OD_{600} = 0.4-0.6) and then transferred to NaCl-containing medium (0.5 M). At the indicated times, cells were harvested, processed and the crude protein extracts analysed by Western blot. Non-stressed cells (time 0) were used as control. Total protein extracts were analyzed by SDS-PAGE and blotting with anti-phospho-p38 antibody (P-TdHog1p). The membrane was then re-blotted with anti-Hog1p antibody as loading control (TdHog1p). **B:** Western blot analysis of YPD-grown cells of the *T. delbrueckii* PYCC5321 strain transferred to YPD-sorbitol at 0.5, 1.0 or 1.5 M final concentration. After 5 min, cells were harvested and analysed for P-TdHog1p levels as described above. **C:** wild-type strains were analysed for growth on YPD (control) or YPD supplemented with 1.0 M sorbitol or 0.5 M NaCl. Plates were inspected after 2-4 days at 30ºC. Representative experiments are shown.

strains, PYCC5321 and PYCC5323, share 100% identity (data not shown). Moreover, non-phosphorylated TdHog1p was easily detected in protein extracts of the PYCC5323 strain by using polyclonal anti-Hog1p (Fig. 2A, loading control).

Effect of TdHOG1 *on growth under NaCl and sorbitol stress*

The above result prompted us to investigate the growth of both strains PYCC5321 and PYCC5323 under osmotic stress conditions. As shown in Fig. 2C, cells of strain PYCC5321 were more resistant to 0.5 M

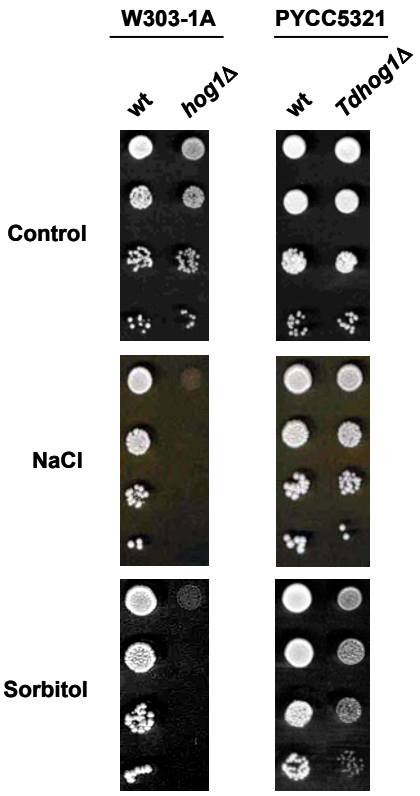

Fig. 3. The HOG-pathway has a minor role in the osmotolerance phenotype of *T. delbrueckii*. Growth of wild-type and *hog1*Δ mutant strains of *S. cerevisiae* W303-1A and *T. delbrueckii* PYCC5321 (*Tdhog1*Δ) was examined on solid YPD (control) or YPD containing 1.0 M sorbitol or 0.5 M NaCl. YPD-pregrown cells were diluted, spotted and incubated as described in Fig. 1. A representative experiment is shown.

NaCl-stress than those of the PYCC5323 strain. When 0.5 M NaCl was replaced by 1.0 M sorbitol, which both give approximately the same water activity, around 0.98 (Rep *et al.*, 1999), strain PYCC5323 showed again a growth defect as compared with strain PYCC5321. However, the phenotypes in either NaCl or sorbitol were by far much less

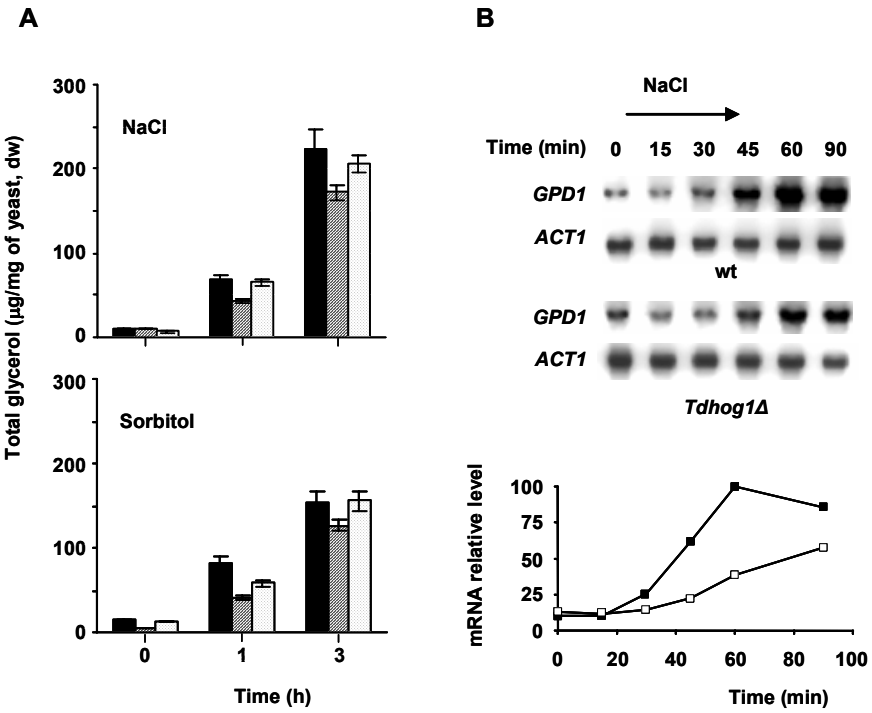

Fig. 4. Glycerol production and *GPD1* induction in wild-type and *Tdhog1*Δ mutant cells of *T. delbrueckii*. A: total glycerol levels after a hyperosmotic shock in cultures of the *T. delbrueckii* strains, PYCC5321 (black bars), *Tdhog1*Δ mutant (PYCC5321 background, grey bars) and PYCC5323 (white bars). Cells were grown in YPD to mid-exponential phase (OD_{600} = 1.5), collected and transferred to the same medium containing 1.0 M NaCl or 1.0 M sorbitol. At the indicated times, aliquots of the cell culture were withdrawn and analysed for glycerol content as described in the Experimental Procedures section. Data represent the mean value of at least three independent experiments. Errors were calculated by using the formula: (1.96 × *SD*) / √*n*, where *n* is the number of measurements. dw, dry weight. **B:** YPD-grown cells of the PYCC5321 wild-type (■) and *Tdhog1*Δ mutant (□) strain were transferred to 1.0 M NaCl-containing medium. Samples were taken at the indicated times and analysed by Northern blot as described in the Experimental Procedures section. Filters were probed for *GPD1* mRNA. The graph in panel B represents quantification of the mRNA levels of *GPD1*, relative to those of *ACT1*.

pronounced than those previously reported for *S. cerevisiae hog1*Δ mutant strains (Albertyn *et al.*, 1994; see also Fig. 3).

In order to confirm the functional role of the *T. delbrueckii* HOG pathway, we constructed a *Tdhog1*Δ mutant of the strain PYCC5321 and cells were tested for their ability to grow on YPD plates supplemented with 0.5 M NaCl or 1.0 M sorbitol. In contrast to the dramatic osmosensitivity of the *S. cerevisiae hog1*Δ mutant, lack of the MAPK had scarce effects on the growth of *T. delbrueckii* cells under osmotic stress (Fig. 3). Hence, a shift to higher osmolarity triggers the activation of the *T. delbrueckii* HOG pathway. However, this event appears to have a marginal role in the adaptation of this yeast to osmotic stress.

Osmostress-induced glycerol overaccumulation and GPD1 *expression*

In *S. cerevisiae*, hyperosmotic shock triggers the Hog1p-dependent transcriptional induction of *GPD1*, the gene for the cytosolic glycerol-3-phosphate dehydrogenase, which in turn enhances the intracellular content of glycerol (Albertyn *et al.*, 1994), the only compatible osmolyte known in this yeast (Blomberg and Adler, 1992). Unlike *Saccharomyces*, *T. delbrueckii* is able to produce small amounts of arabinitol, galactitol and methyl-galactictol in response to osmotic stress, although glycerol is by far the main osmoregulator (Hernandez-Lopez *et al.*, 2003). Consequently, we analysed the kinetics of glycerol production in YPD-cultures of wild-type and *Tdhog1*Δ mutant strains subjected to osmotic stress. As can be seen, lack of the MAPK slightly delayed the accumulation of total glycerol in response to 1.0 M sorbitol (Fig. 4A). Similar results were obtained at 0.5 M NaCl (data not shown) or even at higher salt concentrations, 1.0 M (Fig. 4A). However, after 3 h of incubation at either 1.0 M NaCl or 1.0 M sorbitol, the glycerol content in the culture of the *Tdhog1*Δ mutant enhanced to about 76 and 81% of the wild-type level, respectively (Fig. 4A). Under similar conditions, the glycerol content of *S. cerevisiae hog1*Δ mutant cells has been reported to be about 14% of the wild-type with NaCl and 37% with sucrose (Albertyn *et al.*, 1994). We did the same experiment with the *T. delbrueckii* strain PYCC5323. Except for a certain delay in the osmotic response to sorbitol (see 1 h samples, Fig. 4A), the glycerol levels observed in cultures of this strain were exactly the same as those in the PYCC5321 wild-type strain.

We then examined by Northern blot analysis the expression profile of *GPD1* in NaCl-stressed cells of the wild-type and *Tdhog1*Δ mutant strain. Since the gene encoding for glycerol-3-phosphate dehydrogenase of *T. delbrueckii* has not been cloned, total RNA samples were hybridised with a probe of the *S. cerevisiae GPD1* gene under heterologous conditions. Previous work in our laboratory has shown that this approach allows a reliable quantification of this mRNA (G. Hernandez, F. Randez-Gil, J.A. Prieto and F. Estruch, unpublished results). As expected, expression of *GPD1* was induced in cells of the wild-type exposed to 1.0 M NaCl, with an approximately 10-fold induction within 60 min (Fig. 4B). The absence of TdHog1p

altered the osmostress-induced expression of *GPD1*. However, the effects were less important as compared with those reported in the *S. cerevisiae hog1*Δ strain (Rep *et al.*, 1999). Although the maximal induction was shifted to later time points, the mutant was able to accumulate a *GPD1* mRNA level about 60% of the wild-type (Fig. 4B). In addition, lack of the MAPK did not affect the *GPD1* expression in non-stressed control cells. Similar results were observed in cells exposed to 1.0 M sorbitol (data not shown). Therefore, the *T. delbrueckii* HOG-pathway is involved in the full induction of *GPD1* in response to osmostress. However this effect is small and has an irrelevant contribution in the overaccumulation of glycerol.

Osmostress induced morphology changes in hog1*Δ mutants*

Exposure of wild-type *S. cerevisiae* cells to osmotic stress has no effect on their morphology. In contrast, a *hog1*Δ mutant display under the same conditions, morphological alterations (shmoo-like cells) characteristics of pheromone treated cells (Brewster *et al.*, 1993). Fig. 5 shows the budded morphology of wild-type cells of the *S. cerevisiae* W303-1A strain exposed to 1.0 M sorbitol or 1.0 M NaCl, in contrast to the

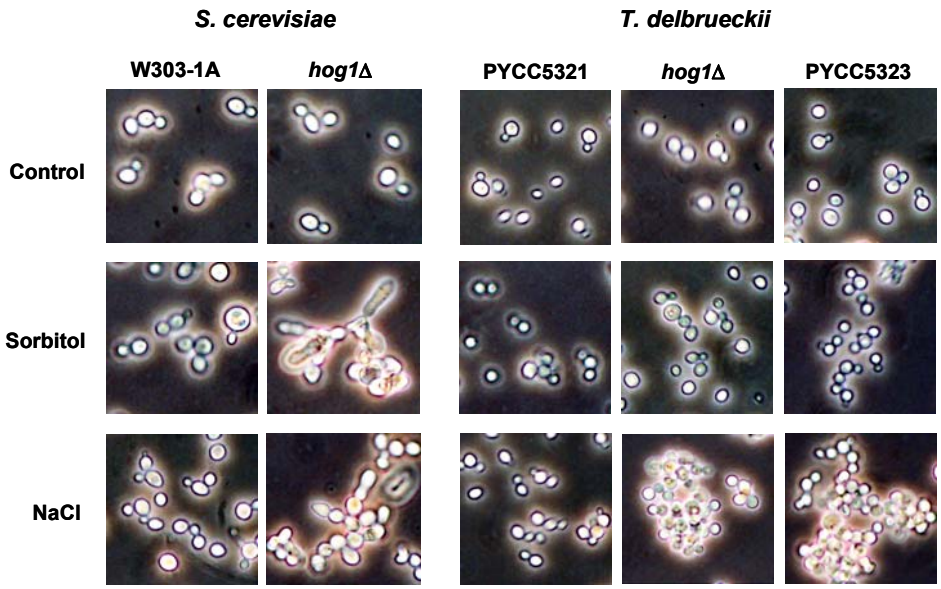

Fig. 5. Effect of TdHog1p on the osmostress-induced morphology changes of *T. delbrueckii*. Wild-type strains of *S. cerevisiae* (W303-1A) and *T. delbrueckii* (PYCC5321, PYCC5323), and corresponding *hog1*Δ mutants, *hog1*Δ and *Tdhog1*Δ (PYCC5321 background) were pre-grown in YPD medium, collected and then transferred (initial OD_{600} = 0.05) to the same medium (control) or YPD plus 1.0 M NaCl or 1.0 M sorbitol. Microphotographs were taken in an optical microscope after 16 h of culture. A representative cluster of cells is shown.

shmoos formed in the *S. cerevisiae hog1*Δ mutant. However, wild-type and *Tdhog1*Δ mutant strains of *T. delbrueckii* displayed a normal morphology in both the presence and absence of 1.0 M sorbitol. Only exposure to 1.0 M NaCl resulted in a clear morphological response, although shmoo-like cells were not observed (Fig. 5). Instead, cells showed a clear inability to separate normally. Thus the response appeared to be dependent of the chemical used. However, when cells were exposed to 2.0 M sorbitol, the result was exactly the same (data not shown). We then examined the morphology of cells of the PYCC5323 strain under the same conditions. As can be seen, lack of TdHog1p phosphorylation produced the same phenotype as that observed in the *Tdhog1*Δ mutant strain (Fig. 5). Again, blocking in cell separation was only evident at the highest osmolalities, 1.0 M NaCl (Fig. 5) or 2.0 M sorbitol (data not shown).

The functional role of T. delbrueckii *Hog1p in response to several stresses*

Additional functions to that of osmostress protection have been identified for HOG homologous pathways of yeast species, including *S. cerevisiae*, *C. albicans* and *S.*

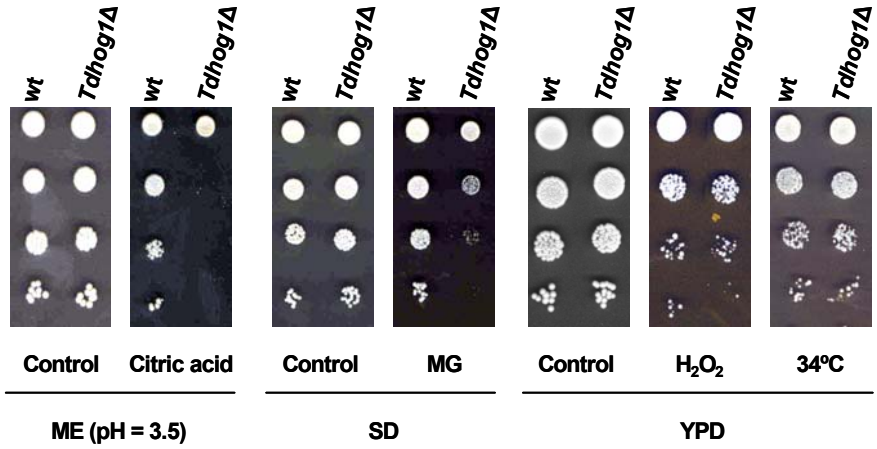

Fig. 6. TdHog1p is required in response to diverse stresses. Exponentially growing cells of the *T. delbrueckii* strains, wild-type PYCC5321 and corresponding *Tdhog1*Δ mutant, were spotted onto ME, SD or YPD plates containing 200 mM citric acid, 20 mM methylglyoxal (MG) and 4 mM H_2O_2, respectively. Control plates lacking the compounds cited above were tested in parallel. Growth at high temperature was inspected onto YPD plates incubated at 34ºC. For the rest, plates were incubated at 30ºC for 2-4 days. A representative experiment is shown.

pombe. Based on this, we analysed the requirement of the TdHog1 protein in the response of *T. delbrueckii* to diverse stimuli, like oxidative stress, high temperature, citric acid and methylglyoxal (Fig. 6). Clearly, the MAPK was essential for tolerance to methylglyoxal and citric acid. However, it appeared to be dispensable for growth at high temperature, 34ºC (Fig. 6). This functional profile is in good consonance with that previously reported for *S. cerevisiae* (Winkler *et al.*, 2002; Lawrence *et al.*, 2004; Aguilera *et al.*, 2005) and *C. albicans* (Alonso-Monge *et al.*, 2003; Smith *et al.*, 2004) Hog1 proteins. However, we also noted that the *T. delbrueckii* MAPK was not required for the response to oxidative stress (4 mM H_2O_2). This is in sharp contrast with the situation in other yeasts (Gacto *et al.*, 2003; Alonso-Monge *et al.*, 2003; Haghnazari and Heyer, 2004). Hence, the *T. delbrueckii* HOG pathway appears to be required under specific environmental conditions.

Stress cross-protection in T. delbrueckii

S. cerevisiae cells exposed to non-lethal doses of one stress develop tolerance to higher doses of the same stress, as well as to unrelated stresses, phenomena described as induced tolerance and cross-protection, respectively (Lewis *et al.*, 1995; Estruch, 2000). It is commonly accepted that yeasts, other than *Saccharomyces*, display induced tolerance. However, the existence of cross-protection mechanisms is not always evident and it has frequently been the object of controversy (Enjalbert *et al.*, 2003). The function of a general stress response pathway as responsible of this phenomenon in non-*Saccharomyces* yeasts is also unclear. Thus, recent evidences indicate that stress cross-protection in *C. albicans* depends specifically on the HOG-pathway (Smith *et al.*, 2004). Therefore we were interested to investigate this phenomenon in the osmotolerant yeast *T. delbrueckii*.

The results of plate growth presented in Fig. 7A demonstrate that *T. delbrueckii* is unable to grow at 37ºC (control). Therefore, we choose this growth inhibitory condition to test if the presence of sorbitol in the culture medium would provide stress cross-protection to *T. delbrueckii* cells. As shown in Fig. 7A, formation of colonies of the wild-type strain PYCC5321 was not detected after 4 days at 0.5 M sorbitol and 37ºC. However, this phenotype was suppressed when cells were challenged to 1.0 or 1.5 M sorbitol (Fig. 7A). Nevertheless, we rationalised that this phenomenon might not imply a previous adaptation of the cells by osmotic stress. Reduction of water activity by sorbitol or salts is indeed an effective way to reduce thermal death, as it has been demonstrated for osmotolerant yeasts as *D. hansenii* (Almagro *et al.*, 2000). Consequently, we investigated the implication of TdHog1p in this phenomenon. As can be seen, the thermal protection afforded by high concentrations of sorbitol was retained in the *Tdhog1*Δ strain. Nevertheless, growth of mutant cells was clearly impaired at 37ºC as compared with the wild-type, whereas at 30ºC, the differences were less pronounced, in particular at 1.0 M sorbitol (Fig. 7A). Hence, adaptation to thermal stress appears to be dependent upon TdHog1p. Presumably

growth at high temperature is also improved by water activity reduction mechanisms, being more pronounced at high osmolyte concentrations.

To investigate more in deep the role of TdHog1p in the above findings, YPD-grown cells were exposed for 3 h to 1.5 M sorbitol at 30ºC, and then transferred to 37ºC-prewarm YPD fresh medium lacking sorbitol (Fig. 7B). As can be seen, pretreatment of wild-type cells with an osmotic stress provided higher growth ability at 37ºC. However, this effect was lost in *Tdhog1*Δ cells (Fig. 7B). Hence, cross-protection exists in *Torulaspora*, being mediated by the HOG-pathway.

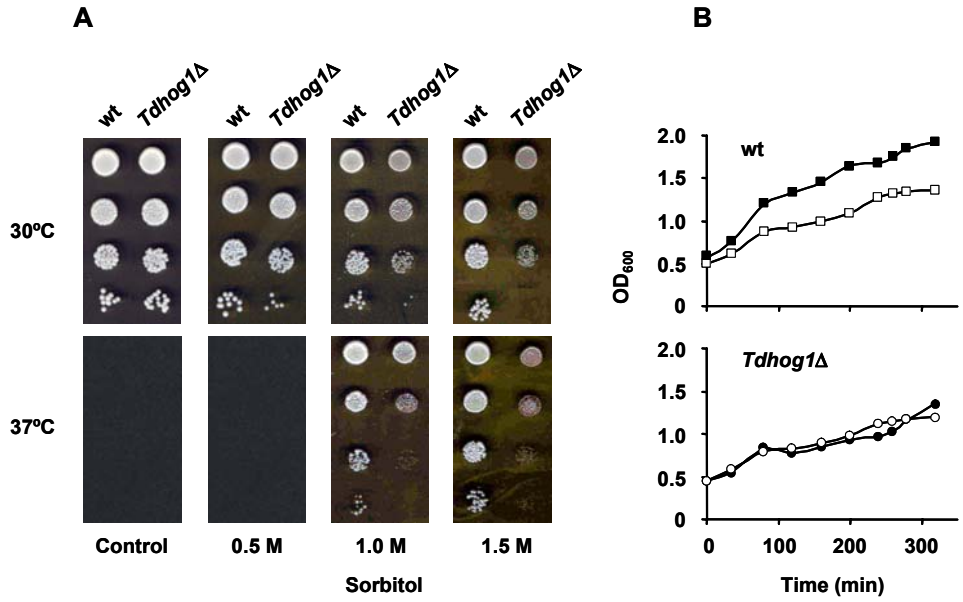

Fig. 7. The HOG-pathway mediates stress cross-protection in *T. delbrueckii*. A: YPD-grown cells of *T. delbrueckii* PYCC5321 wild-type and *Tdhog1*Δ mutant strains were spotted onto YPD plates (control) and YPD plates containing 0.5, 1.0 or 1.5 M sorbitol. Plates were incubated at 30 or 37ºC for 2-4 days. **B:** Exponentially YPD-growing cells of the wild-type (squares) and *Tdhog1*Δ mutant (circles) were either unstressed (open symbols) or exposed to 1.5 M sorbitol (closed symbols) at 30ºC. After 3 h, cells were washed, shifted to 37ºC-prewarmed YPD medium lacking sorbitol and the OD_{600} was measured at regular intervals. A representative experiment is shown. Independent experiments revealed identical kinetics of growth.

Discussion

Phylogenetically, *T. delbrueckii* and *S. cerevisiae* are closely related, as it has been previously demonstrated by sequence comparisons (Belloch *et al.*, 2000; Hernandez-Lopez *et al.*, 2002). Despite this genealogical relationship, *T. delbrueckii* is able to survive and proliferate at high osmolyte concentrations, a property not shared by *S. cerevisiae* (Hernandez-Lopez *et al.*, 2003). As a result, this non-conventional yeast is frequently found in distinct habitats than *S. cerevisiae*, between them cane molasses, syrups, honey and high-sugar-containing foods (Kurtzman, 1998). Hence, it would be expected they differ in the regulatory mechanisms and molecular targets that allow for adaptability to high osmolarity environments.

These differences were first evidenced in our analysis of the functional response of the *T. delbrueckii* HOG pathway to osmotic stress. Although the MAPK TdHog1p was rapidly phosphorylated after transfer to NaCl or sorbitol-containing media, this fact was not essential for growth of *T. delbrueckii* cells under moderate-stress conditions, 0.5 M NaCl or 1.0 M sorbitol. Only when severe stress conditions were tested, for example 1.5 M NaCl (data not shown), the requirement of a functional HOG pathway was evident. This result is in complete agreement with previous observations in *Z. rouxii hog1*Δ mutant cells, which show NaCl-sensitivity only at concentrations above 2.0 M (Iwaki *et al.*, 1999). Therefore, we predicted that this fact could reflect a distinct role of the HOG pathway in the osmoadaptation mechanism employed by *T. delbrueckii* and *S. cerevisiae*. Consistent with this, neither the osmostress-induced glycerol accumulation nor the expression of *GPD1* was strongly affected by the lack of TdHog1. Similar results were observed in *T. delbrueckii* cells displaying an insensitive HOG pathway. This is in sharp contrast with the situation in *S. cerevisiae*, where Hog1p is the main regulator of these responses (Albertyn *et al.*, 1994; Rep *et al.*, 1999). Nevertheless, we noted that glycerol production and *GPD1* mRNA levels were upregulated upon osmotic shock. Therefore, a sudden change in the water activity of the environment is perceived by *T. delbrueckii* as a stressful condition. Yet *T. delbrueckii*, as *S. cerevisiae*, must be often exposed to significant osmolarity fluctuations in natural habitats. The adaptation to these changes requires therefore of a strict regulatory system that allows adaptability avoiding a continuous stimulation of the response. Glycerol production is by far one of the most energy-requiring processes (van Dijken and Scheffers, 1986). Hence, both yeasts appear to follow a similar strategy in response to osmotic stress. However, they have diverged in the regulatory mechanisms that control glycerol accumulation.

The differential specialization of Hog1p functions in *S. cerevisiae* and *T. delbrueckii*, was further evidenced by analysis of the osmostress-induced morphological changes in the *Tdhog1*Δ strain. High osmolarity induces an aberrant morphology in *S. cerevisiae hog1*Δ mutants that resembles that of cells exposed to pheromones, shmoos or pear-shaped cells (Brewster and Gustin, 1994). This phenomenon is

consequence of the inappropriate activation of the pheromone pathway, a MAPK cascade that prepares cells for mating (Leberer *et al.*, 1997), which shares components with the HOG pathway, such as Ste20p, Ste50p and Ste11p (Gustin *et al.*, 1998). As we demonstrated, shmoos were not formed by *Tdhog1*Δ mutant cells exposed to sorbitol or NaCl. These data suggest that a cross-talk between the HOG and pheromone response MAPK pathways does not exist in *T. delbrueckii* or that is independent of TdHog1p. Indeed, previous studies in *S. cerevisiae* indicate that Hog1p prevents cross-talk between these pathways (O'Rourke and Herskowitz, 1998). Nevertheless, further work is required to identify upstream elements of the *T. delbrueckii* HOG pathway and clarify the activation mechanisms operating in this yeast.

Regarding the morphological effects of osmotic stress, we noted however that *Tdhog1*Δ mutant cells remain attached after budding at high osmolyte concentrations, 1.0 M NaCl or 2.0 M sorbitol, a phenotype that was missing in the wild-type strain. This apparent cell division defect resulted in large branched aggregates, similar to those observed for chitinase-minus *S. cerevisiae* strains with random budding pattern (Kuranda and Robins, 1991). This phenotype has also been reported in *hog1*Δ mutant cells of *C. albicans* subjected to 1.0 M NaCl, suggesting a link between cell wall metabolism and the activity of CaHog1p in this pathogenic yeast (Alonso-Monge *et al.*, 1999). Such a relationship has been also suggested in *S. cerevisiae*, since components of the HOG pathway appear to be involved in cell wall maintenance (Alonso-Monge *et al.*, 2001). At the light of our results this connexion appears to be extended to the *T. delbrueckii* HOG pathway.

Another aspect of relevance addressed in our work is the functional role of TdHog1p under stress conditions other than high osmolarity. Interestingly, TdHog1p has no apparent function at supraoptimal temperatures or at high levels of H_2O_2. The lack of TdHog1p activity at high temperature can account for the earlier observation that *S. cerevisiae* Hog1p is not essential for growth at 37ºC (Winkler *et al.*, 2002). Recently, Smith *et al.* (2004) also showed that *C. albicans* Hog1p is not activated by temperature upshifts. However, the finding that TdHog1p is not required in response to oxidative stress was quite unexpected. Previous reports have demonstrated that the HOG-pathway provides protection against this stressful condition in *S. cerevisiae* (Haghnazari and Heyer, 2004), as well as in other yeasts and fungi species, like *C. albicans* (Alonso-Monge *et al.*, 2003), *S. pombe* (Buck *et al.*, 2001) and *C. neoformans* (Bahn *et al.*, 2005). Although we have no obvious explanation for this result, it could reflect a larger ability of this yeast to cope with high levels of reactive oxygen species. Consistent with this, we found that *T. delbrueckii* was able to grow at high relative levels of H_2O_2 (4 mM) as compared with other yeasts and in particular with *S. cerevisiae*. Thus, the *T. delbrueckii* HOG pathway appears to have evolved not to respond to oxidative stress.

Our work also leads to the observation of stress cross-protection in *T. delbrueckii*, suggesting the existence in this yeast of a general stress response (Estruch, 2000). In *S. cerevisiae* the general stress response is mediated by a common pathway, the cAMP-protein kinase A (PKA) pathway (Thevelein and de Winde, 1999), which controls the activity and nuclear localization of the transcriptional factors *MSN2|MSN4* (Martínez-Pastor *et al.*, 1996). In addition, *S. cerevisiae* uses strategies of co-induction of defence mechanisms. Instead of a common pathway, different stresses control a common set of genes via different signalling pathways and transcription factors (Gasch *et al.*, 2000; Causton *et al.*, 2001). For example, osmotic shock induces in a Hog1p-dependent manner, the rapid and strong expression of *HSP104*, the most important chaperone providing thermotolerance in budding yeast (Lindquist and Kim, 1996). This appeared to be indeed the strategy employed by *T. delbrueckii*. Far of the classical concept of general stress response, we observed that growth of *T. delbrueckii* at 37ºC, a temperature that inhibits the proliferation of this yeast, was dependent on the activity of TdHog1p coupled with the thermal protection provided by sorbitol. Furthermore, we find that pre-treatment with sorbitol provides a growth advantage at 37ºC for wild-type cells, but not for *Tdhog1*Δ mutant cells. Therefore, stress-cross protection exists in *T. delbrueckii*, but this is mediated by TdHog1p. Similarly, Smith *et al.* (2004) reported Hog1p-dependent stress cross-protection in *C. albicans*, a result that fits well with the lack of functional role of homologues of *S. cerevisiae MSN2|MSN4* in this yeast (Nicholls *et al.*, 2004).

Overall the results presented in this study make emphasis in the divergence of a classical stress signalling pathway between different yeasts. Our current knowledge indicates that the HOG pathway has evolved in different yeasts in a niche-specific manner (Smith *et al.*, 2004; Bahn *et al.*, 2005). We also have demonstrated that changes in the Hog1p-*GPD1* relationships have occurred during the evolutionary divergence of *S. cerevisiae* and *T. delbrueckii*. While it is easy to image different mechanisms through which gene expression regulation evolves (Gasch *et al.*, 2004), it is much difficult to understand how this events determine the adaptability of yeasts to changing environments.

Experimental procedures

Yeast strains and culture conditions

T. delbrueckii strains PYCC5321 and PYCC5323 (Almeida and Pais, 1996) and *S. cerevisiae* wild type W303-1A (*MAT*α *leu2-3112 ura3-1 trp1-1 his3-1,/15 ade2-1 can1-100 GAL SUC2 mal0*) (Thomas and Rothstein, 1989) were used throughout this work. *S. cerevisiae hog1*Δ and *T. delbrueckii Tdhog1*Δ mutant strains were constructed as described below. The *E. coli* strain DH10B was used as host for plasmid construction. Yeast cells were cultured at 30ºC in defined media, YPD (1% yeast extract, 2% peptone, 2% glucose) or SD (0.67% yeast nitrogen base without amino acids [DIFCO] plus 2% glucose) supplemented with the appropriate auxotrophic requirements (Sherman *et al.*, 1986). Citric acid sensitivity was monitored

Table 1. Oligonucleotides used in this study

Primer	Sequence 5´ to 3´	Comments
FR3	ACCACTAACGAGGAATTC	*S. cerevisiae HOG1* probe, Southern blot
FR4	TAGTGGAAACATAGCCTG	*S. cerevisiae HOG1* probe, Southern blot
FR5	GTAACGAGTAGAAACATAAC	Diagnostic PCR (*Tdhog1*Δ strain), Sequencing
FR6	GTAAAACGACGGCCAGT	Sequencing primer
FR7	TACGCTGAAATTTGTGACTTC	Sequencing primer
FR25	ATCACGATCCTACTGACGAAC	Sequencing primer
FR26	TCTTGGCCAAGAGGACGT	Diagnostic PCR, *Tdhog1*Δ strain
FR58	AGTGAGTGAGTCTAGAAGACACTG	Disruption cassette construction, Diagnostic PCR (*Tdhog1*Δ strain), YEpTdHOG1 and YCpTdHOG1 plasmid construction
FR59	TGGCCTGGTAAAGCTTCATAAGTC	Disruption cassette construction, Diagnostic PCR (*Tdhog1*Δ strain), YEpTdHOG1 and YCpTdHOG1 plasmid construction
FR75	AGTTAAGTGCGCAGAAAG	Diagnostic PCR, *Tdhog1*Δ strain
FR76	GTCAAGGAGGGTATTCTGG	Diagnostic PCR, *Tdhog1*Δ strain
FR272	AAGGGAAAACAGGGAAAACTAC AACTATCGTATATAATAGCATAG GCCACTAGTGGATCTG	*hog1*Δ strain construction
FR273	AAGTAAGAATGAGTGGTTAGGG ACATTAAAAAAACACGTTTACA GCTGAAGCTTCGTACGC	*hog1*Δ strain construction
FR282	CTCCAAGACTTTGCCCTG	Diagnostic PCR, *hog1*Δ strain
FR119	GGCATGCTCTCTGGTTACCCTA	YCpScHOG1plasmid construction
FR120	CCTCGAGCTCACTATATACGTAAATAC	YCpScHOG1plasmid construction
FR390	GGTATGTTCTAGCGCTTG	*S. cerevisiae ACT1* probe, Northern blot
FR391	TCTGGGGCTCTGAATCTT	*S. cerevisiae ACT1* probe, Northern blot
FR392	TTGAATGCTGGTAGAAAG	*S. cerevisiae GPD1* probe, Northern blot
FR393	TGACCGAATCTGATGATC	*S. cerevisiae GPD1* probe, Northern blot

in malt extract (ME) medium as reported (Lawrence *et al.*, 2004). *T. delbrueckii* transformants containing the nourseothricin (natMX4) resistant module were selected on YPD agar plates containing 10 mg/l of nourseothricin (clonNAT, WERNE Bioagents, Germany). Selection of G-418-resistant *S. cerevisiae* transformants was carried out onto YPD plates supplemented with 200 mg/l Geneticin® (G-418 sulphate, Gibco BRL, Germany). *E. coli* was grown in Luria Bertani (LB) medium (1% peptone, 0.5% yeast extract, 0.5% NaCl) supplemented with ampicillin (50 mg/l). Antibiotics were filter-sterilised and added to autoclaved medium.

Stress sensitivity tests

For stress experiments, cells were grown at 30ºC to mid-exponential phase, collected and transferred to fresh medium containing NaCl or sorbitol at the indicated concentration. Plate phenotype experiments were made by diluting the cultures to OD_{600} = 0.3 and streaking cells onto SD- or YPD-agar solid media containing the stressor to be tested. More-detailed sensitivity assays were performed by spotting (3 μl) 10-fold serial dilutions of the cell culture. Unless indicated colony growth was inspected after 2-4 days of incubation at 30ºC.

Strain and plasmid construction

Plasmid pMJH28, carrying a DNA fragment containing the *HOG1* gene from *T. delbrueckii* (*TdHOG1*) and flanking regions around this gene, was isolated from a genomic library (Hernandez-Lopez *et al.*, 2002) by complementation in *S. cerevisiae* of the osmosensitivity phenotype of a *hog1*Δ mutant strain (see Results). Multicopy and centromeric plasmids containing the isolated *TdHOG1* gene were constructed by cloning a 1,541 bp PCR-fragment, which includes the complete open reading frame plus 143 bp upstream from the ATG and 140 bp of the 3'-UTR. Amplification was carried out under standard conditions, using synthetic oligonucleotides (Table 1) and plasmid pMJH28 as template. The amplified fragment was digested with *Xba*I/*Hin*dIII, and ligated into the vectors YEplac195 and YCplac33 (Gietz and Sugino, 1988) previously digested with the same set of enzymes, resulting in plasmids YEpTdHOG1 and YCpTdHOG1, respectively.

Construction of the *TdHOG1* disruption cassette containing the nourseothricin-resistance natMX4 module (Goldstein and McCusker, 1999) was performed by restriction. First, the PCR-amplified fragment of *TdHOG1* obtained as described above, was cloned into the pGEM®-T Easy vector (Promega) resulting in plasmid pGEM-TdHOG1. This was digested with the restriction endonucleases *Eco*RV and *Bam*HI, and the released fragment was replaced by the natMX4 module obtained from plasmid pAG25 (Goldstein *et al.*, 1999) by digestion with the same enzymes. Finally, the resulting plasmid, pGEM-TdHOG1-natMX4 was treated with *Xba*I and *Hind*III. This digestion releases the disruption cassette, which contains the natMX4 module flanked by long fragments homologous to the *TdHOG1* gene.

The *S. cerevisiae HOG1* disruption cassette was constructed by PCR, using the short flanking homology technique, SF-PCR (Wach *et al.*, 1994). Briefly, we designed two oligonucleotides containing at their 3'-end a segment homologous to sequences left and right of the *loxP-kanMX-loxP* module on plasmid pUG6 (Güldener *et al.*, 1996) and a segment at the 5'-end homologous to the *S. cerevisiae HOG1* gene (Table 1).

Detection of the correct gene disruption of *HOG1* and *TdHOG1* were done by diagnostic PCR, using whole yeast cells (Huxley *et al.*, 1990) from isolated colonies, and oligonucleotides designed to bind outside or inside of the replaced *HOG1* and *TdHOG1* sequences and within the marker modules (Table 1). Because of the high GC content of the nourseothricin acetyltransferase-encoding gene *nat1* from *Streptomyces noursei* (Krugel *et al.*, 1993), the PCR-reaction mixture of the natMX4 module contained 5% DMSO, as previously described (Goldstein and McCusker, 1999). Verification was also carried out by western blot analysis using polyclonal anti-Hog1 as described below.

Transformation of *T. delbrueckii* and *S. cerevisiae* was performed by the lithium acetate method (Ito *et al.*, 1983). Cultures transformed with the dominant marker were grown for 4 h in YPD at 30ºC before plating on selective medium. *E. coli* was

transformed by electroporation following the manufacturer's instructions (Eppendorf).

DNA manipulations

Southern blot analysis of *TdHOG1* was carried out using standard techniques, except that hybridizations were carried out at 37ºC. Genomic DNA from *T. delbrueckii* cells was prepared as described by Sherman *et al.* (1986). A PCR-amplified DNA fragment containing a partial sequence of *S. cerevisiae HOG1* gene (+2 to +538) was used as probe. PCR was carried out using specific oligonucleotides (Table 1) and genomic DNA as template. The probe was labelled with the DIG-High Prime Kit (Boehringer Mannheim) following the manufacturer's instructions. DIG-labelled DNA was detected using anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) and the chemiluminescent substrate CPD-Star (Roche).

Total RNA from *T. delbrueckii* cells was prepared as described (Sherman *et al.*, 1986). Equal amounts of RNA (30 μg) were separated in 1% (w/v) agarose gels, containing formaldehyde (2.5% v/v), transferred to a Nylon membrane and hybridised with a ^{32}P-labelled probe of *S. cerevisiae GPD1* gene (Aguilera *et al.*, 2005). A PCR-amplified fragment of the *S. cerevisiae ACT1* gene (+10 to +1,066) was used as the loading control. Probes were radiolabeled with the random primer kit Ready to Go (Amersham Biosciences, Chalfont-St Giles, England) and [α^{32}P]dCTP (Amersham Biosciences). After hybridization overnight at 35ºC, the filters were rinsed once with 50 ml of 2 x SSC, 0.1% SDS, and once with 50 ml of 0.2 x SSC, 0.1% SDS 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 (Fuji, FLA-3000). Spot intensities were quantified with the 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 *GPD1* mRNA for each sample analyzed was set at 100.

TdHog1p phosphorylation assay

To prepare whole-cell extracts for TdHog1p detection, *T. delbrueckii* cells were harvested by filtration, transferred to a tube containing 300 μl of SDS loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 20% glycerol) and boiled at 95ºC for 10 min. Ten μl of each sample was separated by SDS–PAGE and blotted onto nitrocellulose membranes. Filters were blocked with 5% BSA in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Tween-20).

Dual phosphorylated TdHog1p (P-TdHog1) was detected by an antibody specific to phosphorylated p38 MAPK (Cat# 9215, Cell signaling, Beverly, MA). A rabbit polyclonal antibody raised against a recombinant protein corresponding to the carboxy terminus (221-435) of *S. cerevisiae* Hog1p was used as a loading control (Cat# sc-9079, Santa Cruz Biotechnology, Santa Cruz, CA). The antisera were applied at 1:1000 (phosphorylated Hog1p) and 1:6000

(total Hog1p) dilutions according to the manufacturer's instructions. As secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit (1:2000, Cat# 7074, Cell Signaling), was used. Blots were developed using the ECL Western blotting detection kit from Amersham Biosciences. Films were scanned in a HP scanjet 5370c (Hewlett-Packard, Palo Alto, CA) and quantified with the Image Gauge software version 3.12 (Fuji). Intensity values of P-TdHog1p were corrected with respect to the total TdHog1p level. The highest relative value for each sample analyzed was set at 100.

Glycerol determination

To determine total glycerol content, aliquots (1 ml) of the *T. delbrueckii* cultures were removed, placed in an Eppendorf tube and boiled for 10 min. After cooling on ice, the samples were centrifuged at 15,300 x g for 10 min (4 ºC), and the supernatants assayed for glycerol content. Glycerol was measured colorimetrically with a commercial kit (Roche Molecular Biochemicals, Mannheim, Germany), following the manufacturer's instructions. Values are expressed as μg of glycerol per mg of yeast cells (dry weight). Growth over the time course of the experiment was estimated by measuring as the OD_{600} (OD_{600} = 1 equals 0.21 mg cells dry weight/ml).

Sequencing and sequence analysis

DNA sequencing was performed in both strands by the dideoxychain termination procedure (Sanger *et al.*, 1977). Analysis of sequence data was carried out using the DNAMAN sequence analysis software (Lynnon BioSoft). Similarity searches were performed using the BLAST server at the Munich Information centre for Protein Sequences. Search of TdHog1p domains was carried out by scanning the sequence against protein profile databases, PROSITE and Pfam. Multiple sequence alignment was done using the ClustalW program (Thompson *et al.*, 1994).

GenBank accession number

The nucleotide sequence for *TdHOG1* has been deposited in the GenBank database (available at http://www.ncbi.nlm.nih.gov/Genbank/index.html) receiving the accession number DQ020519.

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References

Albertyn, J., Hohmann, S., Thevelein, J.M., Prior, B.A. (1994) *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. *Mol Cell Biol* **14**: 4135-4144.

Almagro, A., Prista, C., Castro, S., Quintas, C., Madeira-Lopes, A., Ramos, J., Loureiro-Dias, M.C. (2000) Effects of salts on *Debaryomyces hansenii* and *Saccharomyces cerevisiae* under stress conditions. *Int J Food Microbiol* **56:** 191-197.

Almeida, M.J., Pais, C.S. (1996) Characterization of the yeast population from traditional corn and rye bread doughs. *Lett Appl Microbiol* **23**: 154-158.

Alonso-Monge, R., Navarro-Garcia, F., Molero, G., Diez-Orejas, R., Gustin, M., Pla, J., Sanchez, M., Nombela, C. (1999) Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. *J Bacteriol* **181:** 3058-3068.

Alonso-Monge, R., Real, E., Wojda, I., Bebelman, J.P., Mager, W.H., Siderius, M. 2001. Hyperosmotic stress response and regulation of cell wall integrity in *Saccharomyces cerevisiae* share common functional aspects. *Mol Microbiol* **41:** 717-730.

Alonso-Monge, R., Navarro-Garcia, F., Roman, E., Negredo, A.I., Eximan, B., Nombela, C., Pla, J. (2003) The Hog1 mitogen-activated protein kinase is essential in the oxidative stress response and chlamydospore formation in *Candida albicans*. *Eukaryot Cell* **2:** 351-361.

Aguilera, J., Rodriguez-Vargas, S., Prieto, J.A. (2005) The HOG MAP kinase pathway is required for the induction of methylglyoxal-responsive genes and determines methylglyoxal resistance in *Saccharomyces cerevisiae*. *Mol Microbiol* **56:** 228-239.

Bahn, Y.S., Kojima, K., Cox, G.M., Heitman, J. (2005) Specialization of the HOG pathway and its impact on differentiation and virulence of *Cryptococcus neoformans*. *Mol Biol Cell* **16:** 2285-2300.

Bansal, P.K., Mondal, A.K. (2000) Isolation and sequence of the *HOG1* homologue from *Debaryomyces hansenii* by complementation of the *hog1*Delta strain of *Saccharomyces cerevisiae*. *Yeast* **16:** 81-88.

Belloch, C., Querol, A., García. M.D., Barrio. E. (2000) Phylogeny of the genus *Kluyveromyces* inferred from the mitochondrial cytochrome-c oxidase II gene. *Int J Syst Evol Microbiol* **50:** 405-416.

Blomberg, A., Adler, L. (1992) Physiology of osmotolerance in fungi. *Adv Microb Phys* **33**: 145-212.

Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E., Gustin, M.C. (1993) An osmosensing signal transduction pathway in yeast. *Science* **259**: 1760-1763.

Brewster, J.L., Gustin, M.C. (1994) Positioning of cell growth and division after osmotic stress requires a MAP kinase pathway. *Yeast* **10:** 425-439.

Buck, V., Quinn, J., Soto Pino, T., Martin, H., Saldanha, J., Makino, K., Morgan, B.A., Millar, J.B. (2001) Peroxide sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway. *Mol Biol Cell* **12:** 407-419.

Cano, E., Mahadevan, L.C. (1995) Parallel signal processing among mammalian MAPKs. *Trends Biochem Sci* **20:** 117-122.

Causton, H.C., Ren, B., Koh, S.S., Harbison, C.T., Kanin, E., Jennings, E.G., Lee, T.I.,

True, H.L., Lander, E.S., Young, R.A. (2001) Remodeling of yeast genome expression in response to environmental changes. *Mol Biol Cell* **12:** 323-337.

Enjalbert, B., Nantel, A., Whiteway, M. (2003) Stress-induced gene expression in *Candida albicans*: absence of a general stress response. *Mol Biol Cell* **14:** 1460-1467.

Estruch, F. (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiol Rev* **24:** 469-486.

Ferrigno, P., Posas, F., Koepp, D., Saito, H., Silver, P.A. (1998) Regulated núcleo/cytoplasmatic exchange of *HOG1* MAPK requires the importin beta homologos *NMD5* and *XPO1*. *EMBO J* **17:** 5606-5614.

Gacto, M., Soto, T., Vicente-Soler, T., Villa, T.G., Cansado, J. (2003) Learning from yeasts: intracellular sensing of stress conditions. *Int Microbiol* **6:** 211-219.

Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D., Brown, P.O. (2000) Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell* **11:** 4241-4257.

Gasch, A.P., Moses, A.M., Chiang, D.Y., Fraser, H.B., Berardini, M., Eisen, M.B. (2004) Conservation and evolution of cis-regulatory systems in ascomycete fungi. *PLoS Biol* **2:** e398.

Gietz, R.D., Sugino, A. (1988) New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74:** 527-534.

Goldstein, A.L., McCusker, J.H. (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15**: 1541-1553.

Güldener, U., Heck, S., Fielder, T., Beinhauer, J., Hegemann, J.H. (1996) A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24:** 2519-2524.

Gustin, M.C., Albertyn, J., Alexander, M., Davenport, K. (1998) MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **62**: 1264-300.

Han, K.H., Prade, R.A. (2002) Osmotic stress-coupled maintenance of polar growth in *Aspergillus nidulans*. *Mol Microbiol* **43:** 1065-1078.

Haghnazari, E., and Heyer, W.D. (2004) The Hog1 MAP kinase pathway and the Mec1 DNA damage checkpoint pathway independently control the cellular responses to hydrogen peroxide. *DNA Repair* **3:** 769-776.

Hernandez-Lopez, M.J., Prieto, J.A., Randez-Gil, F. (2002) Isolation and characterization of the gene *URA3* encoding the orotidine-5'-phosphate decarboxylase from *Torulaspora delbrueckii*. *Yeast* **19**: 1431-1435.

Hernandez-Lopez, M.J., Prieto, J.A., Randez-Gil, F. (2003) Osmotolerance and leavening ability in sweet and frozen sweet dough. Comparative analysis between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* baker's yeast strains. *Antonie van Leeuwenhoek* **84**: 125-134.

Huxley, C., Green, E.D., Dunham, I. (1990) Rapid assessment of *S. cerevisiae* mating type by PCR. *Trends Genet* **6:** 236.

Ito, H., Fukuda, Y., Murata, K., Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153:** 163-168.

Iwaki, T., Tamai, Y., Watanabe, Y. (1999) Two putative MAP kinase genes, *ZrHOG1* and *ZrHOG2*, cloned from the salt-tolerant yeast *Zygosaccharomyces rouxii* are functionally homologous to the *Saccharomyces cerevisiae HOG1* gene. *Microbiology* **145:** 241-248.

Kawasaki, L., Sanchez, O., Shiozaki, K., Aguirre, J. (2002) SakA MAP kinase is involved in stress signal transduction, sexual development and spore viability in *Aspergillus nidulans*. *Mol Microbiol* **45:** 1153-1163.

Krugel, H., Fiedler, G., Smith, C., Baumberg, S. (1993) Sequence and transcriptional analysis of the nourseothricin acetyltransferase-encoding gene *nat1* from *Streptomyces noursei*. *Gene* **127:** 127-131.

Kuranda, M.J., Robbins, P.W. (1991) Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *J Biol Chem* **266:** 19758-19767.

Kurtzman, C.P. (1998) *Torulaspora* Lindner. In The Yeasts. A taxonomyc study. Kurtzman, C.P., and Fell, J.W. (eds.). Amsterdam: Elsevier Science, pp. 404-408.

Lawrence, C.L., Botting, C.H., Antrobus, R., and Coote, P.J. (2004) Evidence of a new role for the high-osmolarity glycerol mitogen-activated protein kinase pathway in yeast: regulating adaptation to citric acid stress. *Mol Cell Biol* **24:** 3307-3323.

Leberer, E., Thomas, D.Y., Whiteway, M. (1997) Pheromone signalling and polarized morphogenesis in yeast. *Curr Opin Genet Dev* **7:** 59-66.

Lewis, J.G., Learmonth, R.P., Watson, K. (1995) Induction of heat, freezing and salt tolerance by heat and salt shock in *Saccharomyces cerevisiae*. *Microbiology* **141:** 687-694.

Lindquist, S., Kim, G. (1996) Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. *Proc Natl Acad Sci USA* **93:** 5301-5306.

Martinez-Pastor, M.T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H., Estruch, F. (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *EMBO J* **15:** 2227-2235.

Millar, J.B., Buck, V., Wilkinson, M.G. (1995) Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. *Genes Dev* **9:** 2117-2130.

Nicholls, S., Straffon, M., Enjalbert, B., Nantel, A., Macaskill, S., Whiteway, M., Brown, A.J. (2004) Msn2- and Msn4-like transcription factors play no obvious roles in the stress responses of the fungal pathogen *Candida albicans*. *Eukaryot Cell* **3:** 1111-1123.

O'Rourke, S.M., Herskowitz, I. (1998) The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK

pathways in *Saccharomyces cerevisiae*. *Genes Dev* **12:** 2874-2886.

O'Rourke, S.M., Herskowitz, I. (2004) Unique and redundant roles for HOG MAPK pathway components as revealed by whole-genome expression analysis. *Mol Biol Cell* **15:** 532-542.

Randez-Gil, F., Aguilera, J., Codón, A., Rincón, A.M., Estruch, F., Prieto, J.A. (2003) Baker's yeast: challenges and future prospects. In Functional Genetics of Industrial Yeasts. de Winde, J.H. (ed.). Heidelberg: Springer-Verlag, pp. 57-97.

Reiser, V., Ruis, H., Ammerer, G. (1999) Kinase activity-dependent nuclear export opposes stress-induced nuclear accumulation and retention of Hog1 mitogen-activated protein kinase in the budding yeast *Saccharomyces cerevisiae*. *Mol Biol Cell* **10**: 1147-1161.

Rep, M., Albertyn, J., Thevelein, J.M., Prior, B.A., Hohmann S. (1999) Different signalling pathways contribute to the control of *GPD1* gene expression by osmotic stress in *Saccharomyces cerevisiae*. *Microbiology* **145:** 715-727.

Rep, M., Krantz, M., Thevelein, J.M., Hohmann, S. (2000) The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. *J Biol Chem* **275**: 8290-8300.

Rusnak, F., Mertz, P. (2000) Calcineurin: form and function. *Physiol Rev* **80:** 1483-1521.

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

San Jose, C., Monge, R.A., Perez-Diaz, R., Pla, J., Nombela, C. (1996) The mitogen-activated protein kinase homolog *HOG1* gene controls glycerol accumulation in the pathogenic fungus *Candida albicans*. *J Bacteriol* **178:** 5850-5852.

Sherman, F., Fink, G.R., and Hicks, J.B. (1986) In Methods in Yeast Genetics. New York: Cold Spring Harbor Laboratory Press.

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

Tanoue, T., Adachi, M., Moriguchi, T., Nishida, E. (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat Cell Biol* **2:** 110-116.

Thevelein, J.M., de Winde, J.H. (1999) Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. *Mol Microbiol* **33:** 904-918.

Thomas, B.J., and Rothstein, R. (1989) Elevated recombination rates in transcriptionally active DNA. *Cell* **56:** 619-630.

Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994) Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673-4680.

van Dijken, J.P., Scheffers, W.A. (1986) Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiol Rev* **32:** 199-224.

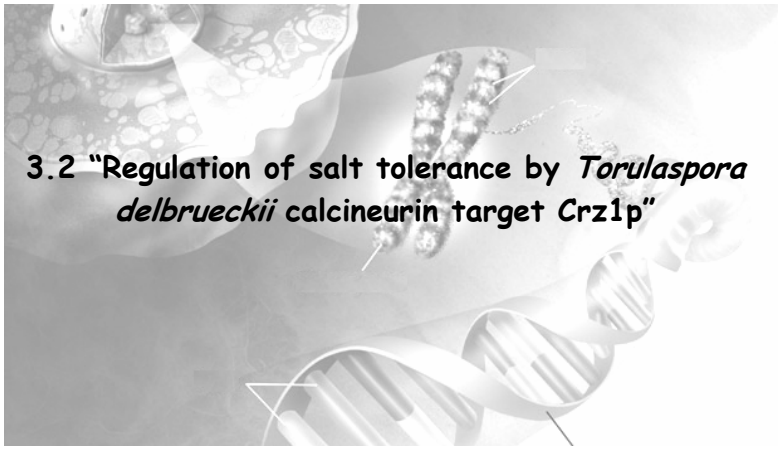
van Wuytswinkel, O., Reiser, V., Siderius, M., Kelders, M.C., Ammerer, G., Ruis, H., Mager, W.H. (2000) Response of *Saccharomyces cerevisiae* to severe osmotic stress: evidence for a novel activation mechanism of the HOG MAP kinase pathway. *Mol Microbiol* **37**: 382-397.

Wach, A., Brachat, A., Pohlmann, R., Philippsen, P. (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10:** 1793-808.

Westfall, P.J., Ballon, D.R., Thorner, J. (2004) When the stress of your environment makes you go HOG wild. *Science* **306:** 1511-1512.

Winkler, A. Arkind, C. Mattison, C.P., Burkholder, A., Knoche, K., and Ota, I. (2002) Heat stress activates the yeast high-osmolarity glycerol mitogen-activated protein kinase pathway, and protein tyrosine phosphatases are essential under heat stress. *Eukaryot Cell* **1:** 163-173.

Zhang, Y., Lamm, R., Pillonel, C., Lam, S., Xu, J.R. (2002) Osmoregulation and fungicide resistance: the *Neurospora crassa os-2* gene encodes a *HOG1* mitogen-activated protein kinase homologue. *Appl Environ Microbiol* **68:** 532-538.



3.2 "Regulation of salt tolerance by *Torulaspora delbrueckii* calcineurin target Crz1p"

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Regulation of salt tolerance by *Torulaspora delbrueckii* calcineurin target Crz1

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SUMMARY

Recently, the academic interest in the yeast *Torulaspora delbrueckii* has increased notably due to its high resistance to several stresses, including salt and osmotic resistance. However, the molecular mechanisms underlying these unusual properties are poorly understood. In *Sacharomycces cerevisiae*, the high salt response is mediated by calcineurin, a conserved Ca^{+2}/calmoduline-modulated protein phosphatase that regulate the transcriptional factor Crz1p. Here, we cloned the *T. delbrueckii TdCRZ1* gene, which encodes a putative zinc finger transcription factor homologous to Crz1p. Consistent with this, overexpression of *TdCRZ1* 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 distinct phenotypes to those previously observed in *S. cerevisiae crz1*Δ mutants. Quite remarkable, *Tdcrz1*-null cells were insensitive to high Na^+ and more Li^+-tolerant than wild-type cells. In complete agreement, TdCrz1p was not required for the salt-induced transcriptional activation of the *TdENA1* gene, encoding a putative P-type ATPase homologous to the main *S. cerevisiae* Na^+-pump *ENA1*. Furthermore, *T. delbrueckii* cells were insensitive to the immunosuppressant FK506 either in the presence or absence of NaCl. Only on high Ca^{2+}/Mn^{2+} media, signaling through the calcineurin/Crz1 pathway appeared to be essential. Hence, *T. delbrueckii* and *S. cerevisiae* differ in the regulatory circuits and mechanisms that drive the adaptive response to salt stress.

Capítulo 3

INTRODUCCTION

Exposure of cells to saline stress implies both a specific cation toxicity and osmotic stress. Sodium and lithium ions are particularly toxic to cells of most living organisms due to their ability to inhibit specific metabolic pathways. Therefore, regulation of intracellular ion content is a main issue in the cellular reprogramming of almost all organisms subjected to salt stress (4, 56).

The high degree of evolutionary conservation of stress pathways between higher eukaryotes and *Saccharomyces cerevisiae* and the genetic advantages of budding yeast have made of this organism a model system for studying stress responses (24). Yeast genes involved in salt tolerance have been identified by their ability in protecting cells at increasing gene dosage or by the growth defects of yeast mutants at elevated ion concentrations (51). Thus, studies in yeast have covered basic mechanisms of ion homeostasis and have identified key genes in the maintenance of a high K^+/Na^+ ratio (47, 52). Protein kinases and signaling pathways involved in the salt response have been also identified and characterised (50). Despite of these advances, we are far to understand completely the mechanisms, the nature of signaling pathways and the function of gene targets that allow cells to adapt to salt stress. On top of this, there is evidence that signaling pathways and stress responses have evolved in different organisms, including yeasts, in a niche-dependent manner (8, 58). It is clear for example, that *S. cerevisiae* is not the best model of salt-resistant microorganism. Non-conventional yeasts like *Zygosaccharomyces rouxii*, *Debaryomyces hansenii* or *Torulaspora delbrueckii* are by far more resistant to the combined effect of ion toxicity and osmotic stress (10, 32). Thus, the identification and characterization of the cellular mechanisms regulating salt tolerance in these non-*Saccharomyces* species is 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 (62), one of the five MAP (for Mitogen Activated Protein) kinase cascades known in *S. cerevisiae* (16) and the calcineurin/Crz1p pathway (19). Calcineurin is a highly conserved Ca^{+2}/calmodulin-dependent Ser/Thr protein phosphatase type 2B (30, 48). 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 gene 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 ions Na^+/Li^+, Mn^{+2} and OH^- (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 of calmodulin, allowing it to bind and activate calcineurin (39) that in turn, dephosphorylates the transcriptional factor Crz1p (41, 59). Dephosphorylation of Crz1p causes its nuclear import (60), and binding to a consensus DNA sequence (42), the calcineurine-dependent response element, CDRE (59), found in the promoter of most salt-responsive genes (65). In consonance with this, cells lacking Crz1p display hypersensitivity to α-factor, Mn^{2+} or Li^{+} (41, 59). Nevertheless, *crz1* and calcineurin mutant cells show opposite phenotypes under specific conditions, such as exposure to ions Ca^{2+} and OH^{-} (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* (13). 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). From them, only the *prz1*$^{+}$ gene from *S. pombe* has been studied in detail (34). Like Crz1p, Prz1p acts downstream calcineurin and regulates Ca^{2+} homeostasis. However, *prz1* null cells show distinct phenotypes as those observed in *S. cerevisiae crz1* mutants, such as aberrant cell morphology or hypersensitivity to Cl^{-} (34). Hence, calcineurin and its target Prz1p in fission yeast appear to play functional roles 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 respectively a putative Na^{+}/Li^{+} P-type ATPase and a zinc finger protein homologous to *S. cerevisiae* Crz1p. 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* resulted in enhanced resistance to Li^{+}, whereas 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 in a different manner trough uncovered regulators and molecular circuits.

MATERIALS AND METHODS

Strains, culture media and general methods. *T. delbrueckii* PYCC5321 wild-type strain (1) and *S. cerevisiae* strains (Table 1) were used throughout this work. *T. delbrueckii Tdcrz1*Δ mutant strain (MJHY211) was constructed as described below. Cells were cultured at 30ºC in defined media, YPD (1% yeast extract, 2%

peptone, 2% glucose) or SD (0.5% yeast nitrogen base without amino acids [DIFCO], 0.5%$(NH_4)_2SO_4$, 2% glucose) supplemented with the appropriate auxotrophic requirements (53). *E. coli* was grown in Luria Bertani (LB) medium (1% peptone, 0.5% yeast extract, 0.5% NaCl) supplemented with ampicillin (50 mg/l). Antibiotics were filter-sterilised and added to autoclaved medium. Transformation of yeasts was performed by the lithium acetate method (37). *T. delbrueckii* transformants containing the nourseothricin (natMX4) resistant module were grown for 4 h in YPD at 30ºC before plating on YPD agar plates containing 10 mg/l of nourseothricin (clonNAT, WERNE Bioagents, Germany). *E. coli* was transformed by electroporation following the manufacturer's instructions (Eppendorf).

Stress sensitivity tests. For stress experiments, cells were grown at 30ºC 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 OD_{600} = 0.3 and spotting (3 μl) 10-fold serial dilutions onto SD- or YPD-agar solid media containing NaCl, LiCl, $MnCl_2$ or $CaCl_2$. FK506 (Fujisawa GmbH; 20 mg/ml in 90% ethanol-10% Tween 20) was added at 1 μg/ml, final concentration. Unless indicated colony growth was inspected after 2-4 days of incubation at 30ºC.

Strain and plasmid construction. Plasmids pMJH1 and pMJH14, 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 the strain CEN.PK2-1C. Plasmid YEpTdCRZ1, containing the isolated *TdCRZ1* gene, was constructed by cloning a 1,980-bp-lentgh *Sca*I/*Eco*RI fragment from plasmid YEpMJH14, into the vector YEplac195 (26) previously digested with *Eco*RI/*Sma*I. Construction of the *TdCRZ1* disruption cassette containing the nourseothricin-resistance natMX4 module (27) was performed by restriction. First, a 3'-side fragment of *TdCRZ1* (+1,322 to +1,810) was obtained by PCR using two specific primers FR142 and FR141 (Table 2) and plasmid YEpMJH14 as template. The PCR product was cloned into the pGEM®-T Easy vector (Promega), released then by restriction with *Sal*I/*EcoR*I, and inserted into the pBS plasmid (Stratagene) previously digested with the same set of enzymes. The resulting plasmid pBSCRZ was treated with *Bam*HI/*Eco*RI and used to accommodate the natMX4 module obtained from the *Bam*HI- and *Eco*RI-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 YEpMJH14 as template. The PCR product was inserted into the pGEM®-T Easy vector, released then with *Not*I and *Spe*I, and subcloned into plasmid pBSCRZ-natMX4, obtaining plasmid pBSCRZ-natMX4-CRZ. This was digested with *Eco*RV releasing the *TdCRZ1* disruption cassette, which contains the natMX4 module flanked by 718

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 MAL 2-8^c SUC2*	Entian y Kötter, 1998
YPH499	*MATa ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1*	Sikorski and Hieter, 1989
DD12	same as YPH499, except *cnb1::hisG*	Cyert and Thorner, 1992
ASY472	same as YPH499, except *crz1::loxP-kanMX-loxP*	Stathopoulos and Cyert, 1997
ASY475	same as DD12, except *crz1::loxP-kanMX-loxP*	Stathopoulos and Cyert, 1997
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

TABLE 2. Oligonucleotides used in this study

Primer	Sequence 5´ to 3´	Comments
FR55	ATGACCATGATTACGCCAA	*TdENA1* and *TdCRZ1* sequencing
FR77	GTAAAACGACGGCCAGT	*TdENA1* and *TdCRZ1* sequencing
FR94	AATCATCGGCAACCTTAG	*TdCRZ1* sequencing
FR103	GGCGACATGCTACGACTTC	*TdCRZ1* sequencing
FR136	GTGGTTAAATAGGACATCGC	*TdCRZ1* sequencing
FR90	TGCTACTGAAAAGACAAGG	*TdENA1* sequencing
FR96	GCTTACAGGCGAGGAATT	*TdENA1* sequencing
FR135	TGAGTCCTTGCCTATCGC	*TdENA1* sequencing
FR110	CCTCGTTCTGCTTTGACA	*TdENA1* sequencing
FR117	CAGGATATCAAGGGTAAGCT	*TdENA1* sequencing
FR376	AATGGTTCAGACGTCGC	*TdENA1* sequencing
FR377	CCAGCTGATCACTTCGG	*TdENA1* sequencing
FR101	CCTAAAGCCCAAACTATAACA	*TdCRZ1* sequencing, verify correct targeting of the natMX4 module
FR142	CGAAGTCGACAGCTCAATCA	PCR amplification of *TdCRZ1*-3′-side
FR141	TGAATTCGGGTAAGAAAAGG	PCR amplification of *TdCRZ1*-3′-side
FR140	CATTGAGCTCCTTGGAAGG	PCR amplification of *TdCRZ1*-5′-side
FR139	ATTCGGATCCTAAGTCACTC	PCR amplification of *TdCRZ1*-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	*TdENA1* probe for Northern
FR121	GCTGCACCAACAGACAAAG	*TdENA1* probe for Northern
FR390	GGTATGTTCTAGCGCTTG	*ACT1* probe for Northern
FR391	TCTGGGGCTCTGAATCTT	*ACT1* probe for Northern

and 488 bp (5'- and 3'-side, respectively), homologous to the *TdCRZ1* gene.

Detection of the correct gene disruption of the *TdCRZ1* gene was done 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 *TdCRZ1* sequence, and within the marker module (Table 2).

β-galactosidase assay. Exponentially SD-growing cells (OD_{600} = 0.6-0.8) were collected, resuspended in YPD (pH=5.5) or the same medium supplemented with 0.2 M $CaCl_2$ and incubated at 30ºC and 200 rpm for 45 min. Then, aliquots of the yeast suspension (15 units of OD_{600}) were harvested, washed with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$), centrifuged at 3,000 × *g* for 2 min (4ºC), and the cell pellets were frozen at -20ºC for further analysis. Cell extracts were prepared as previously described (14). Total protein was determined using the Bio-Rad Bradford assay kit and bovine serum albumin as standard protein. The β-galactosidase activity was determined at room temperature using the substrate ONPG (*O*- nitrophenyl–β-D-galactopyranoside) as previously described (46). 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 blot. Total RNA from *T. delbrueckii* cells was prepared as described (53). Equal amounts of RNA (10 μg) were separated in 1% (w/v) agarose gels, containing formaldehyde (2.5% v/v), transferred to a Nylon membrane and hybridised with a ^{32}P-labelled probe of *TdENA1* (+90 to +1,003). A fragment of the *S. cerevisiae ACT1* gene (+10 to +1,066) was used as the loading control. Probes were generated by PCR and radiolabeled with the random primer kit Ready to Go (Amersham Biosciences, Chalfont-St Giles, England) and [α^{32}P]dCTP (Amersham Biosciences). Hybridisation was carried out under standard conditions (53), except for the *ACT1* probe. Briefly, after hybridization overnight at 35ºC, the filters were rinsed once with 50 ml of 2 x SSC, 0.1% SDS, and once with 50 ml of 0.2 x SSC, 0.1% SDS 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 (Fuji, FLA-3000). Spot intensities were quantified with the 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 for each sample analyzed was set at 100.

Sequencing and sequence analysis. DNA sequencing was performed in both strands by the dideoxychain termination procedure (49). Analysis of sequence data was carried out using the DNAMAN sequence analysis software (Lynnon BioSoft). Similarity searches were performed using the BLAST software (3) at the Munich Information Center for Protein Information, MIPS (http:/mips.gsf.de). Search of TdEna1p and TdCrz1p domains was carried out by scanning protein sequences in the ExPASy Molecular Biology Server

(http://www.expasy.ch) from the Swiss Institute of Bioinformatics, SIB (http://www.isb-sib.ch) against the PROSITE database of protein families (54). Multiple sequence alignment was done using the MultAlin software (15) at INRA (http://prodes.toulouse.inra.fr).

GenBank accession numbers. The nucleotide sequences for *TdCRZ1* and *TdENA1* have been deposited in the GenBank database (available at http://www.ncbi.nlm.nih.gov/Genbank/index.html) receiving the 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. cerevisiae* CEN.PK2-1C strain 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 0.5 M NaCl, a salt concentration that inhibits the growth of the host strain. Plasmid-restriction analysis established four plasmids

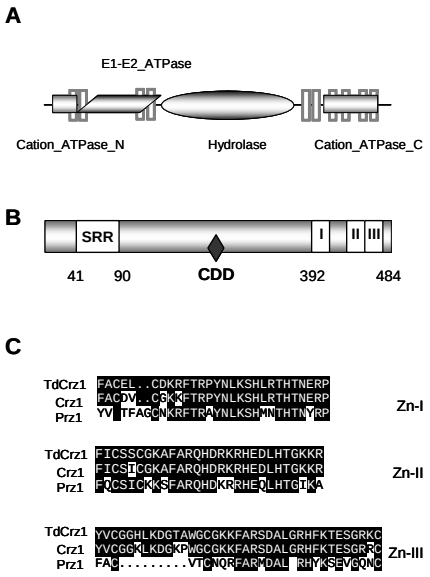

Fig. 1. Conserved protein domains in TdEna1p and TdCrz1p. A: schematic structure of TdEna1p showing the conserved domains for cation transporting ATPase, N-terminal (Cation_ATPase_N) and C-terminal (Cation_ATPase_N), hydrolase, E1-E2 ATPase-associated region (E1-E2_ATPase) and transmembrane segments (showed as vertical boxes). **B:** schematic representation of TdCrz1p. Denoted are the serine-rich region (SRR), the calcineurin-docking domine (CDD) domain and three putative C_2H_2 type zinc finger motifs at the carboxyl terminal. **C:** sequence alignment of the three zinc finger motifs from TdCrz1, Crz1p and Prz1p. Residues conserved in at least two sequences are boxed and highlighted.

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, allowed to identify a 3,273-bp length open reading frame (ORF) (GenBank accession number DQ097181), which encodes for a putative polypeptide with a high similarity with Ena proteins isolated from other yeasts, like *Debaryomyces hansenii* (55% identity), *Schwanniomyces occidentalis* (56%), *Zygosaccharomyces rouxii* (67%) or *S. cerevisiae* (69%). These proteins belong to the large P-type ATPases family, subfamily IID, whose members perform active ion transport across biological membranes (7, 9). In *S. cerevisiae*, three isophorms of Ena proteins (encoded by *ENA1*, *ENA2* and *ENA5* genes) have been characterized. From them, *ENA1* encodes the main ATPase involved in Na^+ extrusion, which function determines tolerance to NaCl (28). Because of this homology, we named the ORF identified in plasmid YEpMJH1, *TdENA1*, the *ENA1* gene from *T. delbrueckii*. Consistent with this, the putative protein TdEna1 (Fig. 1A) contains the typical ATPase α chains, implied in Na^+ and K^+ transport, responsible of ATP hydrolisis (12), the E1-E2 ATPase domain characteristic of the superfamily P-ATPases (57), as well as one hydrolase and ten transmembrane domains (Fig. 1A).

DNA sequencing of the second plasmid analysed in this work YEpMJH14, revealed a 1,518-bp-length ORF (GenBank accession number DQ097180) that encoded for a protein of 506 amino acids with an overall 36% identity to *S. cerevisiae* Crz1p (41, 59). Accordingly, the gene was designated *TdCRZ1*. As shown in Fig. 1B, the gene product of *TdCRZ1* contains three C_2H_2 type zinc fingers motifs at the carboxyl terminal highly homologous to those of Crz1p and Prz1p (Fig. 1C). Like *S. cerevisiae* Crz1p and *S. pombe* Prz1p, the protein from *T. delbrueckii* displayed a serine-rich region, SRR (residues 41-90, Fig. 1B) essential for protein dephosphorilation by calcineurine (60). Inspection of the protein sequence also showed the presence of a PVISVQ sequence, similar to the calcineurin-docking domines, CDD (Fig. 1B), defined in Crz1p (11) and human nuclear factors of activated T cells, NFAT (5).

In order to confirm that the gene present in the plasmid YEpMJH14 was responsible of the enhanced salt-resistance of the *S. cerevisiae* CEN.PK2-1C strain, we constructed the plasmid YEpTdCRZ1 by subcloning a 1,980-pb fragment containing the whole ORF plus 349-bp of the promoter region and 107-bp corresponding to the 3'-UTR into the plasmid YEplac195 (26). As shown in Fig. 2, overexpression of *TdCRZ1* in the *S. cerevisiae* recipient strain produced a moderate increase in Na^+ tolerance as compared with 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 YEpTdCRZ1 grew up to 20 mM $MnCl_2$, whereas the control strain only showed a residual growth (Fig. 2). Similar results were observed in transformants cells harboring the plasmid pAMS354 (59) that allows a high-copy number expression of the *S. cerevisiae CRZ1* gene (Fig. 2). Hence, our results indicate

that the plasmid YEpMJH14 contains indeed the *T. delbrueckii CRZ1* gene, the homologous to the transcriptional factor Crz1p, whose overexpression in *S. cerevisiae* confers enhanced tolerance to ions Na^+ and Mn^{2+}.

***TdCRZ1* suppress the ion sensitivity of *S. cerevisiae cnb1*Δ and *crz1*Δ mutants**. Overexpression of *S. cerevisiae CRZ1* compensates the enhanced ion-sensitivity of yeast calcineurin mutants, concretely to Na^+, Li^+ and Mn^{2+} (41, 59). Therefore, we were interested to determine if TdCrz1p could affect these calcineurin phenotypes. As shown in Fig. 3, production of the recombinant TdCrz1p increased Mn^{2+} tolerance of a *cnb1*Δ mutant (DD12 strain) 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 the ion-sensitivity produced by the lack of Crz1p (Fig. 3). Like calcineurin mutants, *crz1*-null cells

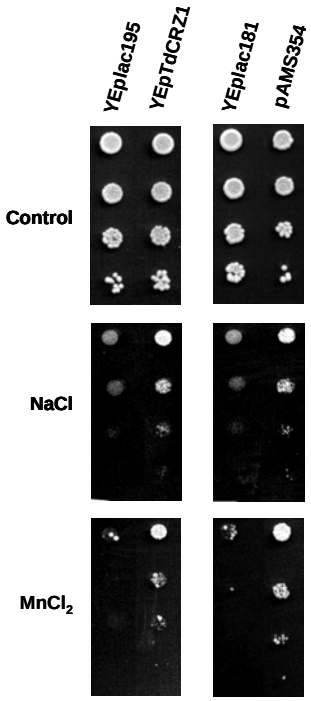

Fig. 2. Overexpression of *TdCRZ1* in *S. cerevisiae* confers enhanced tolerance to ions Na^+ and Mn^{2+}. Mid-exponential grown cultures of the *S. cerevisiae* CEN.PK2-1C strain transformed with plasmids YEpTdCRZ1, pAMS354 (*CRZ1*) or the empty plasmids YEplac195 and YEplac181 were adjusted to OD_{600} = 0.3, diluted ($1\text{-}10^{-3}$) and spotted (3 μl) onto SD plates lacking (control) or containing 1.0 M NaCl or 20 mM $MnCl_2$ (final concentration). Plates were inspected after 2-5 days at 30ºC. A representative experiment is shown.

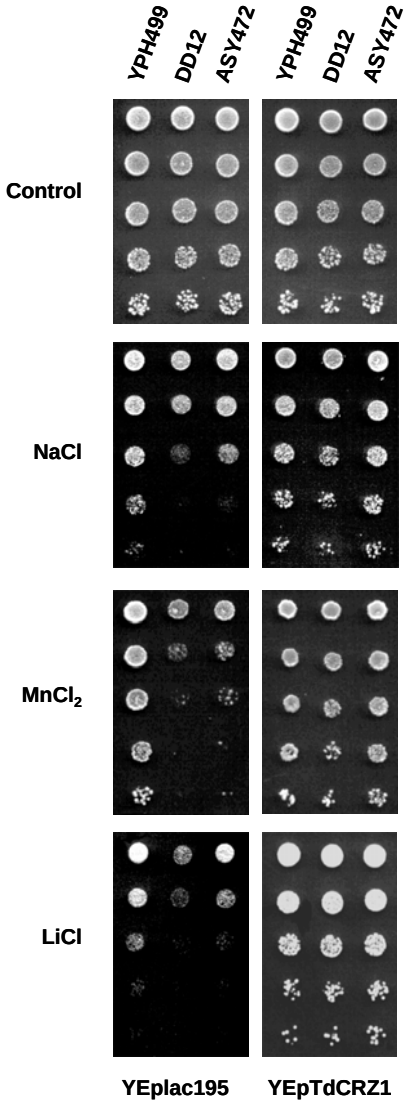

Fig. 3. TdCrz1p restores growth of *S. cerevisiae cnb1*Δ and *crz1*Δ mutants in high salt media. Mid-exponential grown cultures of the *S. cerevisiae* strains YPH499 (wild-type), DD12 (*cnb1*Δ) and ASY472 (*crz1*Δ) transformed with plasmids YEpTdCRZ1 or YEplac195 (empty plasmid), were examined for growth on solid-SD (control) or -SD containing 1.0 M NaCl, 2.5 mM $MnCl_2$ or 0.4 M LiCl. SD-pregrown cells were diluted, spotted and incubated as described in Fig. 2. A representative experiment is shown.

TABLE 3. Overexpression of *TdCRZ1* allows the calcineurin-dependent induction of a 4x-CDRE-*lacZ* gene fusion in response to Ca^{2+}

	β-galactosidase activity[a] (Units/mg protein)	
Strain	Control	Ca^{2+}
ASY832 YEplac195	152.7 ± 43.2	1,336.6 ± 98.1
ASY834 YEplac195	0.9 ± 0.3	1.8 ± 0.4
ASY834 YEpTdCRZ1	111.3 ± 6.2	502.4 ± 24.8
ASY835 YEplac195	1.7 ± 0.2	4.2 ± 1.3
ASY835 YEpTdCRZ1	2.1 ± 0.4	36.2 ± 6.5

[a]Data represent the mean value ± standard error of three independent experiments.

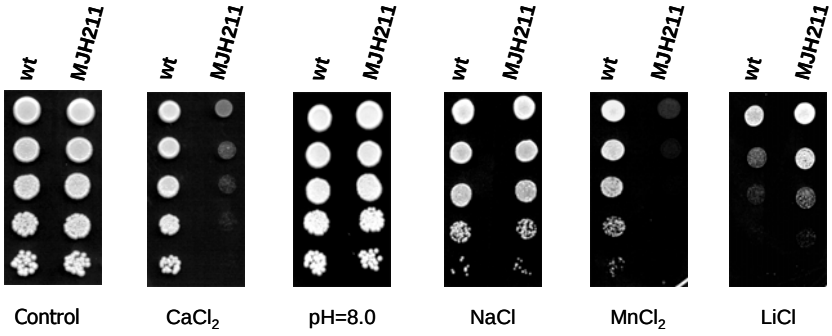

Fig. 4. *Tdcrz1*Δ cells exhibit specific phenotypes in response to diverse ionic stresses. Exponentially growing cultures of the *T. delbrueckii* strains, PYCC5321 wild-type (wt) and corresponding *Tdcrz1*Δ mutant (MJH211), were adjusted to OD_{600} = 0.3, diluted ($1\text{-}10^{-4}$) and spotted (3 μl) onto YPD agar medium (control, pH=5.5), YPD adjusted to pH=8.0 or YPD (pH=5.5) supplemented with 2.0 M NaCl, 0.4 M $CaCl_2$, 10 mM $MnCl_2$ or 0.6 M LiCl. Plates were incubated at 30ºC for 2-5 days. A representative experiment is shown.

show retarded growth at elevated concentrations of Li^+/Na^+ and Mn^{2+} cations (41, 59).

We also tested whether the production of TdCrz1p could activate the expression of a 4x-CDRE::*lacZ* reporter, which contains four tandem copies of the 24-base pair calcineurin-dependent response element (38). Multiple copies of the CDRE increase the calcineurin-dependent transcriptional activation of the reporter gene (59). Thus, 9-fold inductions were observed in wild-type cells carrying an integrated copy of the heterologous construct (ASY832 strain) after 45 min of exposure to 0.2 M $CaCl_2$ (Table 3). Consistent with previous reports (59), CDRE-driven expression was completely dependent on the function of the calcineurin-Crz1p pathway. Thus, no significant β-galactosidase activity could be detected in *S. cerevisiae* cells lacking Crz1p (ASY834 strain) or in the *cnb1*Δ *crz1*Δ double mutant (ASY835 strain) 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 in high Ca^{2+} was lower, 5-fold, than that observed in the wild-type. Nevertheless, the *crz1*Δ mutant transformed with the plasmid pAMS354 that contains the *S. cerevisiae CRZ1* gene (59) also showed a lower induction, around 3-fold (data not shown). As could be expected, overexpression of *TdCRZ1* in the strain lacking both Cnb1p and Crz1p was unable to restore completely the CDRE-mediated expression level observed in the *crz1*Δ single mutant (Table 3). Hence, TdCrz1p is able to mediate the CDRE-driven expression and appears to function downstream of calcineurin in *S. cerevisiae*.

***Tdcrz1*Δ shows conserved and distinct phenotypes to those of *S. cerevisiae crz1*Δ mutants.** To clarify the function of *TdCRZ1* in *T. delbrueckii*, we constructed a *Tdcrz1*-null mutant (MJH211 strain) and cells were analyzed for phenotypes previously reported in *S. cerevisiae* calcineurin and *crz1*Δ mutants (41, 44, 59, 64). On YPD plates containing 10 mM $MnCl_2$ or 0.4 M $CaCl_2$, *Tdcrz1*Δ cells displayed a clear growth defect (Fig. 4). Unlike *S. cerevisiae cnb1*Δ mutants, cells lacking TdCrz1p did not exhibit sensitivity to high pH. These phenotypes are in perfect agreement with those reported for *crz1*Δ mutants (59). However, *T. delbrueckii* mutant cells were indifferent to the presence of 2.0 M NaCl, and remarkably TdCrz1p-deficiency increased Li^+-tolerance (Fig. 4). Either calcineurin or *crz1*Δ mutant cells of *S. cerevisiae* have been reported as sensitive to high concentrations of monovalent cations Na^+ and Li^+ (41, 44, 59).

The *T. delbrueckii* calcineurin-Crz1p pathway plays no role in Na^+ tolerance. The above results led us to investigate the implication of calcineurin in the phenotypes exhibited by *Tdcrz1*Δ cells. Since the *T. delbrueckii* genes for the calcineurin catalytic and regulatory subunits are unknown, the selective deletion of these genes to assess their biological function is not possible. As an alternative, the physiological role of calcineurin was examined in wild-type and MJH211 strains exposed to FK506. The immunosuppressant drug FK506 is a potent

inhibitor of calcineurin (63) and have been extensively used for molecular studies in lower and higher eukaryotes (18, 29, 45). As shown in Fig. 5, addition of FK506 had no effects on growth of *T. delbrueckii* wild-type and *Tdcrz1*Δ mutant cells on YPD medium. This is quite remarkable since FK506 is a potent toxin that inhibits growth of different fungi, including *S. cerevisiae*, under standard growth conditions (6). The effectivenes of the immunosuppressant drug was evidenced in the presence of 0.4 M $CaCl_2$, where no growth could be detected (data not shown). Even at half dosages of Ca^{2+} ions (0.2 M), exposure of wild-type cells to FK506 had dramatic inhibitory effects (Fig. 5). However, this result also was surprising since *S. cerevisiae* calcineurin mutants are more Ca^{2+} tolerant than wild-type cells (64). Moreover, we found that the growth defect of the

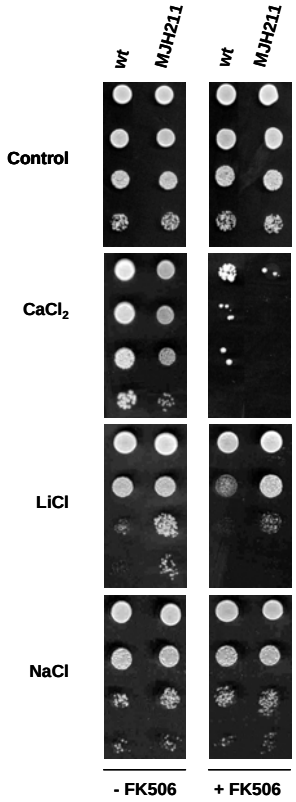

Fig. 5. The *T. delbrueckii* calcineurin-Crz1p pathway is only essential for Ca^{2+}-tolerance. Exponentially growing cultures of the *T. delbrueckii* PYCC5321 wild-type strain (wt) and corresponding *Tdcrz1*Δ mutant (MJH211), were adjusted to OD_{600} = 0.3, diluted ($1\text{-}10^{-3}$) and spotted (3 μl) onto agar medium with or without FK506 in either YPD (control) and YPD supplemented with 0.2 M $CaCl_2$, 2.0 M NaCl or 0.6 M LiCl. Plates were incubated at 30ºC for 2-5 days. A representative experiment is shown

*Tdcrz1*Δ mutant strain was again more pronounced than that observed in the wild-type strain on Ca^{2+}/FK506-containing medium. Thus, our results indicate that Ca^{2+} tolerance in *T. delbrueckii* requires a functional calcineurin-Crz1p pathway.

The addition of FK506 also had effects on growth of cells exposed to Li^+ (Fig. 5). Indeed, either wild-type or *Tdcrz1*Δ cells were less tolerant to this ion in the presence of FK506. Nevertheless, the effect on Li^+-sensitivity was weak as compared with that observed for Ca^{2+}. Furthermore, the growth difference between wild-type and mutant cells noted in the absence of FK506 (Fig. 4 and 5), was still evident in the presence of the immunosuppressant (Fig. 5). Thus, *T. delbrueckii* calcineurin and TdCrz1p appear to function independently and have opposite roles controlling Li^+-tolerance.

We also characterized the properties of wild-type and *Tdcrz1*Δ FK506-treated cells in the presence of NaCl. Unlike *S. cerevisiae*, neither the lack of TdCrz1p nor the exposure to the immunosuppressant had effects in the Na^+ tolerance of this organism (Fig. 5). Hence, the *T. delbrueckii* calcineurin-Crz1p pathway plays distinct and conserved roles to those reported in *S. cerevisiae* and has positive or negative effects in an ion-depending manner.

TdCrz1p is not required for salt-induced transcriptional activation of *TdENA1*. In S. *cerevisiae*, the adaptation to salinity is primarily based on the Na^+/Li^+ extruding ATPase encoded by the gene *ENA1*. Consequently, we were interested to know the effect of *TdCRZ1* deletion on the levels of *TdENA1* mRNA in cells exposed to NaCl or LiCl. Fig. 6 shows a Northern blot analysis of total RNA from wild-type and *Tdcrz1* mutant cells probed with a 913-bp length 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 fall 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. 6).

DISCUSSION

In this study we report for the first time the identification and characterisation of a putative C_2H_2 zinc finger transcriptional factor from the osmotolerant yeast *T. delbrueckii*, TdCrz1p, the homologous of *S. cerevisiae* Crz1p (41, 59) and *S. pombe* Prz1p (34). The budding yeast transcriptional factor Crz1p mediates the Ca^{2+}/calcineurin-dependent induction of genes in response to salt stress and is required for survival under these conditions (19). Similar to its yeasts homologues (11) and mammalian NFATc transcription factors (5), the primary structure of TdCrz1p exhibits motives characteristics of calcineurin-regulated proteins. Overexpression of *TdCRZ1* in *S. cerevisiae* wild-type cells led to improved growth on media containing NaCl or $MnCl_2$. Furthermore, production of TdCrz1p suppress the growth defect at high Na^+/Li^+ or Mn^{2+} of calcineurin and *crz1*Δ mutants and mediates 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 the lack of a functional calcineurin-Crz1p pathway in *S. cerevisiae* and provide tolerance to salt stress.

These results led us to think that TdCrz1p and by extension calcineurin might play a similar role to that of their *S. cerevisiae* counterparts in regulating the salt stress response in *T. delbrueckii*. Far of this notion, our results demonstrate that this signaling pathway has conserved and distinct roles to those described for the *S. cerevisiae* pathway. As shown, *Tdcrz1*-null phenotypes differ of those associated to the Crz1p-deficiency. *S. cerevisiae crz1*Δ mutants are sensitive to extracellular Ca^{2+}, Mn^{2+} and monovalent cations Na^{+} and Li^{+} (59). However, growth of *Tdcrz1*Δ cells was only diminished upon exposure to divalent cations. In *S. cerevisiae*, adaptation to Ca^{2+} requires the calcineurin/Crz1p-dependent induction of genes for vacuolar and secretory Ca^{2+} pumps Pmc1p and Pmr1p

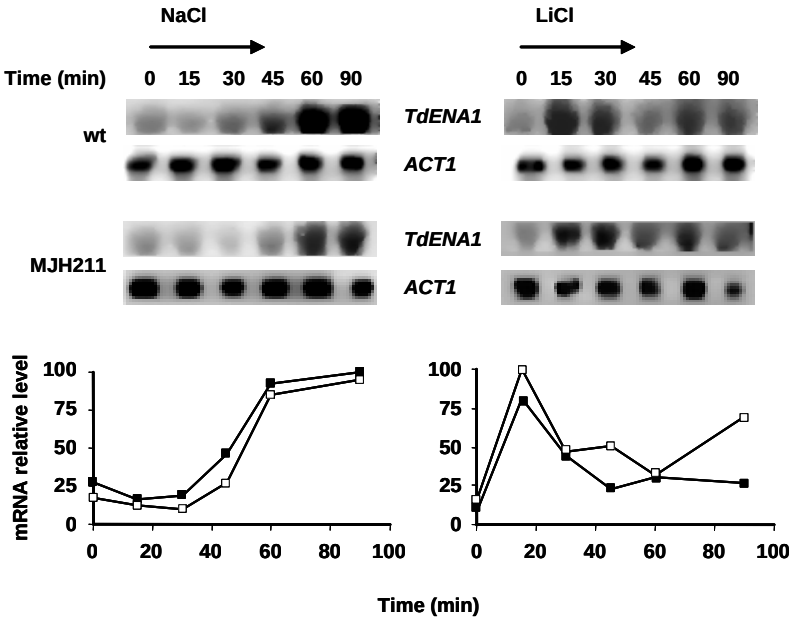

Fig 6. The activity of TdCrz1p is not required for the salt-induced transcriptional activation of the *TdENA1* gene. YPD-grown cells of the *T. delbrueckii* PYCC5321 strain (wt, ■) and corresponding *Tdcrz11*Δ mutant (MJH211, □) were transferred to 1.4 M NaCl or 0.5 M LiCl containing medium. Samples were taken at the indicated times and analysed by Northern blot as described in the Materials and Methods section. Filters were probed for *TdENA1* mRNA. The graphs represent quantification of the mRNA levels of *TdENA1*, relative to those of the *T. delbrueckii ACT1* gene. Results from a representative experiment are shown

(17, 59). 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 has also been reported in *S. pombe* (34). Thus, a common regulatory mechanism *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 are hypersensitive to Ca^{2+}. Hence, unlike in *S. cerevisiae*, calcineurin activation is required for Ca^{2+} tolerance in *T. delbrueckii*. In addition, FK506-treated cells of the *Tdcrz1*Δ strain exhibit a greater degree of sensitivity to Ca^{2+} than wild-type cells. Hence, TdCrz1p must carry out functions in Ca^{2+} tolerance that are independent of calcineurin signaling.

The differences between *S. cerevisiae* and *T. delbrueckii* calcineurin-Crz1p pathways were further evidenced by analysis of their respective phenotypes in response to monovalent cations Na^+/Li^+. In sharp contrast with the situation in *S. cerevisiae*, *Tdcrz1*Δ mutants were insensitive to high external NaCl. Growth of wild-type cells also was unaffected by the combined exposure to Na^+/FK506. In consonance with these phenotypes, TdCrz1p was not required to activate the NaCl-induced expression of *TdENA1*. 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 the wild-type. This fact suggests that Li^+ and Na^+ extrusion in *T.* involving Crz1p/Prz1p homologues appears to control Ca^{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 *delbrueckii* is regulated, at least in part, by independent mechanisms. This is again in striking contrast to the situation in *S. cerevisiae*, where both ions are excluded trough the same calcineurin-regulated Na^+/Li^+-ATPase *ENA1* (40). Moreover, the enhanced Li^+-tolerance observed in the *Tdcrz1*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 execute 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 biological roles of calcineurin as a major player in Ca^{2+}-dependent eukaryotic signal transduction pathways has been evidenced (48). Some of the most prominent research has been devoted to decipher 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 distinct functions in other yeasts and fungi (33, 34, 45). On top of this, stress responses and stress response mechanisms appear to have diverged between different yeasts in a niche-dependent manner. A clear example of this has been the specialization of the HOG pathway toward virulence in pathogenic

fungus (2, 8, 58). Thus, the differences in the biological functions of the calcineurin-Crz1p pathway highlighted in our analysis might reflect the high resistance to salt stress of *T. delbrueckii* compared with *S. cerevisiae*. Further experimentation is required to confirm this possibility and to clarify the regulatory mechanisms operating in this salt-tolerant non-conventional yeast.

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REFERENCES

1. **Almeida, M. J., and C. Pais.** 1996. Leavening ability and freeze tolerance of yeasts isolated from traditional corn and rye bread doughs. Appl. Environ. Microbiol. **62:**4401-4404.
2. **Alonso-Monge, R., F. Navarro-Garcia, G. Molero, R. Diez-Orejas, M. Gustin, J. Pla, M. Sanchez, and C. Nombela.** 1999. Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. J. Bacteriol. **181:**3058-3068.
3. **Altschul, S. F., T. L. Madden, A. A Schaffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389-3402.
4. **Apse, M. P., and E. Blumwald.** 2002. Engineering salt tolerance in plants. Curr. Opin. Biotechnol. **13:**146-150.
5. **Aramburu, J., F. Garcia-Cozar, A. Raghavan, H. Okamura, A. Rao and P. G. Hogan.** 1998. Selective inhibition of NFAT activation by a peptide spanning the calcineurin targeting site of NFAT. Mol. Cell. **5:**627-637.
6. **Arndt, C., M. C. Cruz, M. E. Cardenas, and J. Heitman.** 1999. Secretion of FK506/FK520 and rapamycin by *Streptomyces* inhibits the growth of competing *Saccharomyces cerevisiae* and *Cryptococcus neoformans*. Microbiology **145:**1989-2000.
7. **Axelsen, K. B., and M. G. Palmgren.** 1998. Evolution of substrate specificities in the P-type ATPase superfamily. J. Mol. Evol. **46:**84-101.
8. **Bahn, Y. S., K. Kojima, G. M. Cox, and J. Heitman.** 2005. Specialization of the HOG pathway and its impact on differentiation and virulence of *Cryptococcus neoformans*. Mol. Biol. Cell **16:**2285-2300.
9. **Benito, B., B. Garciadeblás, and A. Rodríguez-Navarro.** 2002. Potassium- or sodium-efflux ATPase, a

key enzyme in the evolution of fungi. Microbiology **148:**933-941.

10. **Blomberg, A., and L. Adler.** 1992. Physiology of osmotolerance in fungi. Adv. Microb. Phys. **33**:145-212.
11. **Boustany, L. M., and M. S Cyert.** 2002 Calcineurin-dependent regulation of Crz1p nuclear export requires Msn5p and a conserved calcineurin docking site. Genes Dev. **16:**608-619.
12. **Broude, N. E., N. N. Modyanov, G. S. Monastyrskaya, and E. D. Sverdlov.** 1989. Advances in Na^+,K^+-ATPase studies: from protein to gene and back to protein. FEBS Lett. **257:**1-9.
13. **Bruno, V. M., and A. P. Mitchell.** 2005. Regulation of azole drug susceptibility by *Candida albicans* protein kinase CK2. Mol. Microbiol. **56:**559-573.
14. **Ciriacy, M.** 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.
15. **Corpet, F.** 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. **16:**10881-10890.
16. **Cowan, K. J., and K. B. Storey.** 2003. Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. J. Exp. Biol. **206:**1107-1115.
17. **Cunningham, K. W., and G. R. Fink.** 1994. Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking *PMC1*, a homolog of plasma membrane Ca^{2+} ATPases. J. Cell. Biol. **124:**351-363.
18. **Cyert, M. S.** 1993. The function of Ca^{+2}/calmodulin-regulated phosphatase in yeast. Adv. Protein Phosphatases **7:**429-443.
19. **Cyert, M. S.** 2003. Calcineurin signaling in *Saccharomyces cerevisiae*: how yeast go crazy in response to stress. Biochem. Biophys. Res. Commun. **311:**1143-1150.
20. **Cyert, M. S., and J. Thorner.** 1992. Regulatory subunit (*CNB1* gene product) of yeast Ca^{2+}/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. Mol. Cell. Biol. **12:**3460-3469.
21. **Cyert, M. S., R. Kunisawa, D. Kaim, and J. Thorner.** 1991. Yeast has homologs (*CNA1* and *CNA2* gene products) of mammalian calcineurin, a calmodulin-regulated phosphoprotein phosphatase. Proc. Natl. Acad. Sci. USA **88:**7376-7380.
22. **Eng, W. K., L. Faucette, M. M. McLaughlin, R. Cafferkey, Y. Koltin, R. A. Morris, P. R. Young, R. K. Johnson, and G. P. Livi.** 1994. The yeast *FKS1* gene encodes a novel membrane protein, mutations in which confer FK506 and cyclosporin A hypersensitivity and calcineurin-dependent growth. Gene **151:**61-71.
23. **Entian, K. D. and P. Kötter.** 1998. Yeast mutant and plasmid collections, p. 431-449. *In* A. Brown and M. Tuite (ed.), Yeast gene analysis. Methods in

Microbiology, vol. 26. Academic Press, Inc., San Diego, C.A.

24. **Estruch, F.** 2000. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol. Rev. **24:**469-486.
25. **Garrett-Engele, P., B. Moilanen, and M. S. Cyert.** 1995. Calcineurin, the Ca^{2+}/calmodulin dependent protein phosphatase, is essential in yeast mutants with cell integrity defects and in mutants that lack a functional vacuolar H(+)ATPase. Mol. Cell. Biol. **15:** 4103-4114.
26. **Gietz, R. D., and A. Sugino.** 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:**527-534.
27. **Goldstein, A. L., and J. H. McCusker.** 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae.* Yeast **15:**1541-1553.
28. **Haro, R., B. Garciadeblas, and A. Rodriguez-Navarro**. 1991. A novel P-type ATPase from yeast involved in sodium transport. FEBS Lett. **291:**189-191.
29. **Heitman, J., A. Koller, J. Kunz, R. Henriquez, A. Schmidt, N. R. Movva, and M. N. Hall.** 1993. The immunosuppressant FK506 inhibits amino acid import in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**5010-5019.
30. **Hemenway, C. S., and J. Heitman.** 1999. Calcineurin. Structure, function, and inhibition. Cell Biochem. Biophys. **30:**115-151.
31. **Hernandez-Lopez, M. J., J. A. Prieto, and F. Randez-Gil.** 2002. Isolation and characterization of the gene *URA3* encoding the orotidine-5'-phosphate decarboxylase from *Torulaspora delbrueckii*. Yeast **19:**1431-1435.
32. **Hernandez-Lopez, M. J, J. A. Prieto, and F. Randez-Gil.** 2003. Osmotolerance and leavening ability in sweet and frozen sweet dough. Comparative analysis between *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* baker's yeast strains. Antonie Van Leeuwenhoek **84:**125-134.
33. **Higuchi, S., J. Tamura, P. R. Giri, J. W. Polli, and R. L. Kincaid.** 1991. Calmodulin-dependent protein phosphatase from *Neurospora crassa.* Molecular cloning and expression of recombinant catalytic subunit. J. Biol. Chem. **266:**18104-18112.
34. **Hirayama, S., R. Sugiura, Y. Lu, T. Maeda, K. Kawagishi, M. Yokoyama, H. Tohda, Y. Giga-Hama, H. Shuntoh, and T. Kuno.** 2003. Zinc finger protein Prz1 regulates Ca^{2+} 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. **Hohmann, S.** 2002. Osmotic stress signaling and osmoadaptation in

yeasts. Microbiol. Mol. Biol. Rev. **66:**300-372.

36. **Huxley, C., E. D. Green, and I. 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.
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, R. Serrano 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 Ca^{++}/calmodulin binding proteins in *Aspergillus nidulans* cell cycle regulation. Prog. Cell Cycle Res. **2:**217-228.
46. **Platt, T., B. Muller-Hill, and J. H. Miller.** 1972. Assay of □-galactosidase, p. 351-355. *In* J. H. Miller (ed.), Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
47. **Rodriguez-Navarro, A.** 2000. Potasium transport in fungi and plants. Biochim. Biophys. Acta **1469:**1-30.
48. **Rusnak, F., and P. Mertz.** 2000. Calcineurin: form and function. Physiol. Rev. **4:**1483-1521.
49. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463-5467.
50. **Serrano, R., and A. Rodriguez-Navarro.** 2001. Ion homeostasis

during salt stress in plants. Curr. Opin. Cell. Biol. **13:**399-404.

51. **Serrano, R., J. A. Márquez, and G. Ríos.** 1997. Crucial factors in salt stress tolerance, p. 147-169. *In* S. Hohmann and W. H. Mager (ed.), Yeast Stress Responses. Springer-Verlag, Heidelberg.
52. **Serrano, R., J. Mulet, G. Rios, J. Marquez, I. de Larrinoa, M. Leube, I. Mendizabal, A. Pascual-Ahuir, M. Proft, R. Ros, and C. Montesinos.** 1999. A glimpse of the mechanisms of ion homeostasis during salt stress. J. Exp. Bot. **50:**1023-1036.
53. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
54. **Sigrist, C. J., L. Cerutti, N. Hulo, A. Gattiker, L. Falquet, M. Pagni, A. Bairoch, and P. Bucher.** 2002. PROSITE: a documented database using patterns and profiles as motif descriptors. Brief Bioinform. **3:**265-274.
55. **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.
56. **Sleator, R. D, and C. Hill.** 2001. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiol. Rev. **26:**49-71.
57. **Smith, D. L., T. Tao, and M. E. Maguire.** 1993. Membrane topology of a P-type ATPase. The MgtB magnesium transport protein of *Salmonella typhimurium*. J. Biol. Chem. **268:**22469-22479.
58. **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.
59. **Stathopoulos, A. M, and M. S. Cyert.** 1997. Calcineurin acts through the *CRZ1*/*TCN1*-encoded transcription factor to regulate gene expression in yeast. Genes Dev. **11:**3432-3444.
60. **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.
61. **Sugiura, R., S. O. Sio, H. Shuntoh, and T. Kuno.** 2001. Molecular genetic analysis of the calcineurin signaling pathways. Cell. Mol. Life Sci. **58**:278-288.
62. **Westfall, P. J., D. R. Ballon, and J. Thorner.** 2004. When the stress of your environment makes you go HOG wild. Science **5701:**1511-1512.
63. **Wiederrecht, G., E. Lam, S. Hung, M. Martin, and N. Sigal.** 1993 The mechanism of action of FK-506 and cyclosporin A. Ann. NY Acad. Sci. **696:**9-19.
64. **Withee, J. L., J. Mulholland, R. Jeng, and M. S. Cyert.** 1997 An essential role of the yeast pheromone-

induced Ca^{2+} signal is to activate calcineurin. Mol. Biol. Cell. **8:**263-277.

65. **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.

Resumen de resultados

El desarrollo de la industria de panadería y bollería congelada unido a la sensibilidad al proceso de congelación-descongelación de las cepas de levadura de *S. cerevisiae* (Attfield, 1997; Rández-Gil y col., 1999) y a la reducción de su capacidad fermentativa en este tipo de masas con el tiempo de almacenamiento (Gelinas y col., 1993; Hatano y col., 1996), justifica la búsqueda de nuevas cepas que combinen características de crioresistencia y osmotolerancia y cuyo uso suponga un beneficio económico al industrial sin detrimento de la calidad de los productos.

En el capítulo 1 de este trabajo se evalúa la posible utilización de dos cepas de *T. delbrueckii*, PYCC5321 y PYCC5323, para la elaboración de masas congeladas y azucaradas. El primer objetivo de este trabajo fue determinar su capacidad para crecer, en los medios industriales utilizados para la propagación de levaduras y evaluar su capacidad fermentativa en masas. Se observó que ambas cepas de *T. delbrueckii* son capaces de crecer en medios de melaza, con velocidades y rendimientos similares a los obtenidos para dos cepas control utilizadas comercialmente en panadería, Cinta Roja y Plus Vital. La comparación de la capacidad fermentativa de *T. delbrueckii* PYCC5321 y PYCC5323 con las cepas control de *S. cerevisiae*, mostró que ambas cepas producen niveles de CO_2 en masas saladas similares e incluso superiores que la cepa Cinta Roja (utilizada habitualmente para la elaboración de este tipo de masa). En el caso de masas dulces, las dos cepas de *T. delbrueckii* produjeron niveles de CO_2 superiores a los obtenidos para la cepa Plus Vital (utilizada habitualmente para la elaboración de masas azucaradas). Esta mayor capacidad fermentativa es evidente cuando la adición de azúcar a la masa es muy elevada, del 20%. En este caso, ambas cepas de *T. delbrueckii* producen niveles de CO_2 tres veces superiores al observado en la cepa Plus Vital.

Otro de los parámetros que determina el potencial uso de una cepa de levadura para la fermentación de distintos tipos de masa, es el nivel de actividad de enzimas como son la maltasa e invertasa. Como se ha comentado en la introducción, una levadura candidata a ser utilizada para la elaboración de

una masa panaria debe presentar una actividad maltasa elevada; mientras que en el caso de una masa azucarada, la levadura debe de tener niveles de invertasa reducidos, para evitar la liberación de grandes cantidades de glucosa y fructosa a partir de la sacarosa añadida en la formulación, lo cual genera un aumento de la presión osmótica del medio. El análisis del nivel de actividad maltasa en las dos cepas de *T. delbrueckii* de estudio, reveló que estos eran menores que los detectados en las cepas comerciales de levadura de panadería, no correlacionándose este resultado con la capacidad fermentativa mostrada. En el caso de la actividad invertasa, las dos cepas de *T. delbrueckii* tienen una actividad reducida, similar a la detectada en la cepa de Plus Vital. La inesperada baja producción de gas que mostraba la cepa Plus Vital en masas dulces, nos llevó a analizar el posible efecto de la adición de sal en estas formulaciones como fuente, tanto de estrés osmótico como iónico. Al evaluar la producción de gas en masas sin adición de sal, las tres cepas (*T. delbrueckii* PYCC5321, PYCC5323 y Plus Vital) presentan una capacidad fermentativa similar en masas incluso conteniendo un 20% de azúcar. Como cabía esperar, la adición de sal, produce un descenso en la capacidad fermentativa, siendo éste efecto más pronunciado en la cepa Plus Vital, la cual presenta un periodo de latencia muy superior y niveles de producción máxima de CO_2 inferiores a los detectados en las dos cepas de *T. delbrueckii*. Con respecto a éstas, la cepa PYCC5321 mostró una cinética similar de producción de gas en masas con o sin sal añadida.

A la vista de estos resultados, decidimos estudiar el fenotipo de osmotolerancia y resistencia a distintos iones que presentaban las cuatro cepas analizadas. Mientras que *T. delbrueckii* PYCC5321 mostró un claro fenotipo tanto de osmotolerancia como de resistencia a la toxicidad del Na^+, la cepa PYCC5323 resultó ser osmosensible, ya que es incapaz de crecer en presencia de iones en el medio de ensayo. Este resultado indica que la elevada capacidad fermentativa presentada por las dos cepas de *T. delbrueckii* en masas con un 20% de azúcar y en presencia de sal, no se correlaciona con el fenotipo de osmotolerancia observado en placa.

La resistencia a la congelación es una característica esencial de aquellas levaduras que se pretenden usar tanto en masas congeladas, como en masas congeladas y además azucaradas. La congelación del agua da lugar a un aumento de la concentración de los electrolitos que había disueltos en ella previamente a la congelación (Hatano y col., 1996; Myers y Attfield, 1999); por otro lado el almacenamiento de masas en congelación, supone la formación y crecimiento de cristales de hielo, éstos provocan daños celulares por rotura de membranas con la consecuente pérdida de la organización subcelular (Grout y col., 1990). Hemos analizado el efecto de estos dos fenómenos en la capacidad de producción de CO_2 de las cuatro cepas de estudio. En el caso de las dos cepas comerciales de *S. cerevisiae*, tiene lugar un descenso significativo en su capacidad fermentativa (40%), debido al hecho de congelar y descongelar la masa. Este efecto es menos significativo en el caso de las masas elaboradas con las cepas de *T. delbrueckii*, especialmente en el caso de PYCC5321, cuya capacidad fermentativa parece no verse alterada por el proceso de congelación-descongelación. Cuando analizamos el efecto del almacenamiento en congelación, éste parece afectar de igual modo a las cuatro cepas, en todos los casos se observa un descenso gradual en su capacidad fermentativa con el tiempo de almacenamiento.

Por último en este trabajo se ha intentado correlacionar los fenotipos de osmotolerancia y crioresistencia, con factores celulares descritos en la bibliografía como implicados en éstos fenómenos. Concretamente se ha analizado el contenido en glicerol y trehalosa, así como la cinética de movilización de esta última por la presencia de azúcares fermentables en el medio de cultivo. La comparación de la cinética de producción de glicerol intracelular en las cuatro cepas, cuando son sometidas estrés osmótico, muestra una diferencia clara entre *T. delbrueckii* y *S. cerevisiae.* Las primeras alcanzan niveles máximos de glicerol intracelular a tiempos cortos, de 3 a 6 horas tras la adición de sal al medio, frente a las 24 a 36 horas observadas en *S. cerevisiae*. Además los valores máximos alcanzados son menores que en *S.*

cerevisiae. Esta característica se analiza con más profundidad en el capítulo 3. Con respecto a los niveles de trehalosa acumulados durante la propagación de la levadura en los medios de melazas, se observa que inicialmente, estos son menores en el caso de *T. delbrueckii*, aunque su movilización por parte de esta levadura es más lenta que en el caso de *S. cerevisiae*. Desde que la levadura es mezclada con el resto de ingredientes durante el amasado, hasta que la masa es congelada, transcurre un periodo de tiempo suficiente para que se produzca la movilización de trehalosa. Por tanto el contenido real de ésta, antes de congelar la masa, es superior en *T. delbrueckii* que en *S. cerevisiae*. Éste hecho, junto a la elevada osmotolerancia presentada por las dos cepas de *T. delbrueckii* PYCC5321 y PYCC5323, podría explicar la mayor tolerancia de estas a la congelación, en el que se ha postulado que la trehalosa cumple un papel importante como crioprotector.

En los últimos años las técnicas de ingeniería genética han abierto la posibilidad de construir nuevas cepas de levadura de panadería con características mejoradas o capaces de producir proteínas que modifiquen el aroma, la reología de la masa o la vida media del pan (para una revisión ver, Rández-Gil y col., 1993). Un claro ejemplo ha sido la obtención de cepas de levadura recombinantes capaces de expresar enzimas heterólogas empleadas habitualmente como mejorantes panarios (Monfort y col., 1997; Monfort y col., 1999).

El empleo de cepas de *T. delbrueckii* en panificación, convierte a esta levadura en un potencial huésped para la expresión de proteínas de interés en este proceso industrial. Sin embargo, el uso de técnicas de ADN-recombinante en este microorganismo es limitado, ya que se carece de las herramientas moleculares necesarias. La mayoría de cepas de *T. delbrueckii* son protótrofas, en consecuencia, su transformación mediante complementación de marcadores auxotróficos, el método comúnmente utilizado en *S. cerevisiae*, no puede ser empleado. Esto implica que la manipulación genética de estas cepas sólo pueda llevarse a cabo mediante el uso de marcadores dominantes de resistencia a

drogas, limitándose así su utilización industrial (Linko y col., 1997). En este escenario, la obtención de cepas auxótrofas de *T. delbrueckii* se convierte en un objetivo prioritario por lo que en el capítulo 2 de este trabajo se presenta la construcción de una genoteca de *T. delbrueckii* PYCC5321, que nos ha permitido el aislamiento, secuenciación y caracterización del gen *URA3* de *T. delbrueckii* mediante complementación de un mutante de *S. cerevisiae* para éste gen.

TdURA3, codifica para una proteína de 264 aminoácidos con motivos típicos de la familia de las oroditina-5´-fosfato descarboxilasas que catalizan el último paso en la síntesis de novo de pirimidinas. Esta proteína presenta un 83% de homología la proteína Ura3 de *S. cerevisiae*. La obtención de mutantes auxótrofos se puede abordar desde dos estrategias distintas, mediante la selección de mutantes espontáneos o bien mediante la disrupción del gen diana por técnicas de manipulación genética. En este trabajo, se muestra la obtención de mutantes auxótrofos para uracilo en la cepa de *T. delbrueckii* PYCC5323 mediante las dos estrategias mencionadas. La adición al medio de cultivo de ácido 5-fluoroorotico (5-FOA), permitió la selección de colonias incapaces de crecer en ausencia de uracilo en el medio. Este mutante es particularmente interesante para su uso industrial, puesto que se puede considerar una levadura de grado alimentario, ya que no lleva ningún fragmento de ADN foráneo.

La interrupción del gen *TdURA3* se abordó mediante técnicas habitualmente utilizadas en *S. cerevisiae*, concretamente se empleó un "casete de disrupción" que contiene un gen de resistencia a nourseotricina (Golstein y McCusker, 1999) flanqueado por zonas homologas al gen *TdURA3*. Con ello se pretendía comprobar si los protocolos habitualmente utilizados para *S. cerevisiae* son óptimos para la manipulación de *T. delbrueckii.* En efecto, se obtuvo una cepa interrumpida en el gen *TdURA3,* a partir de la cual comprobamos la existencia de una copia única de este gen en esta levadura. Este hecho nos llevó a

plantearnos la posibilidad que las cepas de *T. delbrueckii* con las que estábamos trabajando fueran haploides. Análisis mediante citometría de flujo (datos no incluidos en el manuscrito de Tesis), revelaron que ambas cepas de *T. delbrueckii* lo eran. Este resultado, nos ofrece la posibilidad de manipular la ploidía de este microorganismo y estudiar el efecto que esto tiene en parámetros industriales de relevancia como robustez, poder fermentativo o tolerancia a diferentes condiciones de estrés.

En el capítulo 2 también se abordó la posibilidad de utilizar *T. delbrueckii* como hospedador para la expresión de proteínas heterólogas de interés en el proceso de panificación. En concreto se estudió la producción de xilanasa X-24 mediante la expresión del gen *xlnB* de *Aspergillus nidulans* (Pérez-González y col., 1996). Encontrándose que *T. delbrueckii*, es capaz de sintetizar y secretar la proteína heteróloga al mismo nivel que la cepa de panadería control utilizada, HS13 (*ura3*)

La alta osmoresistencia y tolerancia a la presencia de sodio mostrada por *T. delbrueckii* PYCC5321, nos ha llevado a proponer a esta cepa como modelo de estudio de la respuesta a estrés osmótico e iónico en levadura de panadería. En este sentido, el capítulo 3 recoge la identificación de genes de *T. delbrueckii* implicados en resistencia a estrés osmótico y salino, como son *TdHOG1, TdCRZ1 y TdENA1*.

En el primer apartado de este capítulo se presenta el aislamiento del gen *TdHOG1* mediante complementación en un mutante *hog1*Δ de *S. cerevisiae*. La proteína TdHog1 tiene una alta homología (88%) de secuencia con Hog1p de *S. cerevisiae* y, si bien restablece la osmotolerancia de un mutante *hog1*Δ de esta especie, no es capaz de mejorar la osmotolerancia de la misma cuando es expresada en una cepa salvaje. La proteína TdHog1p es fosforilada bajo condiciones suaves de estrés (0.5M de NaCl) y su cinética de fosforilación es similar a la que presenta Hog1p en *S. cerevisiae.* La clonación del gen *TdHOG1*

nos ha permitido la construcción de la correspondiente cepa *hog1*Δ en *T. delbrueckii* PYCC5321, en la que hemos estudiado la implicación de la ruta HOG en tolerancia a estrés en esta especie de levadura. El comportamiento de este mutante bajo diferentes condiciones de estrés osmótico y salino indica que, si bien es más sensible que su cepa parental, el efecto de la mutación no es tan severo como en el caso de *S. cerevisiae,* ya que se necesitan concentraciones muy elevadas de sorbitol o NaCl para observar un defecto en su crecimiento. Por tanto, aunque la ruta HOG en *T. delbrueckii* está implicada en su osmotolerancia, parece tener un papel menor que en el caso de *S. cerevisiae*. En este sentido, cuando analizamos el nivel de inducción de *GPD1* en células de *T. delbrueckii* transferidas a un medio con NaCl (1M), se observa un aumento en los niveles de ARNm de 10 veces. Esta inducción resultó ser dependiente de TdHog1p, pero es menor que la observada en *S. cerevisiae* (Rep y col., 1999b). Este hecho se ve reflejado en los diferentes niveles de glicerol producidos por estas levaduras tras ser sometidas a un choque osmótico. Así, mientras que en *S. cerevisiae* una cepa *hog1*Δ presenta tan solo un 14% de la producción de glicerol total tras tres horas de crecimiento en un medio con 1M de NaCl (Albertyn y col., 1994), un mutante *hog1*Δ de *T. delbrueckii* produce niveles de un 76% con respecto a la cepa salvaje.

El diferente papel en estrés osmótico de la ruta HOG en *T. delbrueckii* nos llevo a estudiar su implicación en la respuesta a otros tipos de estreses. Así, hemos comprobado en *T. delbrueckii,* que al igual que en otras especies de levadura, la ruta HOG está claramente implicada en tolerancia a ácido cítrico y methylglioxal, mientras que no interviene en la respuesta a choque térmico (Winkler y col., 2002; Lawrence y col., 2004; Aguilera y col., 2005; Alonso-Mongue y col., 2003; Smith y col., 2004). Sin embargo, y a diferencia de lo que ocurre en *S. cerevisiae* y *C. albicans* (Gacto y col., 2003; Alonso-Mongue y col., 2003; Haghnazari y Heyer, 2004) esta ruta no está implicada en la respuesta a estrés oxidativo. En este mismo trabajo también hemos descrito un fenómeno de protección cruzada en *T. delbrueckii*. Aunque esta levadura es incapaz de

crecer a 37º C, sí lo hace cuando hay sorbitol o NaCl en el medio de cultivo. Además esta protección es dependiente de la existencia de una proteína Hog1p funcional, ya que sólo las cepas salvajes, preestresadas osmóticamente, son capaces de crecer a 37ºC.

En el segundo apartado del capítulo 3 se describe la identificación de genes de *T. delbrueckii* cuya sobreexpresión mejora la tolerancia a sal de *S. cerevisiae*. Para ello se llevó a cabo un rastreo de una genoteca de *T. delbrueckii* PYCC5321, en una cepa de *S. cerevisiae*, CEN.PK2-1C, que presenta un claro fenotipo de sensibilidad a Na^+, seleccionándose aquellas células que mostraron un mejor crecimiento en presencia de NaCl. Siguiendo esta metodología, se aislaron varios plásmidos que confieren dicha ventaja de crecimiento. En este trabajo se describe los resultados obtenidos con dos de estos plásmidos, los denominados YEpMJH1 y YEpMJH14. Tras su secuenciación, encontramos una pauta completa de lectura abierta en cada uno de ellos, que mostró alta homología con los genes *ENA1* (*ENA2* y *ENA5*) y *CRZ1* de *S. cerevisiae*, respectivamente.

En *S. cerevisiae* el gen *ENA1* codifica la principal ATPasa tipo-P de esta levadura, implicada en el flujo hacia el exterior de iones Na^+ (Haro y col., 1991). Su expresión es inducida tanto por estrés osmótico como por estrés iónico y depende de varias rutas de transducción de la señal, entre ellas de la ruta calcineurina-Crz1p (Stathopoulos y Cyert, 1997). El sistema de selección empleado en este trabajo, nos había permitido clonar en *T. delbrueckii* un posible factor de transcripción clave en estrés iónico y uno de sus bien conocidos genes diana, TdCrz1p y TdEna1p, por lo que centramos nuestros estudios en caracterizar la funcionalidad de TdCrz1p en la respuesta a estrés salino en esta levadura osmotolerante.

Al igual que ocurre con la sobreexpresión de *CRZ1*, un mayor número de copias del correspondiente homólogo de *T. delbrueckii*, mejora la osmotolerancia

de una cepa salvaje de *S. cerevisiae* y suprime la sensibilidad a Na^+, Li^+ y Mn^{2+} tanto de una cepa mutante en la subunidad reguladora de la calcineurina (*cnb1*Δ) como de un mutante *crz1*Δ (Matheos y col., 1997; Stathopoulos y Cyert, 1997). Además, TdCrz1p es capaz de unirse a las secuencias consenso CDRE descritas en los promotores de genes que están bajo el control de Crz1p en *S. cerevisiae* (Stahopoulos y Cyert, 1997).

A la vista de estos resultados, procedimos a la obtención del correspondiente mutante interrumpido en *TdCRZ1* para analizar su implicación en distintas condiciones estrés iónico. En *T. delbrueckii*, células que carecen de este gen resultaron sensibles a Ca^{2+} y Mn^{2+}, coincidiendo con el fenotipo descrito para un mutante nulo en *S. cerevisiae* (Stahopoulos y Cyert, 1997), mientras que su capacidad de crecimiento no se ve afectada por la presencia de Na^+ o a pH básico. Además, sorprendentemente, esta cepa interrumpida muestra una mayor tolerancia a Li^+ que la correspondiente cepa salvaje, lo que sugiere que a diferencia de *S. cerevisiae* que utiliza una misma bomba de iones (Ena1p), para eliminar de la célula tanto Na^+ como Li^+ (Marquez y Serrano, 1996), *T. delbrueckii* debe disponer de distintos sistemas de eflujo de iones; estos posibles transportadores, parecen estar regulados negativamente por *TdCRZ1* o ser independientes de este factor transcripcional. En este sentido, cuando analizamos el patrón de expresión de *TdENA1* en presencia de Li^+ o de Na^+, observamos una cinética de inducción distinta dependiendo del ión presente en el medio. Como habíamos propuesto, esta inducción resultó ser independiente de TdCrz1p.

Llegados a este punto, se decidió estudiar la implicación de la ruta de la calcineurina en la respuesta a estrés salino en *T. delbrueckii*. Como no disponemos en esta levadura de mutantes en calcineurina, procedimos a inhibir su función con el inmunosupresor FK506 (Wiederrecht y col., 1993). Así, en células con esta proteína fosfatasa no funcional, su crecimiento se ve inhibido cuando se añade Ca^{2+} al medio de cultivo, sin embargo no se observa ningún

defecto de crecimiento en condiciones control, ni en presencia de Na^+. Este dato indica que en *T. delbrueckii* la ruta de la calcineurina-Crz1p es esencial para la supervivencia en presencia de Ca^{2+}. Sin embargo, no parece tener ninguna función en la tolerancia a Na^+. Además, la adición de FK506 a un medio con Li^+, pone de manifiesto la implicación de esta ruta de transducción de la señal en la tolerancia a este ión, aunque a diferencia de *S. cerevisiae*, en este caso tiene una función antagónica a TdCrz1p. Todos estos resultados sugieren que la ruta de la calcineurina-Crz1p, juega un papel diferente en tolerancia a estrés salino en esta levadura osmotolerante, al descrito para *S. cerevisiae*.

Discusión General

El gran desarrollo en los últimos años de la industria de bollería congelada requiere del uso de cepas de levadura adaptadas a altas concentraciones de azúcares así como a los procesos de congelación-descongelación de las masas. La obtención de nuevas cepas de *S. cerevisiae* mediante manipulación genética se enfrenta al problema social que existe actualmente con respecto al uso de OMG (organismos modificados genéticamente) por lo que una buena alternativa es el uso de levaduras naturales no convencionales, también conocidas como "non-*Saccharomyces*". Como primer objetivo de este trabajo se planteó el uso de dos cepas de *T. delbrueckii*, PYCC5321 y PYCC5323, en la elaboración de masas congeladas azucaradas. En este sentido, y según los resultados obtenidos en el capítulo 1 de este trabajo, vemos que ambas cepas, no sólo son capaces de proliferar activamente en medios industriales de manera similar a las cepas de *S. cerevisiae*, sino que además, presentan un elevado poder fermentativo en masas no congeladas y, sobre todo, una extraordinaria tolerancia a la congelación. Ambas cepas son, por tanto, candidatas firmes a ser empleadas como levaduras industriales en la elaboración de masas congeladas.

Nuestros resultados muestran que *T. delbrueckii* es capaz de crecer en medios industriales de modo similar a *S. cerevisiae* en condiciones de crecimiento en "batch". Sin embargo, estudios posteriores de crecimiento en "feed-batch" en fermentador, indicaron que *T. delbrueckii* presentaba una velocidad de crecimiento y rendimiento en biomasa inferiores a los mostrados por *S. cerevisiae*. Estos ensayos se realizaron en las condiciones de propagaciones óptimas para *S. cerevisiae*. Estudios recientes han demostrado que *T. delbrueckii* y *S. cerevisiae* se comportan de modo diferente en función de la disponibilidad de glucosa y oxígeno en el medio. El requerimiento de oxígeno por parte de la primera es mayor, lo que sugiere que posee un metabolismo más oxidativo. Se ha relacionado que un aumento en la concentración de oxígeno permite la supervivencia de *T. delbrueckii* en cultivos mixtos con *S. cerevisiae* (Nissen y col., 2004) y además, y pese que se trata

de un microorganismo anaerobio-facultativo, presenta una tasa de crecimiento muy inferior en condiciones estrictas de anaerobiosis con respecto a *S. cerevisiae* (Hanl y col., 2005). Estos resultados sugieren que el comportamiento metabólico de ambas especies es diferente y depende en gran medida de la disponibilidad de nutrientes. Por tanto es necesaria la búsqueda de las condiciones óptimas para el crecimiento de *T. delbrueckii* en fermentadores a nivel industrial. Otro obstáculo a tener en cuenta a la hora de la posible producción industrial de *T. delbrueckii* es la reticencia de los productores de levadura industrial a cambiar sus procesos de producción y adaptarlos a nuevos microorganismos. Con todo lo anterior se hacen necesarios estudios futuros de crecimiento en condiciones industriales, salto de escala e incluso análisis de las características organolépticas de los productos elaborados con *T. delbrueckii,* y de aceptación por parte del consumidor.

Además, la aplicación industrial de *T. delbrueckii* puede estar condicionada por factores tales como su pequeño tamaño celular. El proceso de obtención de levadura, implica un paso de filtración en el cual, habría problemas de colmatación de filtros. Se ha sugerido, como solución a este problema, un aumento de ploidía de *T. delbrueckii* (Sasaki y Oshima, 1987). Estudios preliminares de citometría de flujo realizados en nuestro laboratorio, han confirmado la haploidía de la cepa PYCC5321. La construcción de una cepa diploide a partir de ésta, podría solucionar el problema. Sin embargo, en *S. cerevisiae* se ha observado que cepas con mayor grado de ploidía presentan una menor tolerancia a estrés, en concreto a estrés por congelación (Teunissen y col., 2002). En este sentido, tras la construcción de una cepa diploide de esta levadura, hemos comprobado que se comporta de manera similar a su cepa parental haploide, en cuanto a crecimiento en medios industriales, capacidad fermentativa y tolerancia a estrés.

El uso de cepas recombinantes de levadura capaces de producir proteínas de interés en el proceso de panificación es una estrategia útil para modificar

sus propiedades funcionales (Rández-Gil y col., 1999). Como se muestra en el capítulo 2, gracias a la obtención de una genoteca en la cepa PYCC5321, hemos podido aislar y secuenciar el gen *TdURA3*, un marcador auxotrófico habitualmente empleado como marcador de selección. Esto nos ha permitido la construcción de una cepa Ura$^-$ la cual es capaz de expresar, bajo control del promotor de *ACT1* de *S. cerevisiae*, el cDNA del gen xlbB, que codifica para la xilanasa X24 de *Aspergillus nidulans*. Esto supone un valor añadido para estas cepas en su posible uso industrial. Tanto en el capítulo 2 como en el 3, se muestra como se han obtenido con éxito cepas recombinantes de *T. delbrueckii* como es el caso de la obtención de mutantes mediante el uso de "casetes de disrupción". Es evidente la ventaja de poder obtener cepas en las cuales el gen de interés se halle integrado en su genoma y no en vectores de expresión, los cuales no pueden ser utilizados en cepas comerciales de panadería. Por otro lado, si comparamos el ritmo de producción de xilanasa en esta cepa en un plásmido multicopia, vemos no obstante que es inferior que el producido por una cepa control de *S. cerevisiae* Ura$^-$. Esto nos indica que los promotores de *S. cerevisiae* son funcionales en *T. delbrueckii*, pero no son igual de efectivos. A esto hay que sumar que la frecuencia de transformación y la estabilidad de los plásmidos habitualmente usados en *S. cerevisiae* son menores cuando son utilizados para *T. delbrueckii*. En este trabajo se han aplicado los protocolos de laboratorio habitualmente utilizados para *S. cerevisiae*. Aunque mayoritariamente estos protocolos son válidos para *T. delbrueckii ,* siguen sin ser óptimos, por lo que es necesario, para un futuro uso de este microorganismo como modelo de estudio, la puesta a punto de los distintos protocolos utilizados habitualmente en biología molecular.

Los fenotipos de osmotolerancia y crioresistencia, y en general, la alta resistencia a distintos estreses mostrada por la cepa PYCC5321 nos llevó a proponerla como posible modelo para el estudio de la respuesta a estrés en levaduras de panadería. En concreto, en este trabajo, nos hemos centrado en el estudio de la respuesta a estrés osmótico y salino en esta levadura. Dentro de

nuestros objetivos estaban la búsqueda de genes implicados en osmotolerancia en *T. delbrueckii*, así como de genes de ésta que confirieran mayor osmoresistencia en *S. cerevisiae*. En el primer caso, tal y como se describe en el capítulo 3, hemos aislado y caracterizado el gen *TdHOG1* mediante complementación en un mutante *hog1Δ* de *S. cerevisiae*. En el caso de la búsqueda de genes de *T. delbrueckii* que mejoren la osmotolerancia de *S. cerevisiae*, se han aislado dos genes, *TdCRZ1* y *TdENA1* cuyos homólogos correspondientes en *S. cerevisiae*, codifican un factor transcripcional implicado en la ruta de la calcineurina y uno de sus genes dianas, una Na^+-ATPasa, respectivamente (Stathopoulos y Cyert, 1997; Wieland y col., 1995). Como ya se ha comentado en la introducción, en *S. cerevisiae*, la ruta HOG y la ruta de la calcineurina, son esenciales en la transmisión de señales de estrés osmótico y salino en *S. cerevisiae* y determinan su adaptación a estos estreses medioambientales. La clonación de estos genes y la obtención de cepas mutantes *crz1*Δ y *hog1*Δ en *T. delbrueckii*, nos ha permitido el inicio de la caracterización de estas dos rutas en esta levadura. Los resultados mostrados en este trabajo arrojan que, si bien la ruta HOG juega un papel en la osmotolerancia de *T. delbrueckii*, la implicación de la misma en fenómenos tales como la síntesis de osmolitos o la resistencia a estrés osmótico y a otro tipo de estreses, no siguen los mismos patrones definidos para *S. cerevisiae*. Por tanto, aunque existe esta ruta en *T. delbrueckii*, los mecanismos de regulación de la misma no son iguales que los de *S. cerevisiae*. Del mismo modo, el estudio de los mecanismos de regulación del gen *TdENA1* por parte del factor de transcripción *TdCRZ1*, así como el estudio del fenotipo que muestra una cepa interrumpida para este gen en *T. delbrueckii*, sugieren de nuevo mecanismos de regulación diferentes a los descritos para *S. cerevisiae*. Puesto que *T. delbrueckii* se encuentra en estado natural en hábitats con altas presiones osmóticas, no está de más el pensar que la ruta HOG así como la ruta de la calcineurina, hayan evolucionado como respuesta a una adaptación a éste tipo de ambientes y que los cambios acontecidos sean los responsables, al menos en parte, de la alta osmotolerancia exhibida por esta levadura. El estudio

descrito en este trabajo sugiere que los elementos de estas rutas, su interconexión y regulación podrían diferir de los descritos en otras levaduras. En este escenario, nuestras perspectivas futuras se centran en la identificación, en *T. delbrueckii*, de otros componentes de la ruta HOG y de la ruta de la calcineurina para poder profundizar en la caracterización de las mismas.

Con todo lo expuesto, se confirma la idea de que esta cepa es un buen modelo para el estudio del fenómeno de tolerancia a estrés. El conocimiento a nivel molecular de las rutas que determinan el comportamiento de *T. delbrueckii* frente a estrés osmótico, iónico o otros tipos de estreses puede ayudarnos, no sólo a esclarecer las bases moleculares implicadas en estos fenómenos, sino también a diseñar diferentes estrategias con objeto de construir cepas de *S. cerevisiae* con mayor tolerancia a estrés.

Conclusiones

Los resultados descritos en este trabajo y su discusión posterior, han permitido extraer las siguientes conclusiones:

- Las cepas de *T. delbrueckii* PYCC5321 y PYCC5323, cumplen los requisitos exigidos a una cepa de uso comercial en panificación: crecimiento rápido y alto rendimiento de biomasa en medios de melaza, acoplado a una buena capacidad fermentativa en masas dulces y saladas, tanto frescas como congeladas. Además, esta levadura no convencional es capaz de sintetizar y secretar proteínas heterólogas, lo que potencia su posible uso biotecnológico.

- La cepa de *T. delbrueckii* PYCC5321 muestra un claro fenotipo de osmotolerancia y tolerancia a la toxicidad del sodio y es un modelo útil para la identificación de genes de levadura implicados en tolerancia a estas dos condiciones de estrés.

- La ruta HOG de *T. delbrueckii* es esencial para la tolerancia de esta levadura a diferentes tipos de estreses. Sin embargo, su implicación en la inducción transcripcional de *GPD1* y en la acumulación de glicerol en respuesta a estrés osmótico es escasa.

- En *T.delbrueckii* existe un mecanismo de protección cruzada. Para las condiciones ensayadas en este estudio, dicho mecanismo depende de la actividad de la MAP quinasa TdHog1p

- El gen *TdCRZ1* codifica el homólogo del gen *CRZ1* de *S. cerevisiae*, implicado en la activación vía calcineurina de genes de respuesta a estrés salino.

Conclusiones

- El factor transcripcional *TdCRZ1* está implicado en la respuesta adaptativa de *T. delbrueckii* a la presencia de altas concentraciones de cationes divalentes como Ca^{2+} y Mn^{2+}. Por el contrario, no parece tener un papel funcional en resistencia a NaCl y su ausencia determina una tolerancia exacerbada a Li^{+}.

Bibliografía

(incluida en Introducción, Justificación y Objetivos, Resumen de Resultados y Discusión)

Aguilera J, Rodriguez-Vargas S y Prieto JA (2005) The HOG MAP kinase pathway is required for the induction of methylglyoxal-responsive genes and determines methylglyoxal resistance in *Saccharomyces cerevisiae*. Mol Microbiol, 56:228-239.

Albertyn J, Hohmann S, Thevelein JM y Prior BA (1994) *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high osmolarity glycerol response pathway. Mol Cell Biol, 14:4135-44.

Alepuz PM, Cunningham KW y Estruch F (1997) Glucose repression affects ion homeostasis in yeast through the regulation of the stress-activated *ENA1* gene. Mol Microbiol, 26:91-98.

Alepuz PM, de Nadal E, Zapater M, Ammerer G y Posas F (2003) Osmostress induced transcription by Hot1 depends on a Hog1 mediated recruitment of the RNA Pol II. EMBO J, 22:243-342.

Almeida MJ y Pais CS (1996a) Characterization of the yeast population from traditional corn and rye bread doughs. Lett Appl Microbiol, 23:154-158

Almeida MJ y Pais CS (1996b) Leavening ability and freeze tolerance of yeasts isolated from traditional corn and rye bread doughs. Appl Environ Microbiol, 62: 4401-4404.

Alonso-Monge R, Navarro-Garcia F, Roman E, Negredo AI, Eisman B, Nombela C y Pla J (2003) The Hog1 mitogen-activated protein kinase is essential in the oxidative stress response and chlamydospore formation in Candida albicans. Eukaryot Cell, 2:351-361.

Apse MP y Blumwald E (2002) Engineering salt tolerance in plants. Curr Opin Biotechnol, 2:146-50.

Attfield PV (1997) Stress tolerance: the key to effective strains of industrial baker's yeast. Nat Biotechnol, 15:1351-1357.

Attfield PV y Kletsas S (2000) Hyperosmotic stress response by strains of bakers' yeasts in high sugar concentration medium. Lett Appl Microbiol, 4:323-327.

Bell M, Capone R, Pashtan I, Levitzki A y Engelberg D (2001) Isolation of hyperactive mutants of the MAPK p38/Hog1 that are independent of MAPK kinase activation. J Biol Chem, 276:25351-25358.

Belloch C, Querol A, Garcia MD y Barrio E (2000) Phylogeny of the genus *Kluyveromyces* inferred from the mitochondrial cytochromec oxidase II gene. Int J Syst Evol Microbiol, 1:405-416.

Benito B, Quintero FJ y Rodriguez Navarro A (1997) Overexpression of the sodium ATPase of *Saccharomyces cerevisiae*: conditions for phosphorylation from ATP and Pi. Biochim Biophys Acta, 1328:214-226.

Blomberg A y Adler L (1992) Physiology of osmotolerance in fungi. Adv Microb Phys, 33:145-212

Blomberg A (2000) Metabolic surprises in *Saccharomyces cerevisiae* during adaptation to saline conditions: questions, some answers and a model. FEMS Microbiol Lett, 182:1-8.

Burrows, S (1979) Baker's yeast. Econ. Microbiol. 4:31-64

Clement, P y Loiez A (1983) Strain of yeast for breadmaking and novel strains of yeast tus prepared. US patent, 4,396,632.

Codon AC, Benitez T y Korhola M (1997) Chromosomal reorganization during meiosis of *Saccharomyces cerevisiae* baker's yeasts. Curr Genet, 32:247-259.

Codon AC, Benitez T y Korhola M (1998) Chromosomal polymorphism and adaptation to specific industrial environments of *Saccharomyce*s strains. Appl Microbiol Biotechnol, 49:154-163.

Codon AC, Rincon AM, Moreno Mateos MA, Delgado-Jarana J, Rey M, Limon C,Rosado IV, Cubero B, Penate X, Castrejon F y Benitez T (2003) New *Saccharomyces cerevisiae* baker's yeast displaying enhanced resistance to freezing. J Agric Food Chem, 51:483-491.

Crespo JL, Daicho K, Ushimaru Ty Hall MN (2001) The GATA transcription factors *GLN3* and *GAT1* link TOR to salt stress in *Saccharomyces cerevisiae*. J Biol Chem, 276:34441-34444.

Cyert MS, Kunisawa R, Kaim D y Thorner J (1991) Yeast has homologs *(CNA1* and *CNA2* gene products) of mammalian calcineurin, a calmodulina-regulated phosphoprotein phosphatase. Proc Natl Acad Sci U S A, 88:7376-7380.

Cyert MS y Thorner J (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.

Cyert MS (2003) Calcineurin signaling in Saccharomyces cerevisiae: how yeast go crazy in response to stress. Biochem Biophys Res Commun, 311:1143-1150.

Da Costa MS, Santos H y Galinski EA (1998) An overview of the role and diversity of compatible solutes in Bacteria and Archaea. Adv Biochem Eng Biotechnol, 61:117-153.

de Nadal E, Alepuz PM y Posas F (2002) Dealing with osmostress through MAP kinase activation. EMBO, 3:735-740.

de Nadal E, Casadome L y Posas F (2003) Targeting the MEF2-like transcription factor Smp1 by the stress-activated Hog1 mitogen-activated protein kinase. Mol Cell Biol, 23: 229-237.

de Nadal E, Zapater M, Alepuz PM, Sumoy L, Mas G y Posas F (2004) The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes. Nature , 427:370-374.

Esteve-Zarzoso B, Peris-Toran MJ, Garcia-Maiquez E, Uruburu F y Querol A (2001) Yeast population dynamics during the fermentation and biological aging of sherry wines. Appl Environ Microbiol, 67:2056-2061.

Evans IH (1990) Yeast strains for baking: recent developments. In: Spencer JFT & Spencer DM (Ed) Yeast Technology, (pp 1354).SpringerVerlag, Berlin

Farcasanu IC, Hirata D, Tsuchiya E, Nishiyama F y Miyakawa T (1995) Protein phosphatase 2B of *Saccharomyces cerevisiae* is required for tolerance to manganese, in blocking the entry of ions into the cells. Eur J Biochem, 232:712-717.

Ferrigno P, Posas F, Koepp D, Saito H y Silver PA (1998) Regulated núcleo/cytoplasmatic exchange of HOG1 MAPK requires the importin beta homologos *NMD5* and *XPO1*. EMBO J, 17:5606-5614

Gacto M, Soto T, Vicente-Soler J, Villa TG y Cansado J (2003) Learning from yeasts: intracellular sensing of stress conditions. Int Microbiol, 6:211-219.

Garrett-Engele P, Moilanen B y Cyert MS (1995) Calcineurin, the Ca2+/calmodulindependent protein phosphatase, is essential in yeast mutants with cell integrity defects and in mutants that lack a functional vacuolar H(+)ATPase. Mol Cell Biol, 15:4103-4114.

Gelinas P, Lagiomonière M y Dubord C (1993) Baker´s yeast sampling and frozen dough stability. Cereal Chem, 70:219-225.

Goldstein AL y McCusker JH (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast, 15:1541-1553.

Grout B, Morris J y McLellan M (1990) Cryopreservation and the maintenance of cell lines. Trends Biotechnol, 8:293-297.

Gustin MC, Albertyn J, Alexander M y Davenport K (1998) MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev, 62:1264-1300.

Haghnazari E y Heyer WD (2004) The Hog1 MAP kinase pathway and the Mec1 DNA damage checkpoint pathway independently control the cellular responses to hydrogen peroxide. DNA Repair 7:769-776.

Hahn YS y Kawai H (1990) Isolation and characterization of freeze-tolerant yeasts from nature available for the frozen-dough method. Agric Biol Chem, 54:829-831.

Hanl L, Sommer P y Arneborg N (2005) The effect of decreasing oxygen feed rates on growth and metabolism of *Torulaspora delbrueckii.* Appl Microbiol Biotechnol, 67:113-118

Haro R, Garciadeblas B y Rodriguez-Navarro A (1991) A novel P-type ATPase from yeast involved in sodium transport. FEBS Lett, 291:189-191.

Hatano S, Udou M, Koga N, Honbjoh K y Miyamoto T (1996) Impairment of the glycolytic system and actin in baker´s yeast during frozen storage. Biosci Biotechnol Biochem, 60:61-64.

Hirasawa R y Yokoigawa K (2001) Leavening ability of baker's yeast exposed to hyperosmotic media. FEMS Microbiol Lett, 194:159-162.

Hohmann S (1997) Shaping up: the response of yeast to osmotic stress. En: "Yeast Stress Responses". Hohmann S y Mager WH (eds). Springer New York, N.Y. pp:101-146.

Hohmann S (2002a) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev, 66:300-372.

Hohmann S (2002b) Osmotic adaptation in yeast control of the yeast osmolyte system. Int Rev Cytol, 215:149-187.

Izawa S, Sato M, Yokoigawa K y Inoue Y (2004) Intracellular glycerol influences resistance to freeze stress in *Saccharomyces cerevisiae*: analysis of a quadruple mutant in glycerol dehydrogenase genes and glycerol-enriched cells. Appl Microbiol Biotechnol, 66:108-114.

James SA, Collins MD y Roberts IN (1996) Use of an rRNA internal transcribed spacer region to distinguish phylogenetically closely related species of the genera *Zygosaccharomyces* and *Torulaspora*. Int J Syst Bacteriol. 46:189-194.

Kafadar KA, Zhu H, Snyder M y Cyert MS (2003) Negative regulation of calcineurin signaling by Hrr25p, a yeast homolog of casein kinase I. Genes Dev, 21: 2698-708.

Kafadar KA y Cyert MS (2004) Integration of stress responses: modulation of calcineurin signaling in *Saccharomyces cerevisiae* by protein kinase A. Eukaryot Cell 3:1147-53.

Kim J, Alizadeh P, Harding T, HefnerGravink A y Klionsky DJ (1996) Disruption of the yeast *ATH1* gene confers better survival after dehydration, freezing, and ethanol shock: potential commercial applications. Appl Environ Microbiol, 62:1563-1569.

Ko CH, Buckley AM y Gaber RF (1990) *TRK2* is required for low affinity K+ transport in *Saccharomyces cerevisiae*. Genetics 125:305-312.

Ko CH y Gaber RF (1991) *TRK1* and *TRK2* encode structurally related K+ transporters in *Saccharomyces cerevisiae*. Mol Cell Biol, 8:4266-4273.

Kuno T, Tanaka H, Mukai H, Chang CD, Hiraga K, Miyakawa T y Tanaka C (1991) cDNA cloning of a calcineurin B homolog in *Saccharomyces cerevisiae*. Biochem Biophys Res Commun, 180:1159-1163.

Lawrence CL, Botting CH, Antrobus R y Coote PJ (2004) Evidence of a new role for the high-osmolarity glycerol mitogen-activated protein kinase

pathway in yeast: regulating adaptation to citric acid stress. Mol Cell Biol, 24:3307-3323.

Linko YY, Javanainen P y Linko S (1997) Biotechnology of breadbaking. Trends Food Sci Technol, 8:339-344.

Liu Y, Ishii S, Tokai M, Tsutsumi H, Ohki O, Akada R, Tanaka K, Tsuchiya E, Fukui S y Miyakawa T (1991) The *Saccharomyces cerevisiae* genes (*CMP1* and *CMP2*) encoding calmodulina-binding proteins homologous to the catalytic subunit of mammalian protein phosphatase 2B. Mol Gen Genet, 227:52-59.

Luyten K, Albertyn J, Skibbe WF, Prior BA, Ramos J, Thevelein J y Hohmann S (1995) Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. EMBO J, 14:1360-1371.

Maeda T, Wurgler-Murphy SM y Saito H (1994) A two component system that regulates an osmosensing MAP kinase cascade in yeast. Nature, 369:242-245.

Maeda T, Takekawa M y Saito H (1995) Activation of yeast *PBS2* MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science, 269:554-558.

Marquez JA y Serrano R (1996) Multiple transduction pathways regulate the sodium extrusion gene *PMR2/ENA1* during salt stress in yeast. FEBS Lett, 382:89-92.

Marquez JA, Pascual-Ahuir A, Proft M y Serrano R (1998) The Ssn6-Tup1 repressor complex of *Saccharomyces cerevisiae* is involved in the osmotic induction of HOG-dependent and -independent genes. EMBO J, 17:2543-2553.

Matheos DP, Kingsbury TJ., Ahsan US y Cunningham KW (1997) Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression *in Saccharomyces cerevisiae*. Genes Dev, 11:3445-3458

Mazur P, Morin N, Baginsky W, el-Sherbeini M, Clemas JA, Nielsen JB y Foor F (1995) Differential expression and function of two homologous subunits of yeast 1,3-beta-D-glucan synthase. Mol Cell Biol, 15: 5671-81.

Mendizabal I, Rios G, Mulet JM, Serrano R y de Larrinoa IF (1998) Yeast putative transcription factors involved in salt tolerance. FEBS Lett, 1998 425: 323-328.

Mendizabal I, Pascual-Ahuir A, Serrano R y de Larrinoa IF (2001) Promoter sequences regulated by the calcineurin activated transcription factor Crz1 in the yeast *ENA1* gene. Mol Genet Genomics, 5: 801-811.

Mendoza I, Rubio F, Rodriguez Navarro A y Pardo JM (1994) The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae*. J Biol Chem, 269:8792-8796.

Mizunuma M, Hirata D, Miyahara K, Tsuchiya E y Miyakawa T (1998) Role of calcineurin and Mpk1 in regulating the onset of mitosis in budding yeast. Nature, 392:30-36.

Monfort A, Blasco A, Prieto JA y Sanz, P. (1997) Construction of baker's yeast strains that secrete different xylanolytic enzymes and their use in bread making. J Cereal Sci, 26:195-199.

Monfort A, Blasco A, Sanz P y Prieto JA (1999) Expression of *LIP1* and *LIP2* genes from Geotrichum species in Baker's yeast strains and their application to the bread-making process. J Agric Food Chem, 47:803-808.

Myers DK, Lawlor DTM y Attfield PV (1997) Influence of invertase activity and glycerol synthesis and retention on fermentation of media with sugar concentration by *Saccharomyces cerevisiae*. Appl Envirom Microbiol, 63:145-150.

Myers DK y Attfield PV (1999) Intracellular concentration of exogenous glycerol in *Saccharomyces cerevisiae* provides for improved leavening of frozen sweet doughs. Food Microbiol, 16: 45-51.

Nakagawa S y Ouchi K (1994) Construction from a single parent of baker's yeast strains with high freeze tolerance and fermentative activity in both lean and sweet doughs. Appl Environ Microbiol, 60:3499-3502.

Nakamura T, Liu Y, Hirata D, Namba H, Harada S, Hirokawa T y Miyakawa T (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, 11:4063-4071.

Nissen P, Nielsen D y Arneborg N (2004) The relative glucose uptake abilities of non *Saccharomyces* yeasts play a role in their coexistence with

Saccharomyces cerevisiae in mixed cultures. Appl Microbiol Biotechnol, 64:543-550.

Oda Y y Ouchi K (**1990)** Effect of invertase activity on the leavening ability of yeast in sweet dough. Food Microbiol, 7:241-248.

Oda Y y Tonomura K (1993) Selection of a novel baking strain from the *Torulaspora* yeasts. Biosci Biotech Biochem, 57:1320-1322.

Oda Y y Tonomura K (1995) Electrophoretic karyotyping of the yeast genus *Torulaspora*. Lett Appl Microbiol, 21:190-193.

Oda Y, Yabuki M, Tonomura K y Fukunaga M (1997) Reexamination of yeast strains classified as *Torulaspora delbrueckii* (Lindner). Int J Syst Bacteriol, 47:1102-1106.

O'Rourke SM y Herskowitz I (1998) The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in *Saccharomyces cerevisiae*. Genes Dev, 15:2874-2886.

O'Rourke SM, Herskowitz I y O'Shea EK (2002) Yeast go the whole HOG for the hyperosmotic response. Trends Genet, 18:405-412.

Oura E, Soumalainen H y Viskari R (1979) Breadmaking Econ Microbiol, 4:8714-8716.

Pascual-Ahuir A, Posas F, Serrano R y Proft M (2001) Multiple levels of control regulate the yeast cAMP-response element-binding protein repressor Sko1p in response to stress. J Biol Chem, 276:37373-37378.

Perez-Gonzalez JA, De Graaff LH, Visser J y Ramon D (1996) Molecular cloning and expression in Saccharomyces cerevisiae of two *Aspergillus nidulans* xylanase genes. Appl Environ Microbiol, 62:2179-82.

Philips J y Herskowitz I (1997) Osmotic balance regulates cell fusion during mating in *Saccharomyces cerevisiae*. J Cell Biol, 138:961-974.

Posas F, Wurgler-Murphy SM, Maeda T, Witten EA, Thai TC y Saito H (1996) Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the *SLN1-YPD1-SSK1* "two component" osmosensor. Cell, 86:865-875.

Posas F y Saito H (1998) Activation of the yeast SSK2 MAP kinase kinase kinase by the SSK1 two component response regulator. EMBO J, 17:1385-1394.

Posas F, Witten EA y Saito H (1998) Requirement of *STE50* for osmostress-induced activation of the STE11 mitogen-activated protein kinase kinase kinase in the high-osmolarity glycerol response pathway. Mol Cell Biol, 18:5788-5796.

Posas F, Chambers JR, Heyman JA, Hoeffler JP, de Nadal E y Arino J (2000) The transcriptional response of yeast to saline stress. J Biol Chem, 275:17249-17250.

Pozos TC, Sekler I y Cyert MS (1996) The product of *HUM1*, a novel yeast gene, is required for vacuolar Ca2+/H+ exchange and is related to mammalian Na+/Ca2+ exchangers. Mol Cell Biol, 7:3730-3741.

Proft M y Serrano R (1999) Repressors and upstream repressing sequences of the stress regulated *ENA1* gene in *Saccharomyces cerevisiae*: bZIP protein Sko1p confers HOG dependent osmotic regulation. Mol Cell Biol, 19:537-546.

Proft M y Struhl K (2002) Hog1 kinase converts the Sko1Cyc8Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. Mol Cell, 6:1307-1317.

Raitt DC, Posas F y Saito H (2000) Yeast Cdc42 GTPase and Ste20 PAK-like kinase regulate Sho1-dependent activation of the Hog1 MAPK pathway. EMBO J, 17:4623-4631.

Ramos J, Haro R y Rodriguez-Navarro A (1990) Regulation of potassium fluxes in *Saccharomyces cerevisiae*. Biochim Biophys Acta. 1029:211-217.

Ramos J, Alijo R, Haro R y Rodriguez Navarro A (1994) *TRK2* is not a low affinity potassium transporter in *Saccharomyces cerevisiae*. J Bacteriol, 176:249-252.

Rández-Gil F, Sanz P y Prieto JA (1999) Engineering baker´s yeast: room for improvement. Trends Biotechnol, 17:237-244.

Rández-Gil F, Aguilera J, Codón A, Rincón AM, Estruch F y Prieto JA (2003) Baker´s yeast: challenges and future prospects. In Functional genetics of industrial yeast.Edited by J.H. de Winde. SpringerVerlag Berlin: 57-97.

Reiser V, Ruis H y Ammerer G (1999) Kinase activity dependent nuclear export opposes stress induced nuclear accumulation and retention of

Hog1 mitogen activated protein kinase in the budding yeast *Saccharomyces cerevisiae*. Mol Biol Cell, 10:1147-1161.

Reiser V, Salah SM y Ammerer G (2000) Polarized localization of yeast Pbs2 depends on osmostress, the membrane protein Sho1 and Cdc42. Nat Cell Biol, 9:620-627.

Remize F, Barnavon L y Dequin S (2001) Glycerol export and glycerol 3 phosphate dehydrogenase, but not glycerol phosphatase, are rate limiting for glycerol production in *Saccharomyces cerevisiae*. Metab Eng, 4:301-312.

Rep M, Reiser V, Gartner U, Thevelein JM, Hohmann S, Ammerer G y Ruis H (1999a) Osmotic stress sinduced gene expression in *Saccharomyces cerevisiae* requires Msn1p and the novel nuclear factor Hot1p.Mol Cell Biol, 19:5474-5485.

Rep M, Albertyn J, Thevelein JM, Prior BA y Hohmann S (1999b) Different signalling pathways contribute to the control of *GPD1* gene expression by osmotic stress in *Saccharomyces cerevisiae*. Microbiology, 145:715-727.

Rep M, Krantz M, Thevelein JM y Hohmann S (2000) The transcriptional response of *Saccharomyces cerevisiae* to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway dependent genes. J Biol Chem, 275:8290-8300.

Rios G, Ferrando A y Serrano R (1997) Mechanisms of salt tolerance conferred by overexpression of the *HAL1* gene in *Saccharomyces cerevisiae*. Yeast, 13:515-528.

Rodriguez-Vargas S (2003) Caracterización de la respuesta a estrés por frío en levaduras de panadería. Tesis Doctoral (Universidad de Valencia)

Rusnak F y Mertz P (2000) Calcineurin: form and function. Physiol Rev, 4:1483-1521.

Sakamoto T y Murata N (2002) Regulation of the desaturation of fatty acids and its role in tolerance to cold and salt stress. Curr Opin Microbiol, 5:208-210.

Sales K, Brandt W, Rumbak E y Lindsey G (2000) The LEAlike protein HSP12 in *Saccharomyces cerevisiae* has a plasma membrane location and

protects membranes against desiccation and ethanol induced stress. Biochim Biophys Acta, 1463:267-278.

Sasaki T y Ohshima Y (1987) Induction and characterization of artificial diploids from the haploid yeast *Torulaspora delbrueckii*. Appl Environ Microbiol, 53:1504-1511.

Serrano R (1996) Salt tolerance in plants and microorganisms: toxicity targets and defense responses. Int Rev Cytol, 165:1-52.

Serrano R, Márquez JA y Ríos G (1997) Crucial factors in salt stress tolerance. In: Hohmann S & Mager WH (Ed) Yeast Stress Responses, (pp 147-169). Springer, New York.

Shima J, Hino A, YamadaIyo C, Suzuki Y, Nakajima R, Watanabe H, Mori K y Tacano H (1999) Stress tolerance in doughs of *Saccharomyces cerevisiae* trehalase mutants derived from commercial baker's yeast. Appl Environ Microbiol, 65: 2841-2846

Smith DA, Nicholls S, Morgan BA, Brown AJ y Quinn J (2004) A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans.* Mol Biol Cell, 15:4179-4190.

Stathopoulos AM y Cyert MS (1997) Calcineurin acts through the *CRZ1/TCN1* encoded transcription factor to regulate gene expression in yeast. Genes Dev, 24:3432-3444.

Stathopoulos-Gerontides A, Guo JJ y Cyert MS (1999) Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation. Genes Dev, 13:798-803.

Takagi H, Iwamoto F y Nakamori S (1997) Isolation of freeze tolerant laboratory strains of *Saccharomyces cerevisiae* from praline analogue resistant mutants. Appl Microbiol Biotechnol, 47:405-411.

Tamas MJ, Luyten K, Sutherland FC, Hernandez A, Albertyn J, Valadi H, Li H, Prior BA, Kilian SG, Ramos J, Gustafsson L, Thevelein JM y Hohmann S (1999) Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. Mol Microbiol, 31:1087-110.

Tanghe A, Van Dijck P, Dumortier F, Teunissen A, Hohmann S y Thevelein JM (2002) Aquaporin expression correlates with freeze

tolerance in baker's yeast, and overexpression improves freeze tolerance in industrial strains. Appl Environ Microbiol, 68:5981-5989.

Tao W, Deschenes RJ y Fassler JS (1999) Intracellular glycerol levels modulate the activity of Sln1p, a *Saccharomyces cerevisiae* two component regulator. J Biol Chem, 274:360-367.

Teunissen A, Dumortier F, Gorwa MF, Bauer J, Tanghe A, Loiez A, Smet P, Van Dijck P y Thevelein JM (2002) Isolation and characterization of a freeze tolerant diploid derivative of an industrial baker's yeast strain and its use in frozen doughs. Appl Environ Microbiol, 68:4780-4787.

Thevelein JM y Hohmann S (1995) Trehalose synthase: guard to the gate of glycolysis in yeast?. Trends Biochem Sci, 20:3-10.

Van Dijck P, Gorwa MF, Lemaire K, Teunissen A, Versele M, Colombo S, Dumortier F, Ma P, Tanghe A, Loiez A y Thevelein JM (2000) Characterization of a new set of mutants deficient in fermentation-induced loss of stress resistance for use in frozen dough applications. Int J Food Microbiol, 55: 187-192.

Vaughan Martini A y Martini A (1996) Isolation, purification, and analysis of nuclear DNA in yeast taxonomy. Methods Mol Biol, 53:89-102.

Westfall PJ, Ballon DR y Thorner J (2004) When the stress of your environment makes you go HOG wild. Science, 5701:1511-1512

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

Wieland J, Nitsche AM, Strayle J, Steiner H y Rudolph HK (1995) The *PMR2* gene cluster encodes functionally distinct isoforms of a putative Na+ pump in the yeast plasma membrane. EMBO J, 14:3870-3882

Winkler A, Arkind C, Mattison CP, Burkholder A, Knoche K y Ota I (2002) Heat stress activates the yeast high-osmolarity glycerol mitogen-activated protein kinase pathway, and protein tyrosine phosphatases are essential under heat stress. Eukaryot Cell, 2: 163-173.

Yale J y Bohnert HJ (2001) Transcript expression in *Saccharomyces cerevisiae* at high salinity. J Biol Chem, 276:15996-16007.

Yoshimoto H, Saltsman K, Gasch AP, Li HX, Ogawa N, Botstein D, Brown PO y Cyert MS (2002) Genome wide analysis of gene expression regulated by the calcineurin/Crz1p signaling pathway in *Saccharomyces cerevisiae*. J Biol Chem, 277:31079-31088.