Phenotypic and Genetic Characterization of *Debaryomyces hansenii* Strains Exposed to Cobalt and Saline Stress

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN MARINE SCIENCES
BIOLOGICAL OCEANOGRAPHY

UNIVERSITY OF PUERTO RICO
MAYAGÜEZ CAMPUS
2012

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Abstract

Debaryomyces (*Torulaspora*) *hansenii*, an osmotolerant yeast of mostly marine origin, has become of keen interest for physiological, metabolic, genetic and molecular studies due to its versatility to survive and thrive under conditions that may be unbearable for other microorganisms. Having been isolated from several habitats that exhibit low water activity, high sugar content, limited carbon sources, high salt concentrations, *D. hansenii* is a model organism with appealing characteristics for studies ranging from environmental stresses, industrial metabolic capabilities, ecological implications and biotechnological applications. However, the analyses of various *D. hansenii* strains upon exposure to metal (oxidative) and saline (osmotic) stress, and whether a direct correlation with genotype can be postulated, has not yet been addressed. Therefore, the objectives of this study consisted of: (i) determining the potential of *D. hansenii* as a biosensor of metal contaminated environments using riboflavin (RF) as an indicator and the description of a RF-impaired mutant, (ii) evalulating the phenotypic diversity of over 30 *D. hansenii* strains was upon exposure to cobalt (oxidative) and saline (osmotic) stress over a range of concentrations by growth pattern and RF production, and (iii) profiling the genotype of the functional and inducible genes *FET3* and *ENA1*, which have been associated with cobalt and salt stress, using restriction fragment length polymorphism (RFLP) and subsequent analysis on the possible effect of base substitutions on the predicted translated products.

Growth and RF production were compared between wild-type *D. hansenii* and a RF production-impaired metal-tolerant ura3 mutant in the presence of sublethal cobalt(II) concentrations. *Debaryomyces hansenii* J26 (wild type) exhibited an extended lag phase with an increase in RF synthesis. Supplementation of exogenous uracil shortened the lag phase at the highest concentration of cobalt(II) used, suggesting that uracil has a possible role in metal acclimation. The *D. hansenii* ura3^{-} mutant exhibited a higher level of metal tolerance, no extended lag phase, and no marked increase in RF synthesis. Restoration of uracil phenotype by transformation with *URA3* from *Saccharyomyces cerevisiae* or *D. hansenii* did not restore wild-type characteristics, suggesting a second mutation that impairs RF oversynthesis. These results demonstrate that growth, metal sensitivity, and RF biosynthesis are linked.

Thirty four *D. hansenii* strains isolated from various sources were submitted to cobalt and saline stress to evaluate phenotypic variation by colony growth on agar nutrient media plates supplemented with different concentrations of cobalt(II) and NaCl, respectively. Visible RF production was also monitored for strains exposed to cobalt(II). The results demonstrated that no direct pattern of phenotypic variation among the strains could be correlated to the source of isolation. Upon cobalt stress, most strains (~65%) were described as either highly tolerant (dense growth at 1.5 mM) or tolerant (slightly reduced growth at 1.5 mM Co(II)). Very few (three) strains were characterized as being sensitive. Four different strains produced substantial extracellular RF. When exposed to salt stress, no strains were considered highly tolerant but were mostly described as tolerant (~67%; dense growth up to 2 M NaCl). Overall, the results demonstrate mounting evidence that individual strains may express distinct phenotypes, which may depend on different genetic and molecular mechanisms involved in surviving under harsh conditions.

To profile for genotypic heterogeneity among the 34 *D. hansenii* strains analyzed for phenotypic variability, the DNA sequences of the multicopper oxidase gene *FET3* and the P-type ATPase sodium efflux gene *ENA1* were analyzed using RFLP. Amino acid sequence comparisons of the predicted translated products of each gene were also analyzed to identify conserved regions or domains that may have suffered from base substitutions as detected by RFLP. The results obtained from the *FET3* RFLP profiling revealed six distinct genotype patterns, excluding the type sequence pattern of *D. hansenii* CBS 767^{T}. On the other hand, the *ENA1* RFLP analysis discriminated two patterns that varied from the pattern exhibited by the type strain. Amino acid sequence comparison of the *FET3* predicted translated product of CBS 767^{T} and possible residue changes based on the RFLP analysis revealed that most regions important for ligands were conserved although changes in residues were found close to these areas and in putative transmembrane helices, which may affect its catalytic activity. Results of the comparison of the putative transmembrane helices identified in the *ENA1* predicted translated product showed that at least 3 out of 10 helices may be affected by changes in residues and, thus in conformational structure.

To summarize, the observations in this dissertation provide substantial evidence to support *D. hansenii* as a model organism for understanding the complexity involved in

physiological and metabolic processes of higher level organisms and requires an integrated approach that involves further genomic, transcriptomic, and proteomic analyses.

Resumen

Debaryomyces (Torulaspora) hansenii, una levadura osmotolerante de origen mayormente marina, ha sido de gran interés para los estudios fisiológicos, metabólicos, genéticos y moleculares, debido a su versatilidad en sobrevivir y prosperar en las condiciones que pueden ser insoportable para otros microorganismos. Al ser aislado de varios hábitats que presentan baja actividad de agua, contenido alto de azúcar, fuentes limitadas de carbono, altas concentraciones de sal, *D. hansenii* es un organismo modelo con características atractivas para los estudios desde cambios o estrés ambientales, sus capacidades metabólicas para la industria, implicaciones ecológicas y aplicaciones biotecnológicas. Sin embargo, los análisis de las diversas cepas de *D. hansenii* tras la exposición al estrés por metal (oxidativo) y la sal (osmótica), y si una correlación directa con el genotipo puede ser postulado, aún no se ha reportado. Por lo tanto, los objetivos del presente estudio consistió en: (i) determinar el potencial de *D. hansenii* como un biosensor de ambientes contaminados por metales usando riboflavina (RF) como un indicador y la descripción de un mutante deficiente de RF, (ii) evaluar la diversidad fenotípica de más de 30 cepas de *D. hansenii* al ser expuesto al estrés celular por el cobalto (oxidativo) y la sal (osmótico) a varias concentraciones mediante el patrón de crecimiento y la producción de RF, y (iii) crear un perfil genotipíco de los genes funcionales e inducibles *FET3* y *ENA1*, los cuales han sido asociados al estrés celular causado por el cobalto y el sal, utilizando los polimorfismos en la longitud de los fragmentos de restricción (RFLP) y subsecuentemente un análisis sobre el posible efecto de sustituciones de bases en la predicción de los productos traducidos en proteína.

El crecimiento y la producción de RF se compararon entre una cepa salvaje de *D. hansenii* y la producción de RF alterada de un mutante ura3^{-} tolerante al metal en presencia de concentraciones subletales de cobalto (II). *Debaryomyces hansenii* J26 (cepa salvaje) exhibió una fase de retraso en crecimiento prolongado con un aumento en la síntesis de RF. La suplementación de uracilo exógeno acortó la fase de retraso a la mayor concentración de cobalto (II), lo que sugiere que uracilo tiene un posible rol en la aclimatación por metal en la célula. El mutante ura3^{-} de *D. hansenii* exhibió un mayor nivel de tolerancia a los metales, sin fase de retraaso prolongado, y no aumentó substancialmente la síntesis de RF. Restauración del fenotipo de síntesis de uracilo por transformación con *URA3* de *Saccharyomyces cerevisiae* o *D. hansenii*

no restauró las características de la cepa salvaje, lo que sugiere una segunda mutación que afecta la sobreproducción de RF. Éstos resultados demuestran que el crecimiento, la sensibilidad al metal, y la biosíntesis de RF están vinculados.

Treinta cuatro cepas de *D. hansenii* aisladas de diferentes fuentes fueron sometidos al estrés celular por el cobalto y la sal para evaluar la variación fenotípica por medio del crecimiento de las colonias sobre placas de agar con medios nutritivos suplementados con diferentes concentraciones de cobalto(II) y NaCl, respectivamente. Producción visible de RF se observó también para las cepas expuestos a cobalto(II). Los resultados demostraron que ningún patrón específico de variación fenotípica entre las cepas podría ser correlacionada con la fuente de aislamiento. Con el estrés de cobalto, la mayoría de las cepas (~65%) se describieron como altamente tolerante (un crecimiento denso en 1.5 mM Co(II)) o tolerante (crecimiento reducido ligeramente en 1.5 mM). Muy pocos (tres) de las cepas se caracterizó como sensible. Cuatro cepas diferentes producieron niveles sustanciales de RF extracelular. Al exponerlos al estrés salino, ninguna cepas fue considerada altamente tolerante, pero la mayoría fueron describidos como tolerante (~67%, un crecimiento denso hasta 2 M NaCl). En general, los resultados demuestran un aumento en la evidencia que apoya que las cepas individuales pueden expresar fenotipos distintos, que pueden depender de diferentes mecanismos genéticos y moleculares implicados en la supervivencia de condiciones adversas.

Para crear un perfil de la heterogeneidad genotípica entre las 34 cepas de *D. hansenii* analizadas para variabilidad fenotípica, las secuencias de ADN de los genes que codifican para la oxidasa de multicobre *FET3* y la ATPasa tipo-P del eflujo de sodio *ENA1* fueron analizados utilizando RFLP. Comparaciones de secuencias de aminoácidos de los productos traducidos en proteína de cada gen también se analizaron para identificar regiones conservadas o dominios que podrían sufrir alguna sustitución de base detectada por RFLP. Los resultados obtenidos del perfil de *FET3* reveló seis patrones de genotipos distintos, excluyendo el patrón de secuencia de la cepa tipo *D. hansenii* CBS767^{T}. Por otro lado, el análisis de *ENA1* discriminó dos patrones que variaban del patrón exhibido por la cepa tipo. La comparación de la secuencia de aminoácidos del producto traducido de *FET3* de *D. hansenii* CBS767^{T} y los posibles cambios en los residuos basadas en el análisis de RFLP reveló que la mayoría de las regiones más importantes para los ligandos se conservaron a pesar de cambios encontrados cerca de éstas áreas y en hélices con transmembrana putativos, los cuales pueden afectar su actividad catalítica. Los resultados de la

comparación de las hélices transmembrana putativos identificados en el producto traducido mostró que por lo menos 3 de cada 10 hélices podrían afectar su estructura conformacional.

En resumen, las observaciones en esta tesis doctoral proporcionan evidencia sustancial para apoyar a *D. hansenii* como un organismo modelo para entender la complejidad de los procesos fisiológicos y metabólicos de los organismos de nivel superior y requiere un enfoque mas integrado que involucra análisis genómicos, transcriptómicas y proteómicas.

Copyright

Dedication

I would like to dedicate this work to the person who always encouraged me to never give up on myself, my mother.

Thank you for your unconditional love, undying support, relentless encouragement and lasting care.

Commit to the Lord whatever you do,
and your plans will succeed.

Proverbs 16:3
(Holy Bible, New International Version)

Acknowledgments

I would like to express my appreciation to all of the people who have been involved in making this dissertation possible. First, I would like to thank the president of my committee, Dr. Govind Nadathur, for his support and mentorship throughout the course of my graduate career. Thank you for opening the doors of your laboratory to an aspiring scientist. I will always remember your words when trying to find a sensible solution to a complex problem, "The reason is very simple…" I am also grateful to the other graduate committee members Dr. Michael Cotta, Dr. Jorge Corredor, and Dr. Arup Sen for their help and revision of this manuscript. Special thanks to Dr. Sen for his assistance in providing the oligonucleotides (primers) as a gift for this dissertation.

I convey my sincerest gratitude to the members and alumni of the Molecular Marine Biology Laboratory (MMBL), my extended family. Thanks to William Rosado, faithful friend and lab companion, for your words of encouragement and endearment ("A carrucho is a carrucho…"). Thanks to Edna Justiniano for your enthusiasm, for providing DNA sequences that helped me with my analyses, and for encouraging me to seek the truth in science. Thanks to Dr. Ana Pérez-Matos for your help and revision with the first chapter of this manuscript. My appreciation to my fellow students and lab technicians for sharing their friendship and for making MMBL a place to enjoy learning molecular biology.

I would like to thank the administrative staff and faculty of the Department of Marine Sciences. Thanks to Monserrate Casiano (Taty), Zulma Martínez, Lilivette Valle, and Nilda E. Ramírez for never denying their help with my paperwork and for always sharing a smile. Thanks to the faculty members for their insights and challenges throughout the course of my academic career. I would also like to thank my fellow graduate students of the Department for sharing their friendship and for making Isla Magüeyes unique in all of UPR-Mayagüez.

I would also like to express my appreciation to the UPR – Sea Grant Program in Mayagüez. Thanks to Mr. Ruperto Chaparro, Dr. Manual Valdés-Pizzini, and Dr. Kurt Grove for your financial support during the process of this dissertation and for partially financing my trip to present preliminary data in the *2010 Yeast Genetics & Molecular Biology Meeting* in Vancouver, Canada. I would also like to thank you for giving me the opportunity to be a part of the

Caribbean Regional Research Plan (Assessment). It is a wonderful project that allowed me to expand my skills beyond those of a lab scientist. In addition, thanks to Migdalia Figueroa, María Matos, and Yulissa García for all of your help with paperwork and administrative processes (nothing would get done without all of you).

Finally, I would like to show my deepest gratitude to God for allowing me to fulfill one of my life's goals and for giving me the health to see it through to the end. I would also like to thank my wonderful mother Priscilla Miró for her patience and undying love during the progress of this work, which reflects her endurance and care for me throughout the years. Special thanks to Dr. Nancy Arroyo, my kindred spirit and advisor, for being a part of the process and for always encouraging me to do my best. I must also thank my beloved and dearest David Zapata for his help with the photo editing and for showing me that love can be found in the most difficult of times.

This research was partially funded by the National Institutes of Health - Minority Biomedical Research Support-SCORE Program (S06-08103) and the Alliance for Graduate Education and the Professoriate Fellowship Program (2008-2010).

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Chapter 1: General Introduction

Increasing interest has developed in the extremophilic hemiascomycetous yeast *Debaryomyces* (*Torulaspora*) *hansenii* (Zopf) Lodder et Kreger van Rij (occasionally referred to as *Candida famata* in its anamorph state and because of its genetic similarity to *D. hansenii* var. *hansenii*[48]) due to its expected wealth of biochemical pathways and biotechnological applications. *D. hansenii*, a versatile and highly heterogeneous species, has been isolated from many habitats that include marine fish, seawater, seabird feces, cheese, meat, wine, beer, fruit, soil, high-sugar products, and the digestive tract of a German cockroach [7,13,18,20,50,62,74,80]. Its ability to thrive under such conditions, which mostly constitute low water activity, makes *D. hansenii* an attractive model microorganism for biotechnological applications based on several physiological, ecological, and molecular characteristics [11]. These include: (i) the ability to synthesize various lipolytic and proteolytic enzymes [40,53,75], (ii) the ability to assimilate and ferment several carbon sources [17,47,49,65], (iii) the ability to synthesize, accumulate, and convert lipids [59,60], (iv) the capability to withstand high levels of microbial toxins that may be mediated by active efflux proteins and to produce killer toxins that affect other microbes [32,57,58], and (v) the ability to grow and adapt in media under high osmotic stress [13,31,55]. In 2004, the Génolevures Consortium, an internationally renowned group of laboratories and research centers coordinated by the French National Center for Scientific Research (Centre National de la Recherche Scientifique) with the goal of performing large-scale comparative genomics of the Hemiascomycetous yeast phylum, chose *D. hansenii* strain CBS 767 to be completely sequenced because of its halotolerance and relation to other pathogenic yeasts [24].

The purpose of this dissertation was to describe the phenotypic and genetic characterization of *D. hansenii* strains when exposed to particular factors based on previous reports of its tolerance to conditions that would be considered rather extreme for most microorganisms [2,13,14,37,41]. Based on this main premise, this dissertation is divided into three chapters (Chapters 2, 3 and 4) that are consistent with the style and format of scientific publications. The main objective of each section was: (i) to elucidate the potential use of a *D. hansenii* strain as a biosensor for metal contaminated environments using riboflavin production as an indicator and to describe the contrasting physiological properties of a mutant with the

characteristics of the wild-type strain, (ii) to evaluate the phenotypic diversity of 34 *D. hansenii* strains cultured under environmental stress (i.e., Co(II) and NaCl, respectively), and (iii) to determine the genetic variability of these strains using two functional and inducible genes *FET3* and *ENA1*, which have been associated with Co(II) and NaCl stress in yeast [30,69]. The final section of this dissertation (Chapter 5) includes an overall conclusion of the observations and suggestions for future research prospects for *D. hansenii*.

The study performed in Chapter 2 was based on the characterization of *D. hansenii* as a flavinogenic yeast due to its ability to overproduce riboflavin (vitamin B2), which is visible as a yellow pigment [29,70]. Several genes coding for specific enzymes involved in riboflavin (RF) synthesis have been isolated and sequenced in the anamorph *C. famata* [78]. The increase of RF overproduction in the presence of heavy metals (e.g., Cr(VI), Co(II), and Zn(II)) has suggested that its synthesis can be regulated by these elements [28,29]. Particularly, Co(II) and other toxic metals has been shown to increase RF production of *D. hansenii* [29,73]. Straube [79] (cited by 71) suggests that RF helps to normalize the metabolism of cells by maintaining a favorable level of redox potential due to its ability to chelate metal ions, such as iron, and is known to reduce Fe(III) to Fe(II) in other microbes (e.g., *Helicobacter pylori*) so as to promote iron transport into the cell. Several genes (*RIB80*, *RIB81*, *RIB83*, *RIB84*, *HIT1*, and *RED1*) associated with RF regulation and iron uptake have been identified from the yeast *Meyerozyma (Pichia) guilliermondii*, an hemiascomycete belonging to the same yeast family (Debaryomycetaceae) as *D. hansenii* [27,66-68,71,72]. Cobalt excess or iron deficiency in RF-overproducing and iron-accumulating mutants of *M. guilliermondii* has suggested a state of oxidative stress [10]. In *C. famata*, the introduction of several orthologous copies of transcription factors and homologous genes coding for enzymes of the RF pathway from *D. hansenii* resulted in a RF-overproducing strain [24]. These studies have helped us to understand various dynamics of RF production and its regulation in these yeast species; however, some mechanisms remain unclear.

Based on this information, Chapter 2 is a physiological characterization of the flavinogenic and growth properties of *D. hansenii* when exposed to cobalt stress, which mimics hypoxic conditions [12,29,34]. The purpose of this chapter was to to determine the survival of the wild-type strain *D. hansenii* J26 when exposed to varying $CoSO_4$ (Co(II)) concentrations. A *D. hansenii* cobalt resistant *ura3* mutant (J26-1; unable to synthesize uracil) that did not overproduce RF, contrary to the wild-type characteristics of J26, was also studied. This

investigation was conducted by monitoring growth patterns and quantifying RF production in the presence and absence of supplemented uracil. The observations obtained from this study may serve as a foundation for the potential use of *D. hansenii* as a biosensor using RF as an indicator of metal contaminated or hypoxic environments.

In continuation, the study described in Chapter 3 was performed mostly due to reports on the phenotypic (or physiological) variations in metabolomic assimilation of carbon sources and maximum growth temperature among strains of *D. hansenii* [22,47]. Reports of its ubiquitous nature in various environments [see 11 for a review; 30] have led to the phenotypic identification and description of *D. hansenii* isolates [46,54,55]. Several pathogenic *Candida* species, including *C. famata* (anamorph form of *D. hansenii*), have also been assessed for oxidative sensitivity from the formation of reactive oxygen species and have revealed substantial variability [1]. Variations in phenotype characteristics to different environmental conditions have also been observed in strains of the model yeast species *Saccharomyces cerevisiae* and have been associated with similarities and differences in yeast habitats and in the variability of expressing genes linked to stress resistance [43]. In contrast, the phenotypic diversity of some strains of *D. hansenii* has not been successfully characterized using conventional methods and has resulted in the use of molecular techniques to indicate instraspecific variability [35,36].

The main objective of Chapter 3 was to assess the phenotypic (or physiological) variability of 34 *D. hansenii* strains obtained from different sources of isolation by determining their survival when exposed to metabolically demanding conditions, such as oxidative (or metal) and salt stress, based on the study performed in Chapter 2. This research was conducted by culturing strains in varying concentrations of Co(II) and NaCl for a specific incubation period and were monitored for colony growth and possible RF production when exposed to Co(II). The results from this study provide mounting evidence for the phenotypic diversity of *D. hansenii* strains in relation to their tolerance to stressful environments.

Phenotypic variations, as described in Chapter 3, in response to oxidative (or cobalt) and salt concentrations prompted the analysis of DNA sequence variability in genes associated with these stressful conditions. Although several genes have been correlated to cobalt stress [19,25, 38,45,77], a genome-wide analysis of cobalt detoxification in *S. cerevisiae* revealed that most genes linked to the iron regulon were highly induced at 2 mM Co(II) after 1.5 h of exposure [69]. Of the genes associated with this regulon, the *FET3* gene (ferroxidase or multicopper oxidase) is

involved in the indirect iron uptake by ferroxidation of ferrous iron (Fe^{+2}) following reduction of the insoluble form of ferric iron (Fe^{+3}) by ferrireductases [see 39 for a review]. *FET3* and its homologs among yeast species are considered one of the major components of the high-affinity iron transport system [3,4,16,21,26,53]. Homologous studies on the amino acid sequence of the *FET3* translated product (Fet3p) have revealed sequence conservation and variability that may affect its transport mechanism and enzymatic activity mostly in *S. cerevisiae*, *Schizosaccharomyces pombe* and *Pichia pastoris* [8,9,23,53]. The functional characterization of CaFet34p, one of five candidate ferroxidases in *C. albicans*, was described and successfully expressed in *S. cerevisiae* [79]. However, comparisons of *D. hansenii* Fet3p with the model yeast *S. cerevisiae* and phylogenetically similar yeast *C. albicans* [42] have not been reported.

With regard to osmotic (or saline) stress, *D. hansenii* is well known for its ability to tolerate concentrations up to 25% w/v NaCl [13,44,50]. The accumulation of low molecular weight metabolites, such as glycerol, and cell volume reduction have been considered important mechanisms for the osmotic adjustment of *D. hansenii* [33,76]. The physiological mechanism by which *D. hansenii* tolerates such high levels of salts, such as Na^+, K^+ and Li^+, was also elucidated and compared with *S. cerevisiae*, suggesting that Na^+ substitutes K^+ when K^+ is limited and that sodium protein targets are less susceptible to such conditions [52,55,56]. Although the *NHA1* and *KHA1* has been identified as the encoding gene for the H^+/Na^+ antiporter for extrusion of H^+/Na^+ and K^+ cations in *S. cerevisiae* and *D. hansenii*, respectively, [5,6,15], the P-type ATPase pump encoded by *ENA1* has been suggested as the major player in the detoxification of sodium [30,63]. A genome-wide expression profile of *D. hansenii* CBS 767 revealed that *ENA1* reached peak transcription levels after 3 h of exposure to 2 M NaCl [30]. Although P-type ATPases has been discovered in several organisms and is considered an important player in Na^+, K^+ and H^+ transport [see 61 for a review], a comparison of the homology of the amino acid sequence of *ENA1* among ascomycetes has not been analyzed.

Therefore, the main objective of Chapter 4 was to study the genetic heterogeneity of the iron transport gene *FET3* and the P-type ATPase sodium efflux gene *ENA1* of 34 *D. hansenii* strains from various culture collections using restriction fragment length polymorphism (RFLP), which is a method that identifies and cuts DNA fragments based on specific sequences depending on the type of restriction enzyme used [64]. In addition, an analysis of potential

changes in conserved regions or domains of the predicted translation products of *D. hansenii* Fet3p and Ena1p due to DNA sequence variations revealed in other strains is included.

In summary, the primary goal of this dissertation was to provide a phenotypic and genotypic profile of several *D. hansenii* strains exposed to environmental stress factors that may be considered extreme for other microorganisms. Thus, Chapter 5 is a synopsis of the data presented here and discusses the possible explanations that may elucidate the variations portrayed by these strains. In addition, this data will also complement further research conducive towards comparative and functional genomic or transcriptomic sequence analyses in *D. hansenii*.

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Chapter 2: Impairment of Cobalt-Induced Riboflavin Biosynthesis in a *Debaryomyces hansenii* Mutant

2.1 Introduction

Overproducers of riboflavin (RF) include yeasts from the genus *Candida* [1,12,20,41], *Pichia* [27,35] and *Debaryomyces* [2,19]. Within the *Debaryomyces* spp., *Debaryomyces hansenii* is considered an emerging biotechnological model for yeast systems and has been used industrially for RF overproduction [10,31,36]. RF oversynthesis by *D. hansenii* can be modulated by metals at concentrations toxic to most microorganisms, although the mechanisms of regulation have not been completely elucidated [19,40].

The repression of RF production by iron in flavinogenic yeast [32,41] is well-known, but the mechanisms at the molecular level are still not clear. Several structural RF biosynthesis genes (*RIB* genes) have been isolated from *Candida famata* VKM Y-9 and found to complement RF auxotrophy of another yeast strain, *Pichia guilliermondii* [13,41,42]. Analysis of the upstream regions of several *C. famata* and *D. hansenii RIB* genes revealed putative iron-responsive elements that may be considered potential binding sites of transcription factors involved in iron transport [43].

In contrast to iron, other metals such as Cr(VI), Zn(II), and Co(II) can induce RF synthesis in flavinogenic yeasts [18,19]. It has been suggested that the presence of metals (i.e., Co(II) and Zn(II)) may result in a competition with the iron metabolism of the cell and thereby increase RF production to compensate for the iron deficiency [19]. Although iron transport is not affected by the presence of other metal ions (i.e., Cr(VI)), the oxidation of these metal ions by metabolic intermediates has been considered to account for the local iron deficit resulting in RF oversynthesis in some yeast species [24]. Other studies of flavinogenic yeasts have suggested that extensive flavinogenesis is a resistance mechanism that helps provide for cell survival in the presence of metal [17]. *Pichia guilliermondii* mutants with RF-overproducing and high iron-accumulating properties cultivated under conditions of cobalt excess or iron deficiency suggested a correlation between RF overproduction, iron assimilation, and oxidative stress as evidenced by the production of malondialdehyde [8]. A comparison between flavinogenic and non-

flavinogenic *P. guilliermondii* strains suggested an increased metal tolerance with elevated RF production [24]. *Pichia guilliermondii* mutants have also shown changes in metal sensitivity associated with RF production [7,38]. *Candida famata* RF-overproducing mutants resistant to increased metal concentrations have also been reported [22], but no known mutants of *D. hansenii* associated with metal sensitivity and RF oversynthesis have been described.

The purpose of this study was to evaluate the potential of *D. hansenii* as a biosensor for metal-contaminated environments based on its physiological response. We also report a *D. hansenii* cobalt-resistant *ura3* mutant that does not overproduce RF in contrast to wild-type characteristics of this strain. The behavior of these *D. hansenii* strains in the presence of Co(II) demonstrates an important link among growth, RF production, and metal sensitivity.

2.2 Materials and Methods

2.2.1 Strains and maintenance. *Escherichia coli* XL1 Blue *(Δ*mcrA*) 183,* Δ*(mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, recA, gyrA96, reA1, lac,* λ^-) (Stratagene) was used for plasmid propagation and amplification. *Debaryomyces hansenii* strains used were J26 (wild-type) received as a gift from Dr. L. Adler (University of Göteborg, Sweden) and NRRL Y-7426 (wild-type) obtained from Dr. C. Kurtzman (National Center for Agriculture Utilization Research, US Department of Agriculture, Peoria, Ill.). Strain J26-1 (*ura3)* was isolated by mutagenizing strain J-26 with the alkylating agent *N*-methyl-*N*′-nitro-N-nitrosoguanidine as described by [25] and selected by the positive selection technique utilizing 5-fluoro-orotic acid [6].

Escherichia coli cultures were grown at 37°C with shaking in Luria-Bertani broth (1% tryptone, 0.5% yeast-extract, 1% NaCl, 100 µg/mL ampicillin). *Escherichia coli* strain XL1 Blue and *D. hansenii* J26-1 were transformed by electroporation as described by [3]. Yeast strains were grown at 30°C in yeast-peptone-dextrose (YPD) broth or synthetic complete (SC) broth (2% dextrose, 0.67% yeast nitrogen base without amino acids, 0.02% amino acid mix with 50 µg/mL uracil). Yeast transformants were selected in synthetic dextrose (SD) medium (2% dextrose, 0.67% yeast nitrogen base without amino acids, 0.02% of selected amino acid mix without uracil, 1 M sorbitol, and 2% bacto-agar).

2.2.2 Plasmids. Plasmids pFL61 and pRS314 were purchased from American Type Culture Collection (ATCC 77215 and ATCC 77143, respectively). Total DNA extraction from *D. hansenii* Y-7426 was performed by the method described by [21]. Bacterial plasmid extraction was accomplished with a Qiagen kit (Qiagen®) according to the manufacturer's instructions.

Plasmid pNJ7426, derived from plasmid pRS314, was constructed with a *D. hansenii URA3* gene. Amplification of the *URA3* gene by polymerase chain reaction (PCR) was done using primers DhURA3F (5′-TATCTGCAGCTGATAGCAAGACTGAATTATG-3′), DhURA3Rev (5′-CACGAGCTCTCAACAATCTGATGAACAAGTC-3′) and chromosomal DNA of *D. hansenii* Y-7426 based on coding sequences for the *URA3* gene of *D. hansenii* CBS 767^T [16]. Amplification was performed using a initial denaturation of 5 min at 95°C followed by 30 cycles of denaturation of 1 min at 94°C, annealing of 1 min at 50°C, extension of 2.5 min at 72°C, and a final extension of 7 min at 72°C. The resultant 1.8-kb DNA fragment was cloned into the *Sac*I-*Pst*I sites of pRS314, yielding pNJ7426.

2.2.3 Growth and RF production. Cultures of the *D. hansenii* strains were prepared by inoculating a single colony into 5 mL of SC or SD medium and incubating at 30°C on an orbital shaker (200 rpm) for 18-24 h until log phase. These starter cultures were used to inoculate 250 mL of SC and SD medium with approximately 1.25×10^6 cells each. Cobalt(II) ($CoSO_4 \cdot 7H_2O$, Sigma-Aldrich) concentration gradient ranged from 0.0 mM (control), 0.1 to 0.5 mM at 0.1 mM intervals [19]. Growth rates from three independent readings were measured by optical density at 660 nm using a PerkinElmer UV-vis spectrometer (Lambda Bio). The number of cells per mL was calculated based on the index provided for haplotype yeast cells as described by [11]. The length of lag phase was determined to be less than 5.0×10^6 cells/mL. The lag phase was defined as number of cells instead of time in hours due extended lag phases in the presence of Co(II). RF production was assayed and quantified fluorometrically (from three technical readings) using a BioRad VersaFluor fluorimeter (excitation at 375 nm, emission at 520 nm). Standard curve serial dilutions for RF quantification were obtained using RF (Sigma-Aldrich) dissolved in SC and SD media.

2.3 Results

2.3.1 Growth. *Debaryomyces hansenii* J26 grew well in SD medium (control) reaching stationary phase at 72 h and cell densities of 2.5 x 10^8 cells/mL (Figure 1*a*). Cultures were grown

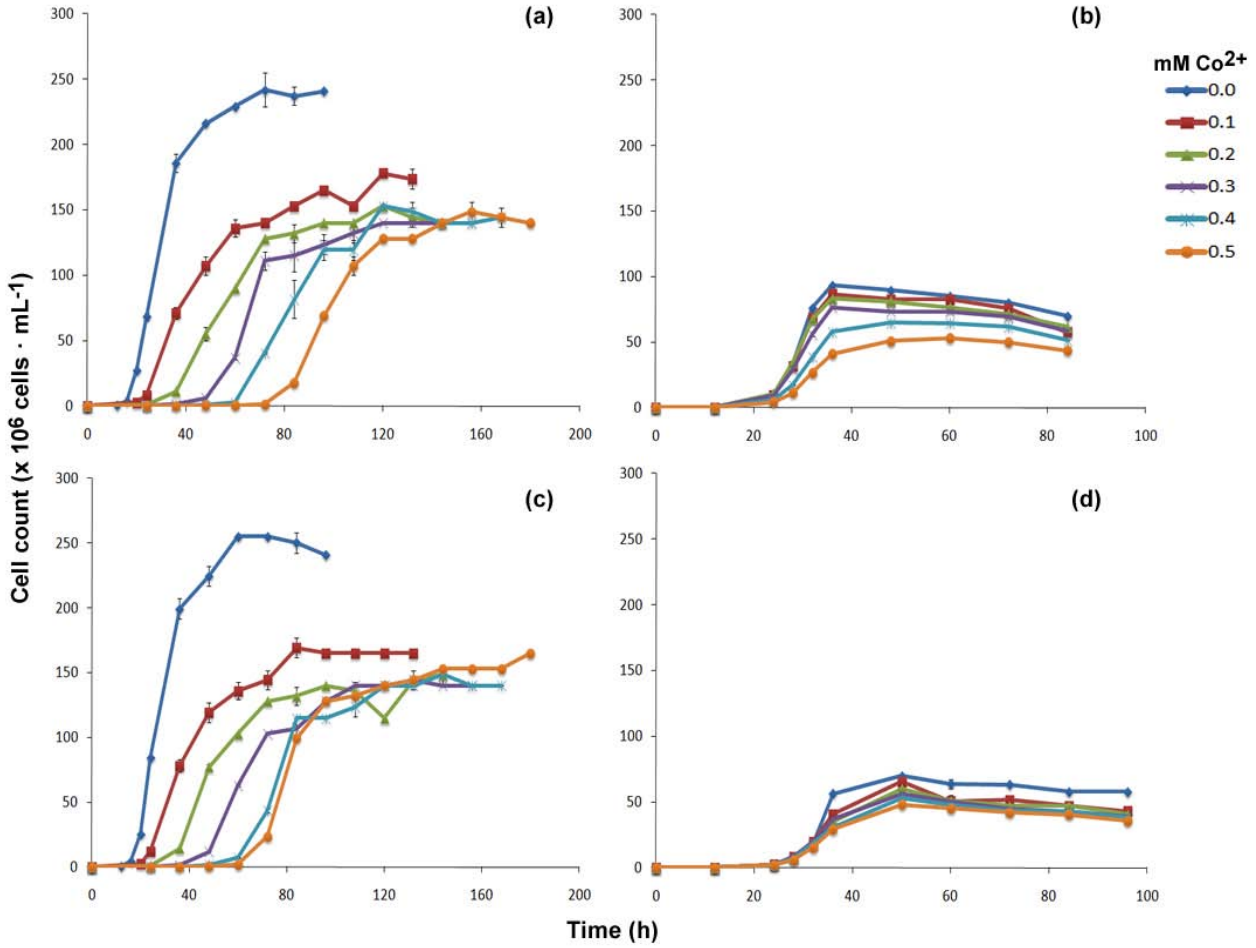

Figure 1. Growth of *Debaryomyces hansenii* strains in synthetic dextrose (SD) or synthetic complete (SC) medium with increasing Co(II) concentrations (mM). (a) J26 in SD, (b) J26-1 in SC, (c) J26 in SC, and (d) J26-1 transformed with pNJ7426 in SD.

in the presence of 0.1 mM Co(II) showed growth inhibition reaching cell densities of approximately 1.5 x 10^8 cells/mL during the stationary phase. Further increase of metal concentrations did not result in a greater inhibition of cell densities. Changes in the acclimation period, however, were observed as an increase in lag phases with increasing Co(II) concentration

resulted in a change in the growth pattern. This extension of the lag phase was directly proportional to metal concentration (Figure 1*a*).

Mutant J26-1 in SC medium grew poorly as compared to the wild type. An extended lag phase typically lasted 36 h reaching a cell density of 9 x 10^7 cells/mL at stationary phase (approximately 40% of wild-type cell yield) (Figure 1*b*). J26-1 did not show significant growth inhibition nor an extension in lag phases with increasing Co(II) concentrations, although lower cell yields of 5.0 x 10^7 cells/mL were observed at the highest concentration of Co(II) compared with control cultures. These results indicated that J26-1 was more resistant to Co(II), or that impaired growth somehow affected metal sensitivity. This could be due to two reasons: (i) the addition of exogenous uracil increases metal resistance and (or) (ii) J26-1 has a second mutation that renders it defective in growth and thereby altered metal sensitivity.

To test the first hypothesis, J26 was grown in SC medium with Co(II). Results indicated that at lower (0.1-0.3 mM) metal concentrations (Figure 1*c*), exogenous uracil had no effect on growth and lag phases, which increased concurrently with increasing metal concentration (Figure 1*c*). However, this pattern was not seen at Co(II) concentrations of 0.4 and 0.5 mM where both lag phases are similar. This suggested that exogenous uracil may be facilitating the acclimation of *D. hansenii* to higher concentrations of Co(II).

To test our second hypothesis, two experiments were done. Initially, we transformed J26-1 with a heterologous *Saccharyomyces cerevisiae URA3* gene (pFL61). Growth of these transformants in SD medium with increasing Co(II) concentrations showed no change from its original growth pattern. Although the *S. cerevisiae URA3* gene was able to restore uracil prototrophy, it did not restore wild-type growth patterns (Figure 2*a*). A second transformation with a homologous *D. hansenii URA3* gene (pNJ7426, see Methods) also restored uracil prototrophy and did not show a change in growth pattern (Figure 1*d*). Both heterologous and homologous transfer of *URA3* genes were used to determine if the homologous *URA3* gene product plays a role other than the synthesis of uracil. Lack of restored wild-type growth characteristics after complementation with *URA3* genes indicates that J26-1 probably has a second mutation that restricts growth and affects acclimation to Co(II).

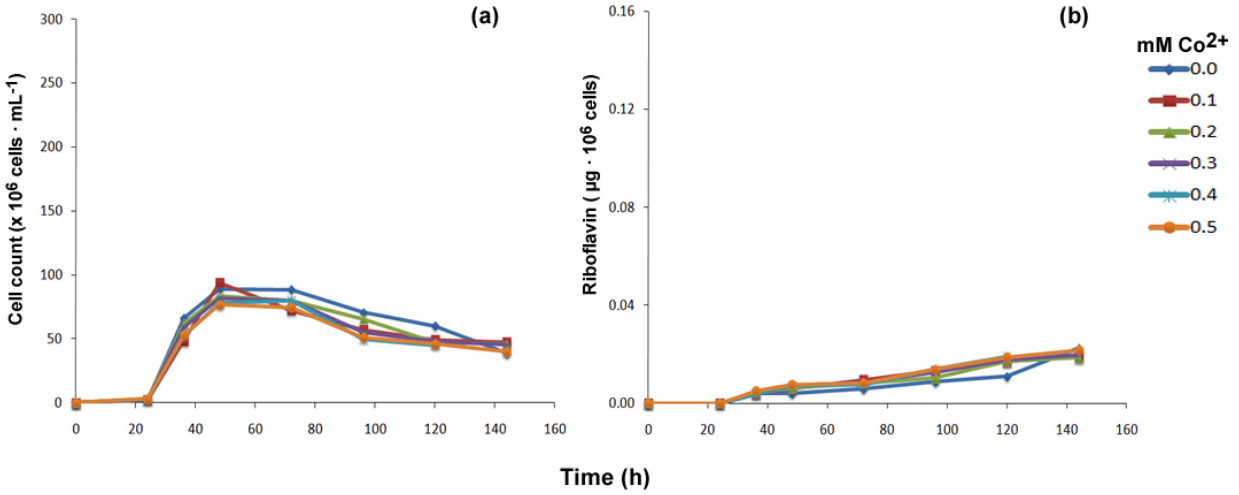

Figure 2. (a) Growth and (b) riboflavin production of *Debaryomyces hansenii* J26-1 transformed with pFL61 in synthetic dextrose medium with increasing Co(II) concentrations (mM).

2.3.2 Riboflavin (RF) production. RF overproduction by J26 was directly proportional to metal concentration although basal concentrations of RF were produced in SD medium at approximately 0.0016 µg/10^6 cells (control) (Figures 3*a* and 4). At higher Co(II) concentrations, RF overproduction was detected during late lag phase and initially showed a sharp increase that eventually stabilized at a lower level during log and stationary phase (Figure 3*a*). An extended lag period observed for RF production also corresponded to the extended lag period seen during growth (Figure 3*a*). When grown in SC medium, a very similar pattern of RF production was observed (Figure 3*c*) with the exception of a shortening of the extended lag period at 0.5 mM.

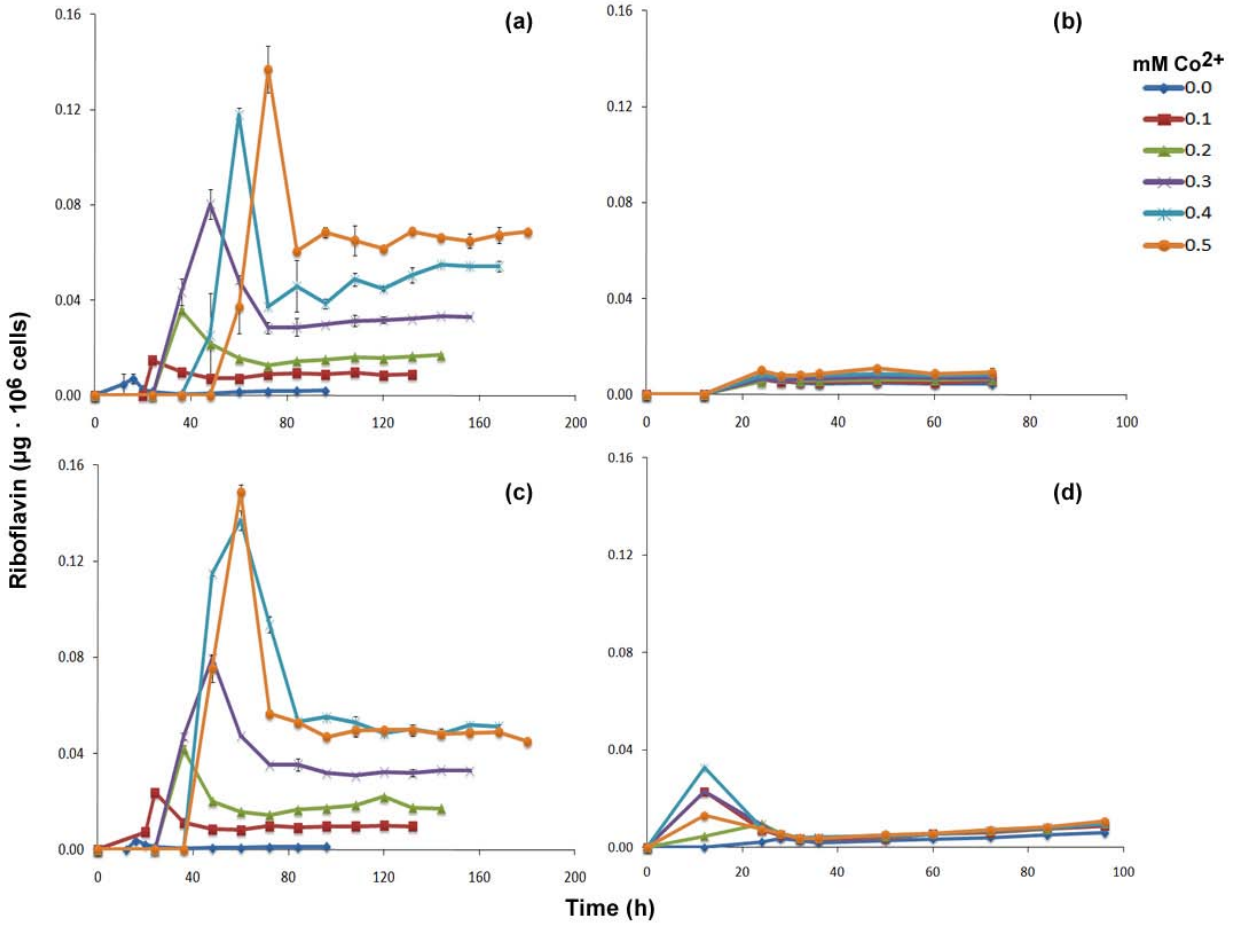

Figure 3. Riboflavin production of *Debaryomyces hansenii* strains in synthetic dextrose (SD) or synthetic complete (SC) medium with increasing Co(II) concentrations (mM). (a) J26 in SD, (b) J26-1 in SC, (c) J26 in SC, and (d) J26-1 transformed with pNJ7426 in SD.

While RF overproduction in the wild type was related to increasing Co(II) concentrations, J26-1 did not produce significant amounts of RF indicating that growth, metal acclimation, as well as RF synthesis, are altered (Figure 3*b*). J26-1, containing plasmids pFL61 and pNJ7426, did not restore wild-type RF production levels, but responded slightly to increasing metal concentrations showing a 3-fold increase at 0.5 mM. J26-1 transformed with the homologous *URA3* gene restored the wild type pattern of an initial peak in RF synthesis and subsequent stabilization at a lower level (Figure 3*d*). This pattern was not restored in J26-1 transformed with a heterologous *URA3* gene (Figure 2*b*).

Figure 4. Riboflavin production (yellow pigment) of *Debaryomyces hansenii* J26 in Co(II) concentrations ranging from 0.0-0.5 mM (left to right, see Methods) in synthetic complete medium.

2.4 Discussion

Debaryomyces hansenii J26 showed a distinctive growth pattern in the presence of Co(II) in both SD and SC media, with an approximate 40% growth inhibition at the lowest concentration (0.1 mM). Further increase in metal concentrations did not enhance inhibition. Increased generation times and decreased final cell yields at stationary phase have been reported in *D. hansenii* and *Candida utilis* when grown with metal ions [19,29]. We find further that increase in metal concentrations causes a concurrent increase in the lag phase of the culture. These results suggest that an acclimation period is necessary for the cell to grow in the presence of Co(II). A similar extension in lag period with increasing metal concentrations has also been observed in *P. guilliermondii*, although there was no growth inhibition [18,24].

Growth patterns of J26 in SD medium (without exogenous uracil) was affected by the presence of Co(II). While in SC medium (with exogenous uracil), metal acclimation may be facilitated at Co(II) higher concentrations. Growth patterns of J26-1 showed significant changes compared to the wild type, including a slower growth rate and lower cell yields (data not shown). This strain was seemingly not affected by Co(II), in that no growth inhibition was observed. These results suggested that exogenous uracil provided a certain level of metal tolerance. This may be possible if the nucleic acid base is able to sequester metal ions, resulting in reduced

toxicity and therefore facilitating acclimation. Reports have shown that uracil can form metal-ligand complexes [4,28]. To test this hypothesis, J26 was grown in uracil-supplemented medium with increasing metal concentrations. Our results indicate that at the highest concentration of Co(II) tested (0.5 mM), there was no increase in lag phase implying that uracil aids in the acclimation process, particularly at this higher level of Co(II). Obligatory supplementation of uracil in J26-1 could thus be aiding in its resistance to metal. A second possible explanation for cobalt resistance in J26-1 may be due to inhibited Co(II) transport, resulting in reduced toxicity levels.

Another hypothesis that could explain the significant change in growth patterns of J26-1, isolated by chemical mutagenesis (see Material and Methods), is the existence of a second mutation that had affected its growth and, as a result, its metal sensitivity. Variations in growth patterns have been observed in another *ura3* auxotrophic strain of *Pichia pastoris* developed for use as a selective marker [26]. This auxotroph also showed significantly reduced growth rates even though cultures were grown in excessive amounts of uracil. However, when this strain was transformed with an appropriate *URA3* vector, wild-type growth rates were restored; therefore, the slow growth rate was due to an inability to transport this nucleic acid base efficiently [26]. Our studies with J26-1 showed no restoration of wild-type growth when transformed with either heterologous or homologous *URA3* genes suggesting the presence of a second mutation affecting growth.

The presence of metals not only causes changes in growth patterns, but can induce RF oversynthesis in flavinogenic yeasts [18,19,41]. RF production has been correlated to growth patterns and the presence of metal. It is oversynthesized mostly during late lag phase until it reaches a peak production and subsequently stabilizing at lower levels [18]. Previous studies on the sensitivity of *P. guilliermondii* to Cr(III) and Cr(VI) have shown that either addition of exogenous RF or RF oversynthesis are associated with an elevated metal tolerance [24]. Our results show no apparent correlation between RF overproduction and increased metal tolerance. *Debaryomyces hansenii* J26-1 showed higher levels of metal tolerance and no significant levels of RF oversynthesis. Exogenous RF addition (50 μg/mL) did not change the growth pattern nor did it affect metal tolerance (data not shown). Transformation of J26-1 with the homologous *URA3* gene restored RF overproduction peaks during lag phase also seen in the wild type strain, even though the levels of RF production were significantly lower. This pattern was not observed

when J26-1 was transformed with the heterologous *URA3* gene, which suggests that the *D. hansenii URA3* gene may be involved in RF production in the presence of metal (data not shown).

Although recent studies have reported the use of molecular techniques in the development of flavinogenic mutants of the anamorph *C. famata*, very little is known about the mechanisms that regulate such flavinogenesis [14,15,23,42]. Alignments of putative amino acid sequences of *C. famata RIB* genes with other flavinogenic yeasts have shown high levels of identity and similarity to *D. hansenii*, *C. albicans*, and *P. guilliermondii* [43]. Substantial overproduction of RF due to the intergration of the *D. hansenii FMN1* gene, which encodes for the RF kinase, and the constitutive *TEF1* promoter in recombinant strains of *C. famata* suggests RF regulation may be controlled by strong promoters in critical genes of the RF pathway [23]. In flavinogenic yeasts, iron ions mostly regulate the transcriptional repression of RF synthesis and the transcripton factor *SEF1* (suppressor of essential function gene *RPM2*) has also been associated with its regulation [see 1 for a review,14,15].

Several genes (*RIB80*, *RIB81*, *RIB83*, *RIB84*, *HIT1* and *RED1*) associated with RF regulation and iron uptake have been characterized from yeast such as *P. guilliermondii* [17,32-34,37,39]. *RIB83* and *RIB84* genes have been associated with a positive regulation of RF synthesis shown by reduced levels of RF production and iron uptake [32,39]. On the other hand, *red* mutants of *P. guilliermondii* with enhanced flavinogenic activity showed an increase in sensitivity to various metal ions including Co(II) [38]. *Red* mutants have also been associated with the ability to reduce 2,3,5-triphenyltetrazolium chloride (TTC) to red formazan, where the presence of reductase activity is observed as the formation of red colonies [38]. TTC has been used to analyze ferric reductase activity in *P. guilliermondii* mutants and has been correlated to iron uptake and RF oversynthesis [17,38]. In our study, *D. hansenii* J26-1 was not able to reduce 2,3,5-triphenyltetrazolium chloride (TTC) (40 μg/mL) to red formazan, whereas J26 formed red colonies (data not shown). The absence of red formazan indicates a mutation or set of mutations that effectively restricts electron transport flow [5]. Whereas *red* mutants of *P. guilliermondii* showed increased levels of RF oversynthesis and metal sensitivity, J26-1 contrasts significantly with impaired RF oversynthesis and decreased metal sensitivity suggesting a second mutation in a gene that is analogous to the *P. guillermondii RIB83* gene involved in reducing RF levels and ferric reductase activity [39].

Several biotechnological methods have helped to elucidate the underlying mechanisms of RF synthesis in flavinogenic yeasts by incorporating or deleting homologous genes associated with oxidative stress and iron transport. The use of insertion mutagenesis in *P. guilliermondii* of the *VMA1* (vacuolar membrane ATPase) genes of *Ashbya gossypii*, and *S. cerevisiae* and the *FES1* (nucleotide exchange factor) and *FRA1* (iron repressor of activation of the iron regulon) genes of *S. cerevisiae* revealed a Δ*fra1-45* mutant capable of accumulating twice the amount of intracelllular iron and seven fold more RF than the parental strain [9]. The identification and deletion of the frataxin homologue gene (*YFH1*), which is involved in iron transport and storage in eukaryotic mitochondria, produced up to 70 times more RF in *P. guilliermondii* and accumulated higher amounts of iron compared to the wild type strain. However, this mutant strain also became more sensitive to hydrogen peroxide, implying that *YFH1* is capable of affecting several phenotypic traits in yeast, including RF production [30].

The physiological response of *D. hansenii ura3* mutant to Co(II) showing an altered RF synthesis, growth pattern, and enhanced metal tolerance suggests an important link that may be related to the presence of uracil. In comparing growth of *D. hansenii* with exogenous uracil, a shortening of the lag phase may be indicative of uracil's role in acclimation and should be considered when examining metal tolerance. However, increasing levels of exogenous uracil at higher metal concentrations may help to provide more information regarding its effect on growth and metal tolerance. In addition, further research on the characterization of a second mutation in *D. hansenii* J26-1 may help provide a more complete analysis of the correlation among growth, RF synthesis, and metal sensitivity.

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Chapter 3: Phenotypic Profiling of *Debaryomyces hansenii* Strains in Response to Cobalt(II) and Saline Stress

3.1 Introduction

The phenotype, or observable characteristics, of an organism has been used traditionally for taxonomic and morphological classification of fungal species. This has been based mostly on the different species concepts described throughout literature, which include the well-known Biological Species Recognition (BSR), the Morphological Species Recognition (MSR), the Ecological Species Recognition (ESR) and the Phylogenetic Species Recognition (PSR) [86]. The BSR (also known as the Biological Species Concept (BSC) as mentioned by [65]) and the MSR have been the predominant criteria for the field of mycology although the Genealogical Concordance Phylogenetic Species Recognition (GCPSR), which involves the use of multiple unassociated genes to assess phylogenetic relationships [64], is becoming the norm for fungal classification [28,32,51,86].

However, the analyses of gene genealogies have not completely elucidated the underlying genetic mechanisms that may influence the phenotypic properties of strains from the same fungal species. Consequently, increasing interest has developed to obtain more information regarding phenotypic traits among strains of the same hemiascomycetous (yeast) species when exposed to different factors that may affect growth [5,55,56,88]. Various studies on phenotypic profiling of natural and mutant hemiascomycete strains (e.g., *Saccharomyces* and *Kluyveromyces* spp.) succumbed to environmental stress or cytotoxic conditions have resulted in the understanding of some metabolic and genetic processes that were otherwise unknown or misinterpreted [14,18, 56,92,93], whereas others remain unclear [55]. A recent study by Jelier et al. [50] evaluated 19 strains of *Saccaharomyces cerevisiae* under 20 different conditions with the aim of linking phenotypic variations with protein-coding variation. However, comparative analyses on the variation of phenotype among yeast species of the same strains, whether natural or mutant, when cultured under stressful conditions are limited and have been mostly associated with effects on metabolic abilities such as trehalose production [35], actin filament organization [19],

fermentation [23,41], antioxidant enzymes [54], and polyamine production [78]. Evidence of variation in physiological processes in different strains *Debaryomyces hansenii* has increased the reassessment of this genus [24,38,67,69]. Based on the evidence of phenotype diversity among strains of the same yeast species, the purpose of this study was to evaluate the phenotypic variations of several variant strains of *D. hansenii* isolated from different sources. To accomplish this objective, these strains were cultured under environmental conditions that resulted in oxidative (Co(II)) and saline (NaCl) stress and were characterized according to tolerance.

Environmental contaminants or factors in the form of toxic substances, especially heavy metals, have caused detrimental effects on human health making metal pollution in aquatic and terrestial systems one of the most potentially severe forms of contamination [59]. Introduction of heavy metals (e.g., Pb, Cu, Cd, Co, Zn, Fe, Ni, Mn, and Cr) to marine ecosystems has been attributed to a number of sources, including anthropogenic activities by means of indutrial processes (metal processing, automobile parts manufacturing, and petroleum), resulting in serious levels of contamination, and thus affecting human health as well as that of other organisms [8,47]. Extraction of mineral deposits in the Wider Caribbean Region (WCR), which includes independent and dependent island states in the Caribbean Sea and mainland territories of South and Central America, has resulted in elevated metal concentrations in sediment deposits [see 26 for a review]. In particular, cobalt ions and inorganic cobalt compounds are normally found in very small concentrations in aquatic environments ranging up to 0.39 ug/L in some oceans [8,52]. These levels have been associated with discharges from industrial processes and other sources of contaminants such as varnishes, paints, and pigments that contain forms of inorganic cobalt [11] and have been linked to human diseases such as contact dermatitis, asthma, and hard metal lung cancer [58,61,90]. However, cobalt, particularly Co(II), is considered an essential trace element for the formation of vitamin B12 (hydroxocobalamin) [11,53], but has also been associated with the ability to substitute divalent metal ions that are important for metabolic processes (e.g., Fe, Mg, Cu, Ca, or Zn), thus resulting in altered genetic and cellular functions [12,62]. It has also been correlated to conditions that mimic hypoxia by substituting iron in a heme protein sensor and stimulating erythropoietin gene expression [33,43]. Cobalt has also been reported as causing defects in the sterol synthesis pathway of the pathogenic fungus *Cryptococcus neoformans* [60], has been associated with the iron regulon, phosphate accumulation, and divalent cation transport in *S. cerevisiae* [21,80,85]. In *Debaryomcyes* spp.,

cobalt has shown to induce riboflavin production [7,29] and reactive oxygen species when induced by other stress factors [69]. However, no analyses on the phenotypic variability of *D. hansenii* (*C. famata*) when exposed to metal (Co(II)) stress have been reported.

Another environmental factor that has been widely studied in yeast species and is capable of causing strenous conditions is osmotic stress in the form of high salt concentrations [3,13, 37,42,72]. One of the most important physiological responses to osmotic stress in yeast is the production and/or uptake of osmolytes, such as glycerol, and several signaling pathways that also control ion transport [see 44-46 for a review]. The underlying mechanisms of the process has been extensively studied in *D. hansenii* [76, see 77 for a review,87]; however, few comparative studies on the phenotypic characterization among strains of *D. hansenii* strains exposed to saline stress have been performed [35,57].

Based on the increasing evidence of the physiological differences among *D. hansenii* strains, the purpose of this study was to determine and characterize the phenotypic diversity of *D. hansenii* by profiling 34 strains of different origins cultured under environmental stress (oxidative and osmotic) with varying concentrations of Co(II) and NaCl, respectively.

3.2 Materials and Methods

3.2.1 Yeast strains and culture media. *Debaryomyces hansenii* (Zopf) Lodder & Kreger-van Rij var. *hansenii* (1985), *D. hansenii* (Zopf) Lodder & Kreger-van Rij var. *fabryi* (Ota) Nakase & M. Suzuki (1985), and *D. hansenii* strains used in this study are described in Table 1.

Table 1. *Debaryomyces hansenii* strains used in this study.

Strain name	Source of Isolation	Culture Collection
IT1	Unknown	Dr. Gianluigi Cardinali (University of Perugia, Italy)
IT2	Unknown	Univ. of Perugia
IT3	Unknown	Univ. of Perugia
IT4	Unknown	Univ. of Perugia
IT5	Unknown	Univ. of Perugia
IT7	Unknown	Univ. of Perugia
var. *hansenii* CBS 767^T	Sherry (Carlsberg Research Lab, Denmark)	Dr. Bernard Dujon (Institut Pasteur and University Pierre & Marie Curie-Paris)

Strain name	Source of Isolation	Culture Collection
CBS 5144	Unknown	Dr. L. Adler (University of Göteborg, Sweden)
CBS 1011	Unknown	Univ. of Göteborg
J6	Swedish estuary	Univ. of Göteborg
var. *hansenii* NRRL Y-7426	Carlsberg Research Lab, Denmark	Dr. C. Kurtzman, National Center for Agriculture Utilization Research (NCAUR), US Department of Agriculture, Peoria, Ill.
var. *hansenii* NRRL Y-1448	Horse-meat sausage, France	NCAUR
var. *hansenii* NRRL Y-1449	Throat of angina patient, France	NCAUR
var. *hansenii* NRRL Y-1454	Kentucky tobacco, Italy	NCAUR
var. *hansenii* NRRL YB-155	Coconut fruit salad	NCAUR
var. *hansenii* NRRL YB-160	Minced ham	NCAUR
var. *hansenii* NRRL YB-162	Pork	NCAUR
var. *hansenii* NRRL YB-163	Meat	NCAUR
var. *hansenii* NRRL YB-164	Meat	NCAUR
var. *hansenii* NRRL YB-165	Meat	NCAUR
var. *hansenii* NRRL YB-221	Camembert cheese	NCAUR
var. *hansenii* NRRL YB-306	Roka cheese spread	NCAUR
var. *hansenii* NRRL YB-398	High moisture corn	NCAUR
var. *fabryi* NRRL YB-327	Soil	NCAUR
var. *fabryi* NRRL Y-17914	Interdigital mycotic lesion, Germany	NCAUR
var. *hansenii* DhhBCS001	CalCOFI station: 125.40 (27°27’5” LN; -114° 42’ 53” LW) Surface seawater (0 m depth)	Dr. Ricardo Vázquez Juárez Centro de Investigaciones Biológicas del Noroeste, S.C. (CIBNOR), La Paz, México
var. *hansenii* DhhBCS002	CalCOFI station: 125.40 (26° 52’ 5” LN; -114° 42’ 53” LW) Seawater (100 m depth)	CIBNOR
var. *hansenii* DhhBCS003	CalCOFI station: 125.40 (26° 52’ 5” LN; -114° 42’ 53” LW) Seawater (100 m depth)	CIBNOR
var. *hansenii* DhhBCS004	CalCOFI station: 130.30 (26° 29’ 2” LN; -113° 29’ 31” LW) Surface seawater (0 m depth)	CIBNOR
var. *hansenii* DhhBCS005	CalCOFI station: 140.35 (24° 35’ 31” LN; -112° 40’ 19” LW) Seawater (100 m depth)	CIBNOR
var. *hansenii* DhhBCS006	CalCOFI station: 127.45 (26° 30’ 36” LN; -114° 48’ 43” LW) Seawater (50 m depth)	CIBNOR
var. *hansenii* DhhBCS007	CalCOFI station: 135.30 (25° 30’ 49” LN; -113° 35’ 26” LW) Seawater (50 m depth)	CIBNOR

Strain name	Source of Isolation	Culture Collection
var. *fabryi* DhfBCS001	CalCOFI station: 131.5.35 (25° 48’ 43” LN; -113° 39’ 25” LW) Seawater (100 m depth)	CIBNOR
var. *fabryi* DhfBCS002	Bahía de la Paz Station (24° 16’ 5” LN; -110° 20’ 10” LW) Surface seawater (0 m depth)	CIBNOR

[T] – type strain. The complete genome sequence of *D. hansenii* CBS767 is presently available online (http://www.genolevures.org/deha.html)

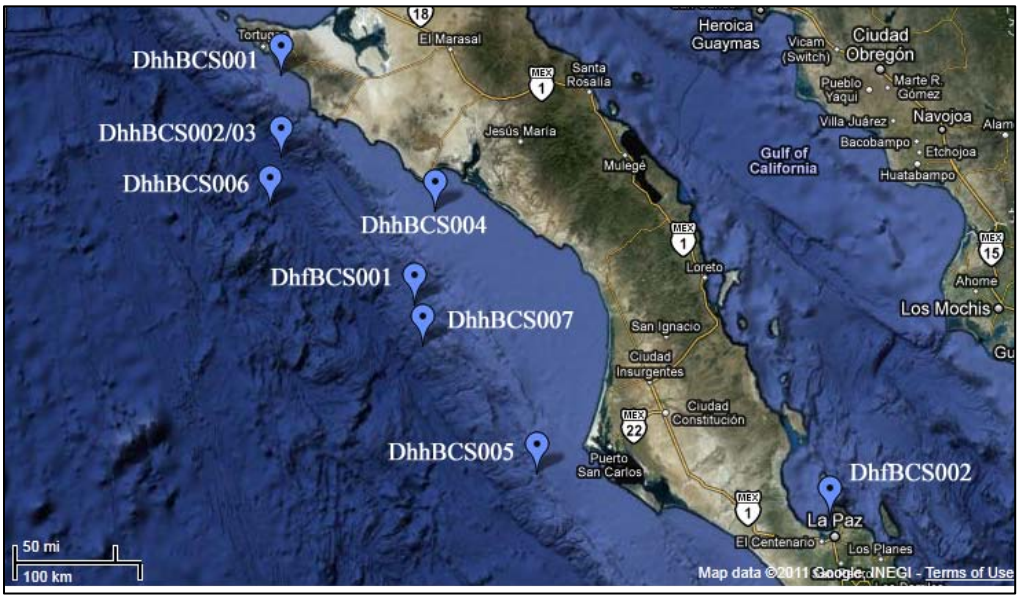

Figure 5. Localities of stations where *Debaryomyces hansenii* strains from CIBNOR (Table 1) were isolated on May 1986 (Source: Google Maps, 2011).

Strains obtained from California Cooperative Oceanic Fisheries Investigations (CalCOFI) stations along the Mexican Pacific west coast and Bahía de la Paz of the Federal State of Baja California Sur, Mexico were originally isolated by D. Hernández-Saavedra and J. L. Ochoa in May 1986 (Figure 5).

The present study consisted of 9 strains isolated from seawater, 8 strains of unknown origin, 6 strains from meat sources, 2 industrial strains, 2 strains from human tissue cultures, 2 strains from cheese, 2 strains from agricultural sources, one strain from fruit, 1 strain from bay (estuarine) water, and 1 strain from soil. All cell cultures were maintained in yeast-peptone-dextrose (YPD; 2% yeast, 2% peptone, 2% dextrose) agar plates (2% agar) or broth and stored as glycerol stocks in -80°C.

3.2.2 Cobalt(II) and saline (NaCl) stress tests. *Debaryomyces hansenii* strains were initially inoculated on YPD plates and incubated at 25°C for 3-4 days. The incubation temperatures of subsequent experiments were conducted at room temperature (RT; 23-24°C) due to noticeable changes in growth in several strains at temperatures of 30°C or higher (data not shown). Therefore, RT was considered appropriate for optimal growth of all strains in order to conduct the stress tests. Strains were then cultivated in 5 mL YPD broth at RT until saturation (2-3 days, approximately 9-10 OD_{660}) for Co(II) and NaCl stress tests. From this starter culture, 10 µL-inoculation drops of a 10^{-3} serial dilution were taken from each strain and were grown in both Co(II) and NaCl plates at RT for 3 days. Strains tested for cobalt tolerance were cultivated in YPD plates supplemented with $CoSO_4 \cdot 7H_2O$ (Sigma-Aldrich) that ranged from 0.1 to 1.5 mM at 0.2 mM intervals. Saline (osmotic) stress tolerance using YPD plates supplemented with NaCl (Fisher Scientific) concentrations ranging from 1 to 2 M at 0.2 M intervals was analyzed. Salt tolerance at 3 and 4 M were also studied. Growth of strains was photographed using a digital camera (Canon PowerShot SD750). Tolerance levels of *D. hansenii* strains to Co(II) and NaCl stress were determined qualitatively based on their ability to grow at a specific range of concentration. Riboflavin (RF) production was detected visibly as a yellow pigment in YPD plates with Co(II).

3.3 Results

3.3.1 Phenotypic variation in *D. hansenii* strains exposed to Co(II) stress. Thirty-four strains of *D. hansenii* (Table 1) were cultivated at increasing concentrations of Co(II) ranging from 0.1 to 1.5 mM (at 0.2 mM intervals) to determine a possible correlation with the source of isolation and to characterize varying levels of tolerance to metal stress. In general, no specific pattern of phenotype variation when exposed to Co(II) was associated with its source of isolation. In Figures 6–9, strains are depicted according to the culture collections described in Table 1. Strains isolated in Italy (University of Perugia, unknown source) showed varying levels of tolerance (Fig. 6), with IT3 being highly sensitive to concentrations higher than 0.7 mM.

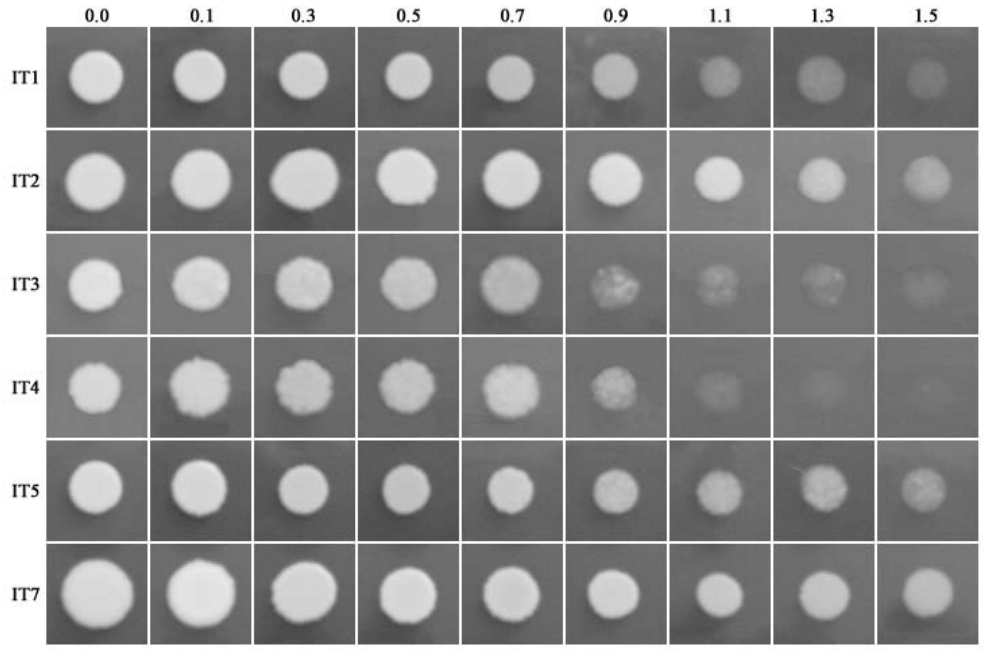

Figure 6. Growth of *Debaryomyces hansenii* strains (unknown source; obtained from the University of Perugia) exposed to different Co(II) concentrations (mM).

Strains from the collections of NCAUR (Peoria, Il.), Institut Pasteur (CBS 767^{T}) and the University of Göteborg showed a wide range of tolerance to Co(II) (Figures 7 and 8). Y-164 was the most sensitive of these collections, with very little growth at 1.1 mM (Figure 7), whereas Y-7426 showed much higher levels of tolerance with growth at 1.5 mM (Figure 6). Interestingly, the strains isolated from seawaters of the Pacific west coast and Bahía de la Paz showed less variability in their levels of Co(II) tolerance, with most able to grow at 1.5 mM (Figure 9), despite differences in depth and locality of their isolation (0, 50, and 100 m; Table 1 and Figure 5). In addition, several strains (DhhBCS002, DhhBCS003 and DhfBCS002) gradually reduced in colony size with increasing Co(II) concentration (Figure 9). With regard to colony morphology, DhhBCS002 changed from having a regular-shaped form and entire margin at 0.0 mM Co(II) to a slightly smaller, irregular-shaped form and undulate margin as the concentration increased.

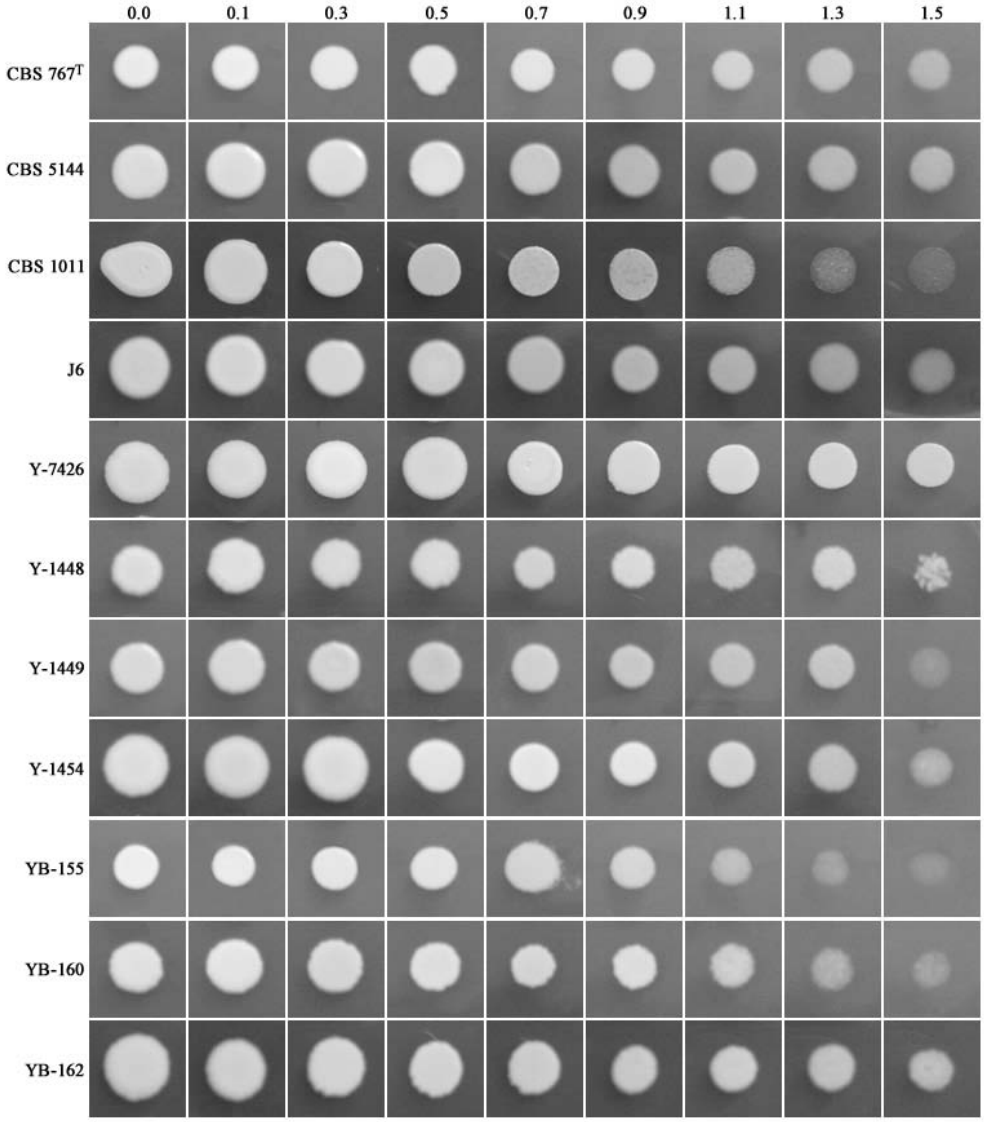

Figure 7. Growth of *Debaryomyces hansenii* strains (vertical axis; isolated from various sources and obtained from the NCAUR Culture Collection as listed in Table 1) exposed to different Co(II) concentrations (horizontal axis; mM).

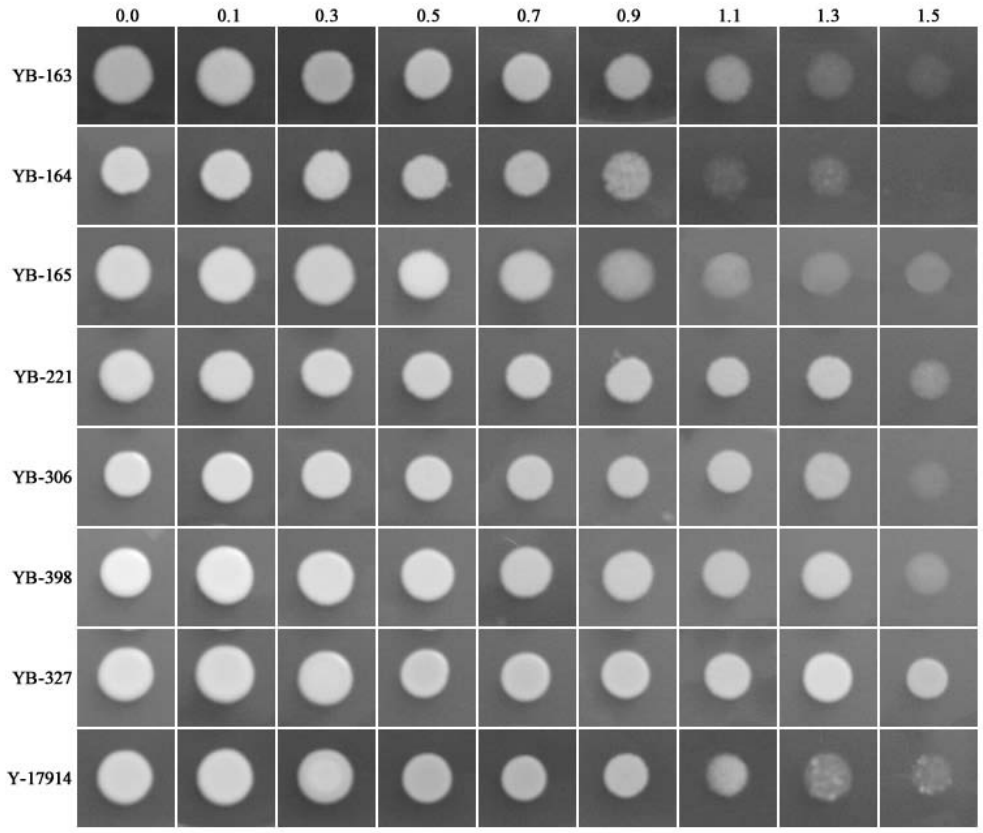

Figure 8. Growth of *Debaryomyces hansenii* strains (vertical axis; isolated from various sources and obtained from the NCAUR Culture Collection as listed in Table 1) exposed to different Co(II) concentrations (horizontal axis; mM).

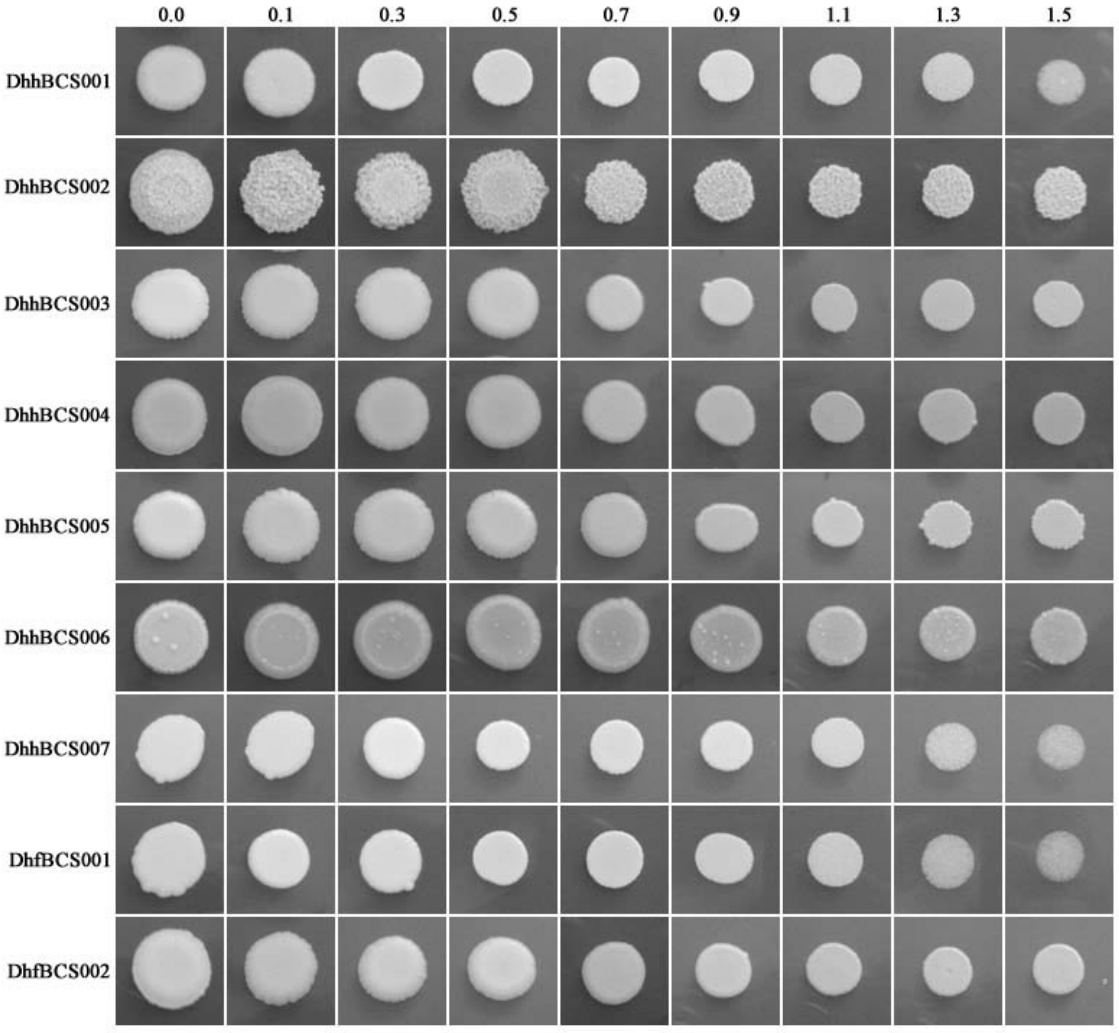

Figure 9. Growth of *Debaryomyces hansenii* strains (vertical axis; isolated from waters of the Mexican Pacific west coast and from Bahía de la Paz, Baja California Sur, Mexico and obtained from the CIBNOR Culture Collection) exposed to different Co(II) concentrations (horizontal axis; mM).

In Figures 10–13, strains were grouped according to levels of tolerance based on a qualitative analysis on the presence of growth at the different Co(II) concentrations tested. Four main categories were identified using this criteria: (i) highly tolerant, (ii) weakly tolerant, (iii) tolerant, and (iv) sensitive. Highly tolerant (HT) strains were considered as those that showed dense colony growth at 1.5 mM (Figure 10). Most of the strains isolated from Pacific seawaters (i.e., DDhhBCS002 – DhhBCS006, and DhfBCS002), although isolated from different depths, were grouped into this category. From the Univ. of Göteborg, only CBS 5144 was found to be HT, whereas only two strains from the NCAUR collection (Y-7426 and YB-327) showed dense colony growth. From the Univ. of Perugia, only IT7 was considered HT.

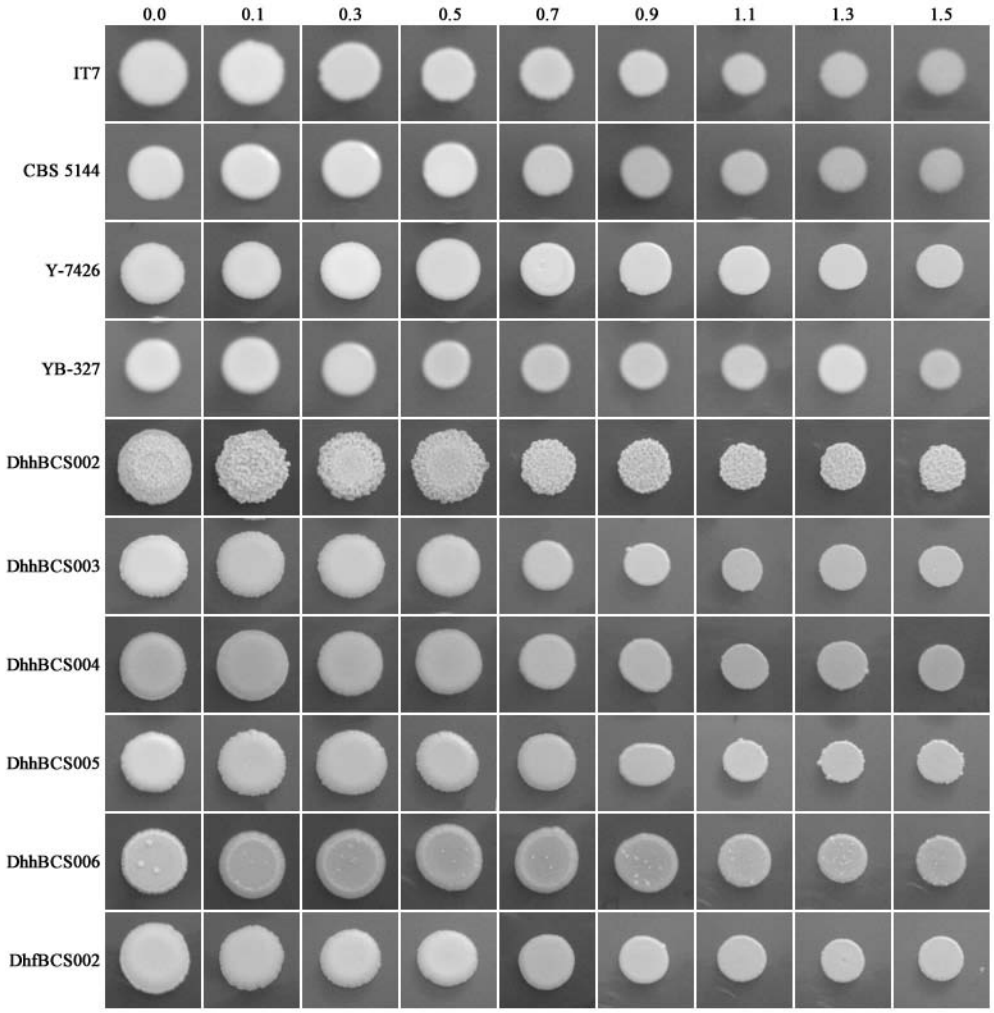

Figure 10. ***Debaryomyces hansenii*** **strains (vertical axis) characterized as highly tolerant (HT) based on dense colony growth at 1.5 mM Co(II) (horizontal axis; mM).**

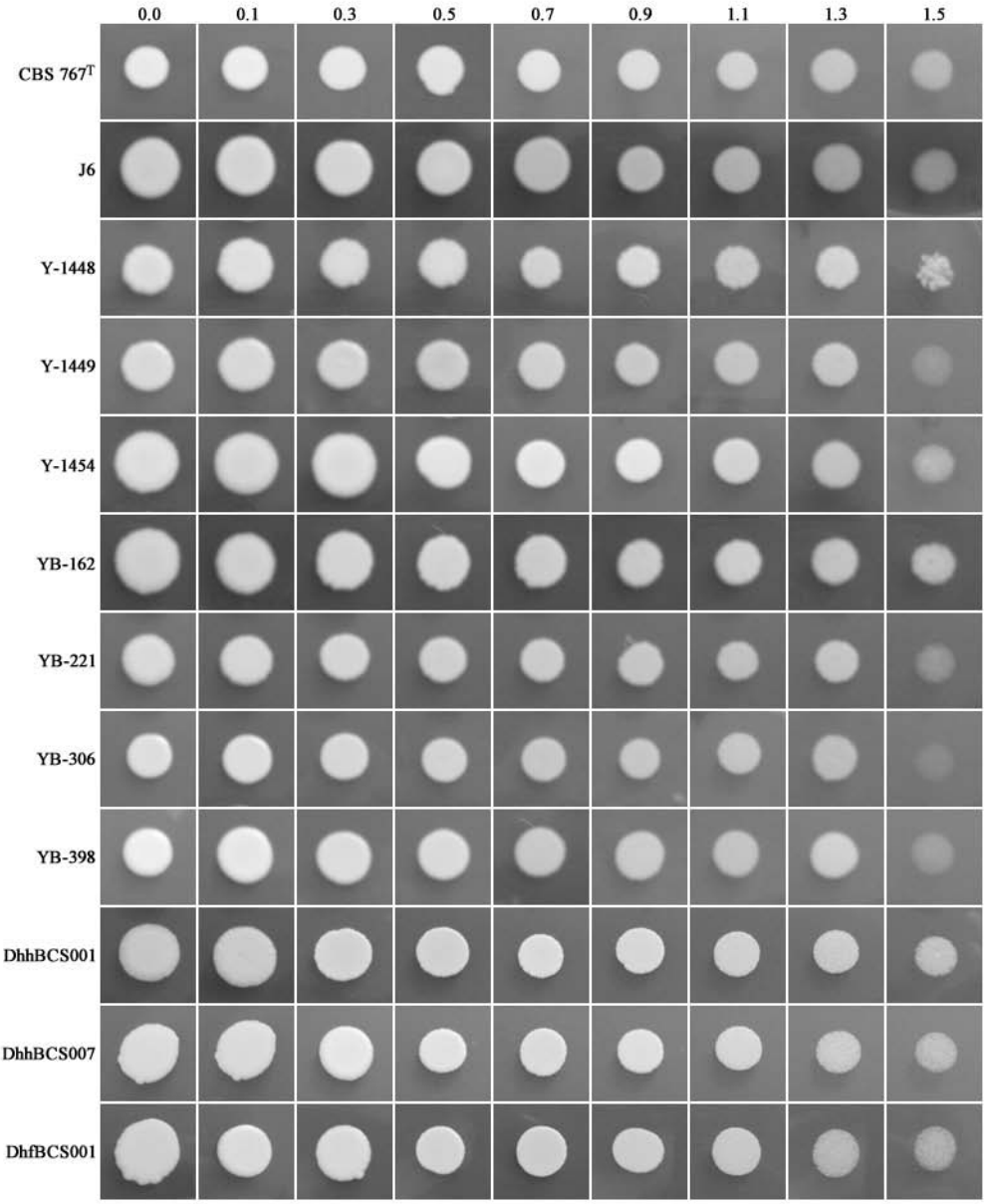

Figure 11. *Debaryomyces hansenii* strains (vertical axis) characterized as tolerant (T) based on slightly reduced growth at 1.5 mM Co(II) (horizontal axis; mM).

Tolerant (T) strains were grouped based on slightly reduced growth at 1.5 mM (Figure 11). Based on this criterion, CBS 767^T was grouped into this category. J6 was the only T strain from the Univ. of Göteborg collection. Strains Y-1448, Y-1449, Y-1454, YB-162, YB-221, YB-306 and YB-398 showed very similar growth densities despite differences in their source of origin (e.g., throat culture, cheese, meat, tobacco and corn). DhhBCS001, DhhBCS007 and

DhfBCS001 (isolated from 0, 50 and 100 m depths, respectively) were also grouped as T strains and showed similar colony morphologies (Figure 11). However, no correlation between tolerance level and isolation depth was found.

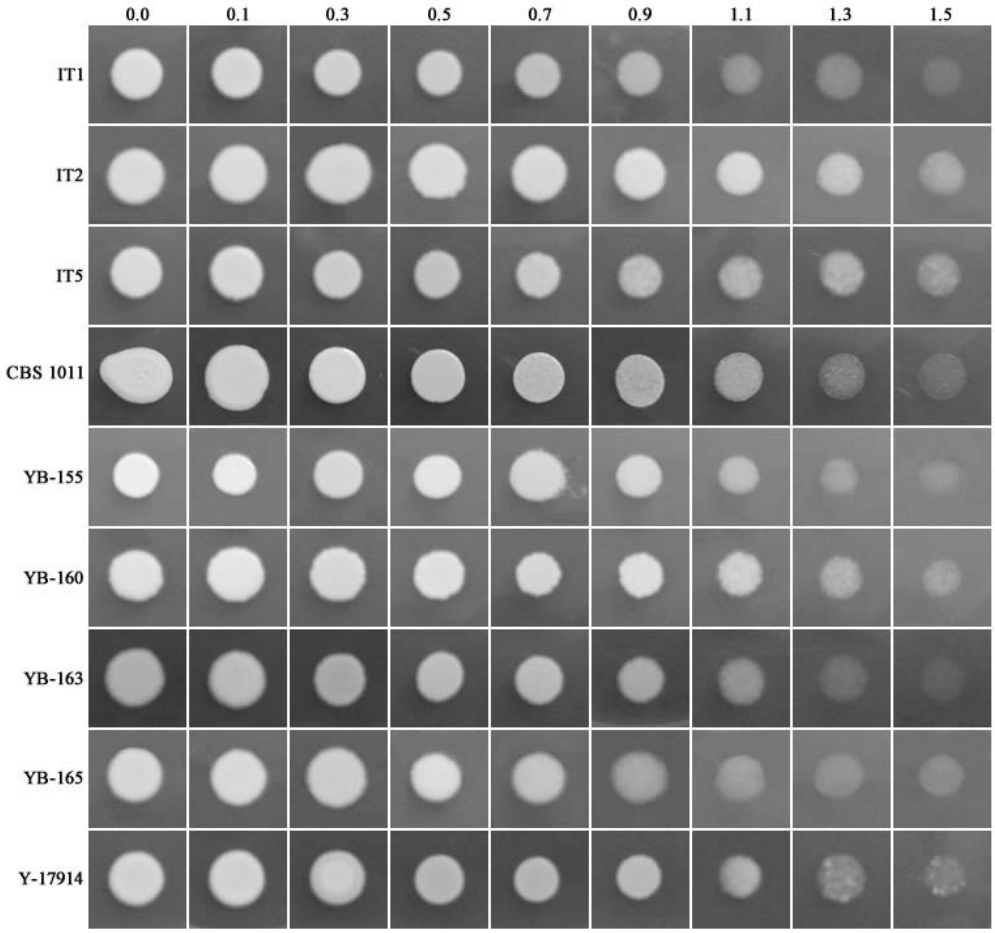

Figure 12. *Debaryomyces hansenii* strains (vertical axis) characterized as weakly tolerant (WT) based on reduced growth at 1.1, 1.3, and 1.5 mM Co(II) (horizontal axis; mM).

Weakly tolerant (WT) strains were grouped based on reduced growth at concentrations from 1.1, 1.3, and 1.5 mM (Figure 12). Although IT2 and IT5 showed similar tolerance levels, their morphologies slightly differed in the formation of umbonate elevations, which was mostly prominent in IT5. Strains YB-155, YB-163 and YB-165 shared similar levels of tolerance. However, Y-17914 showed slightly more growth at 1.1 mM as compared to the other strains of the NCAUR collection that were characterized as WT (Figure 12). In addition, Y-17914 depicted umbonate elevations at 1.3 and 1.5 mM. Strains that were considered sensitive (S) to Co(II) were grouped based on less visible growth at concentrations less than 1.1 mM (Figure 13). Umbonate

elevations were more evident in YB-164, IT3 and IT4 at 0.9 mM Co(II). Strains isolated from Pacific seawater and Bahía de la Paz were not characterized as WT nor S to Co(II) stress.

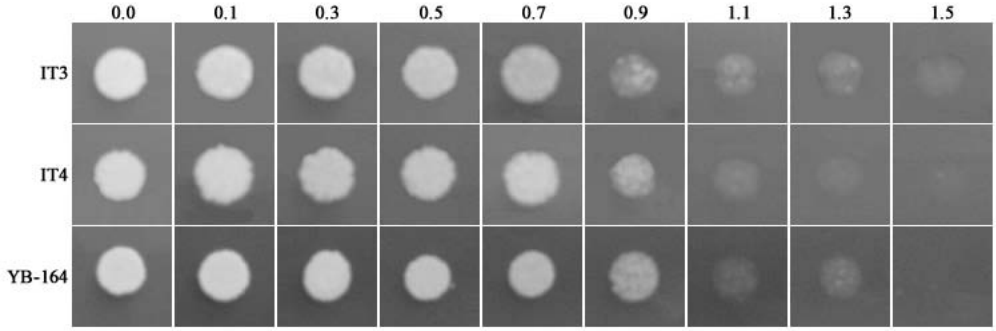

Figure 13. *Debaryomyces hansenii* strains (vertical axis) characterized as sensitive (S) based on reduced growth at concentrations less than 1.1 mM Co(II) (horizontal axis; mM).

RF production was only detected in four strains, i.e., CBS 5144, CBS 1011, J6, and Y-17914 (Figure 14). Of these strains, CBS 1011 and Y-17914 were WT to Co(II) stress (Figure 12), whereas CBS 5144 and J6 exhibited higher levels of tolerance (Figures 10 and 11). Therefore, no direct correlation between RF production and the level of Co(II) tolerance was observed among strains in this study.

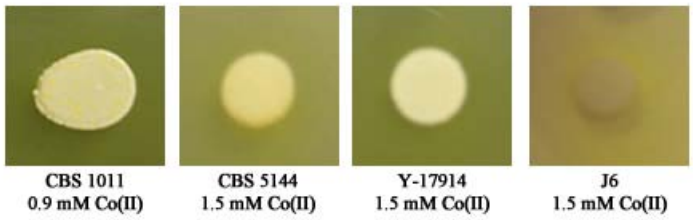

Figure 14. Riboflavin (RF) production of four *D. hansenii* strains when exposed to different Co(II) concentrations. RF production can be seen either as yellow spots within the colony (e.g., CBS 1011) or as a yellowish halo surrounding the colony (e.g., CBS 5144, Y-17914, and J6).

3.3.2 Phenotypic variation of *D. hansenii* strains exposed to saline (NaCl) stress. *Debaryomyces hansenii* strains were exposed to saline (osmotic) stress at increasing NaCl concentrations ranging from 0.0 to 2.0 M at 0.2 M intervals to determine a possible correlation to the source of isolation and to characterize varying levels of tolerance. Tolerance to 3 and 4 M NaCl was also analyzed. Overall, no definite pattern of phenotype variation after exposure to NaCl was associated with its source of origin. However, most of the strains were found to be rather tolerant to this particular environmental stress. In Figures 15–18, strains are depicted according to the culture collection described in Table 1. For all strains studied, no growth was detected at 3 and 4 M after 3 days of incubation. All strains from the Univ. of Perugia showed the same level of tolerance, exhibiting dense colony growth at 2.0 M NaCl (Figure 15). Most strains from the collections of NCAUR (Peoria, Il.), Institut Pasteur (CBS 767^{T}) and the Univ. of Göteborg also showed tolerance to 2 M NaCl, except for Y-7426, Y-1448, Y-162, Y-164, Y-221 and Y-17914 (Figures 16 and 17). Of these strains, no correlation was found with the source of origin (sherry, meat, cheese, and mycotic lesion). Most strains also sustained colony morphology and size despite increasing NaCl concentration, with the exception of Y-165 (Figure 17).

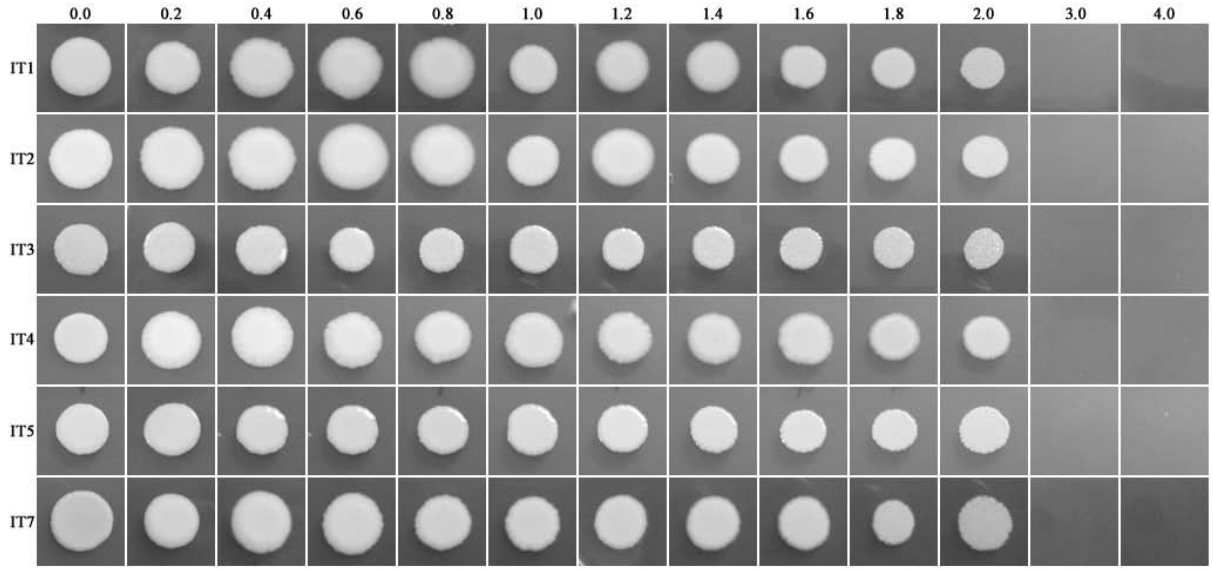

Figure 15. Growth of *D. hansenii* strains (vertical axis; isolated in Italy) exposed to different NaCl concentrations (horizontal axis; M).

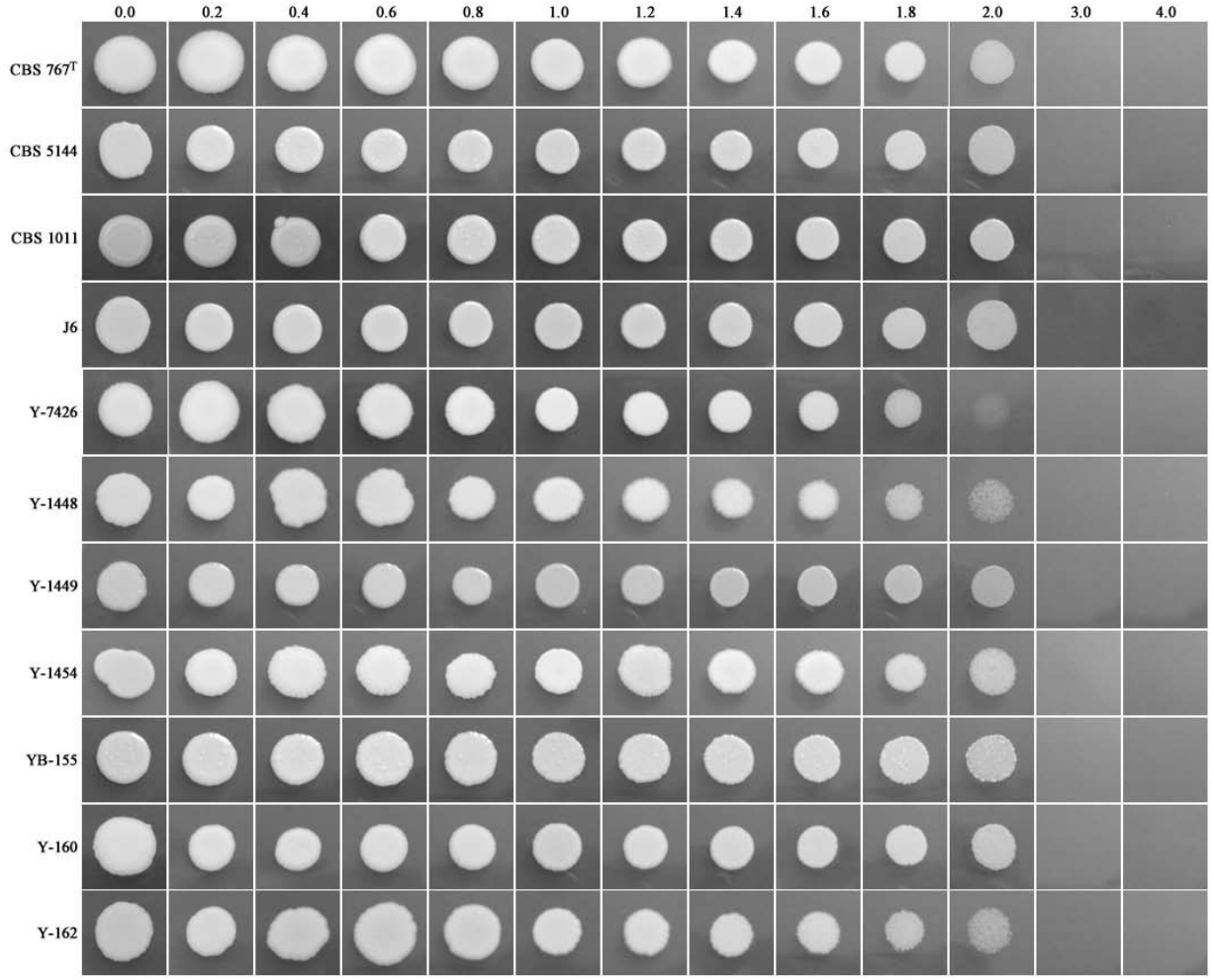

Figure 16. Growth of *D. hansenii* strains (vertical axis; isolated from various sources as listed in Table 1) exposed to different NaCl concentrations (horizontal axis; M).

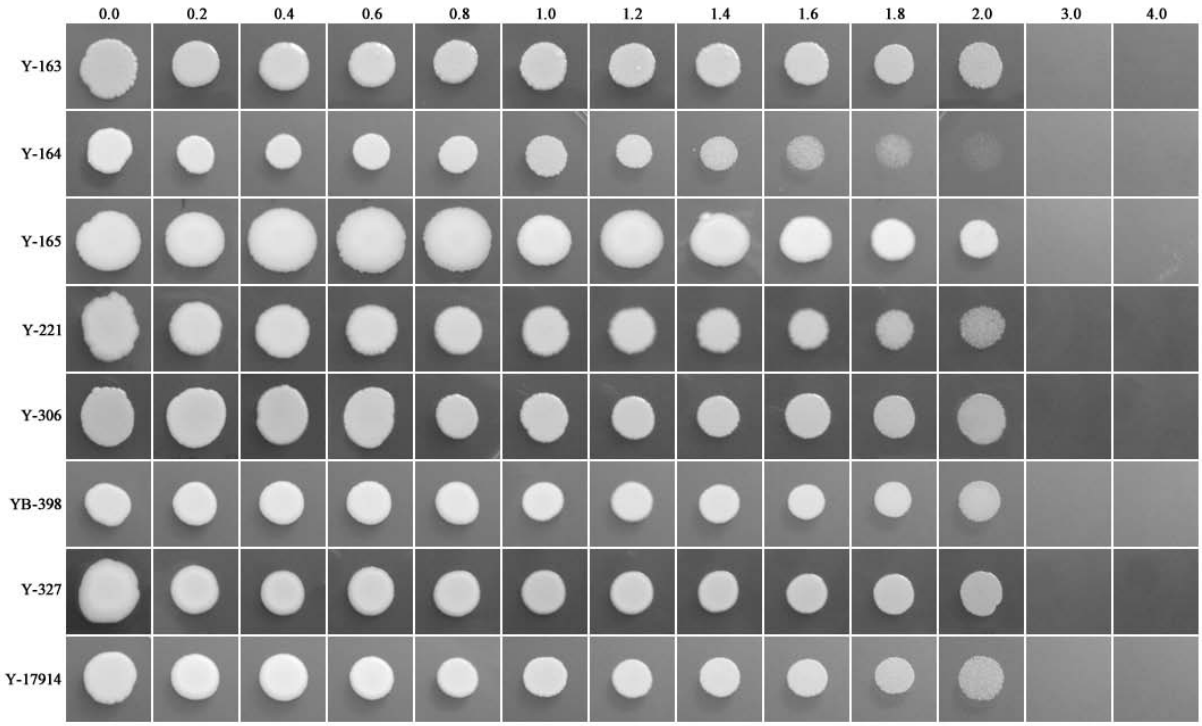

Figure 17. Growth of *D. hansenii* strains (vertical axis; isolated from various sources as listed in Table 1) exposed to different NaCl concentrations (horizontal axis; M).

In contrast, distinct levels of tolerance were observed in the strains isolated off the Pacific coast and at Bahía de la Paz (Figure 18). DhhBCS004 was the most affected by NaCl stress at 1.4 M. Although no direct correlation could be found with the locality (nearshore or offshore) or depth of isolation among these strains, DhhBCS003, DhhBCS005, and DhfBCS001 showed higher levels of NaCl tolerance and were isolated from 100 m depth (Figure 18 and Table 1). Gradual reductions in colony size were observed in most of the strains isolated from seawater, except for DhhBCS001, DhhBCS007, and DhfBCS002 (Figure 18). Changes in colony shape were drastic in DhhBCS002 with increasing NaCl concentration, morphing from a regular-shaped form and entire margin at 0.0 M to a much smaller, irregular-shaped form and undulate margin (Figure 18).

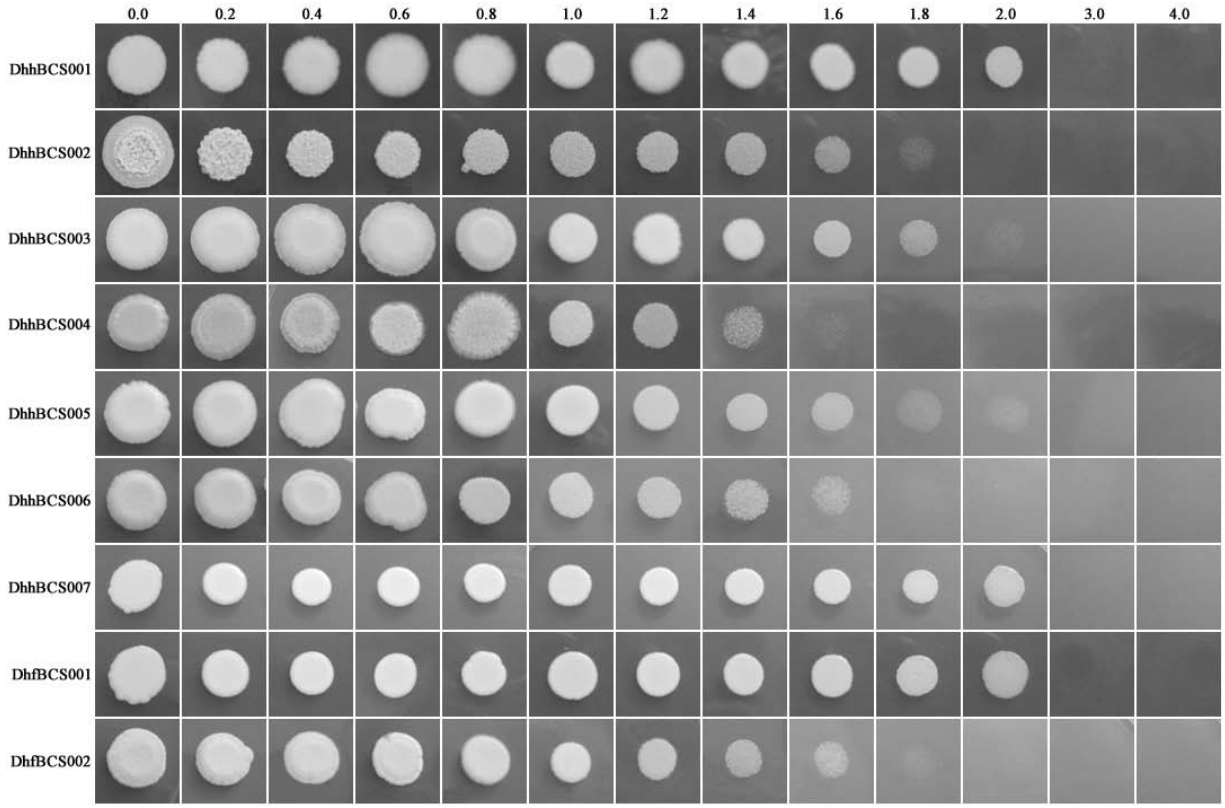

Figure 18. Growth of *Debaryomyces hansenii* strains (vertical axis; isolated from waters of the Mexican Pacific west coast and from Bahía de la Paz, Baja California Sur, Mexico) exposed to different NaCl concentrations (horizontal axis; M).

In Figures 19–21, strains were characterized according to levels of tolerance based on a qualitative analysis on the presence of growth at the different NaCl concentrations tested. Four main groups were identified using this criterion: (i) highly tolerant, (ii) weakly tolerant, (iii) tolerant, and (iv) sensitive. Highly tolerant (HT) strains were considered as those that showed dense colony growth at 3 and 4 M. No strains analyzed in this study were able to grow at these concentrations after 3 days of incubation. Tolerant (T) strains were grouped based on dense colony growth up to 2 M NaCl (Figure 19). Of the 34 strains tested, 23 were characterized as T, which included all of the strains from the Univ. of Perugia, CBS 767^{T}, Univ. of Göteborg and most strains from the NCAUR collection. Based on these results, no particular phenotype was associated with the source of origin. From strains isolated from seawaters, only DhhBCS001, DhhBCS007 and DhfBCS001 were considered T; however, no correlation between tolerance level and isolation depth was found (Figure 19 and Table 1). Weakly tolerant (WT) strains were

considered as those with reduced growth at 1.8 and 2.0 M NaCl (Figure 20). No strains from Italy were included in this group.

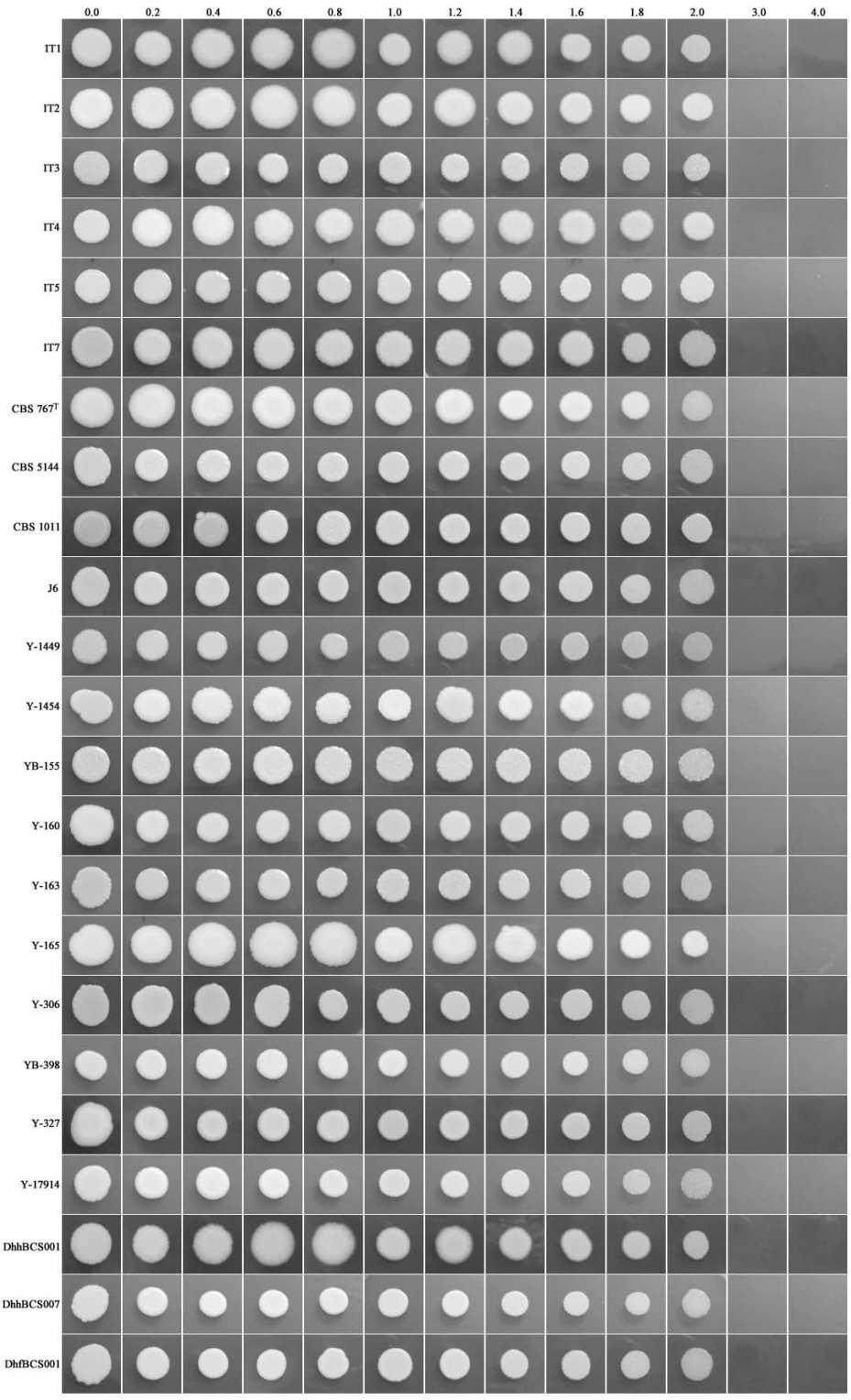

Figure 19. *Debaryomyces hansenii* strains (vertical axis) characterized as tolerant (T) based on dense colony growth at 2.0 M NaCl (horizontal axis; M).

Four strains from the NCAUR collection (Y-7426, Y-1448, Y-162 and Y-221) were characterized as WT, showing similar tolerance levels. DhhBCS003 and DhhBCS005 were also considered WT, although DhhBCS005 showed less visible colony growth at 1.8 M (Figure 20). These strains also showed gradual reductions in colony size with increasing NaCl concentration. No direct association between phenotype and the source of origin (sherry, meat, pork, cheese and seawater) was observed among WT strains. However, both WT strains originating from Pacific seawater samples (DhhBCS003 and DhhBCS005) were isolated from 100 m depth but were not sampled from stations near each other (Figure 5 and Table 1).

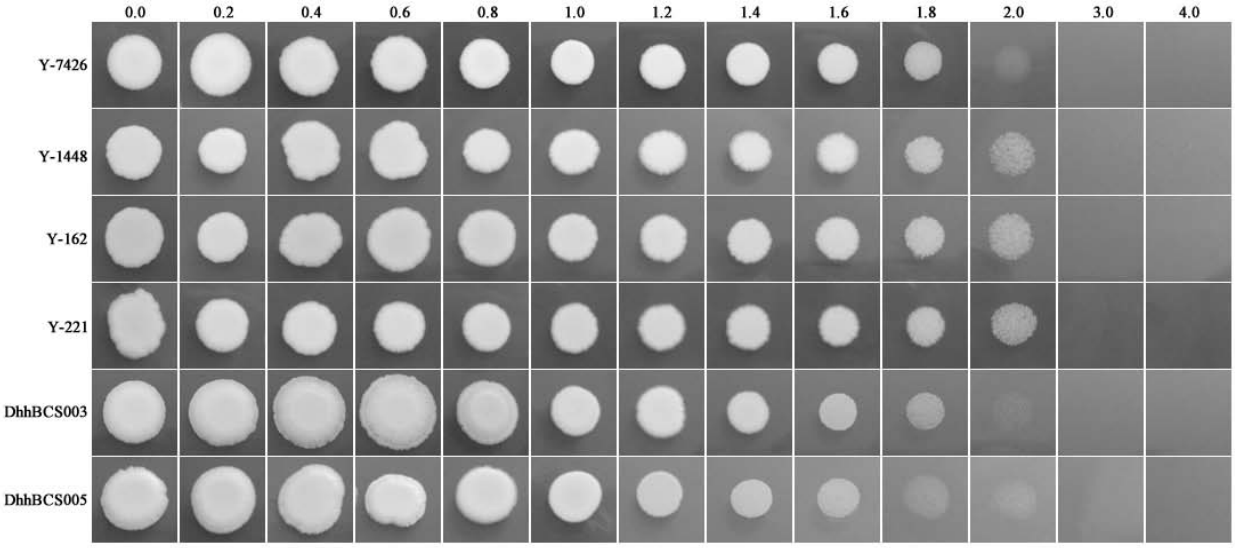

Figure 20. *Debaryomyces hansenii* strains (vertical axis) characterized as weakly tolerant (WT) based on reduced growth at 1.8 and 2.0 M NaCl (horizontal axis; M).

Strains that were considered sensitive (S) to NaCl stress were grouped based on reduced growth at concentrations less than 1.8 M (Figure 21). From the NCAUR collection, only Y-164 (isolated from meat) was included in this group. Interestingly, 4 strains isolated from seawaters (i.e., DhhBCS002, DhhBCS004, DhhBCS006, and DhfBCS002, respectively) were considered S to concentrations higher than 1.2 M (Figure 21). DhhBCS002, DhhBCS004, and DhfBCS002 were isolated at the sea surface (0 m depth), whereas DhhBCS006 was isolated at 50 m. However, DhhBCS002, DhhBCS004, and DhhBCS006 were obtained from stations close to each other along the Pacific coast (Figure 5). DhfBCS002 was the only strain isolated from surface water at Bahía de la Paz (Figure 5 and Table 1). In addition, changes in colony morphology were observed in DhhBCS002 and DhhBCS004 with increasing NaCl concentration (Figure 21).

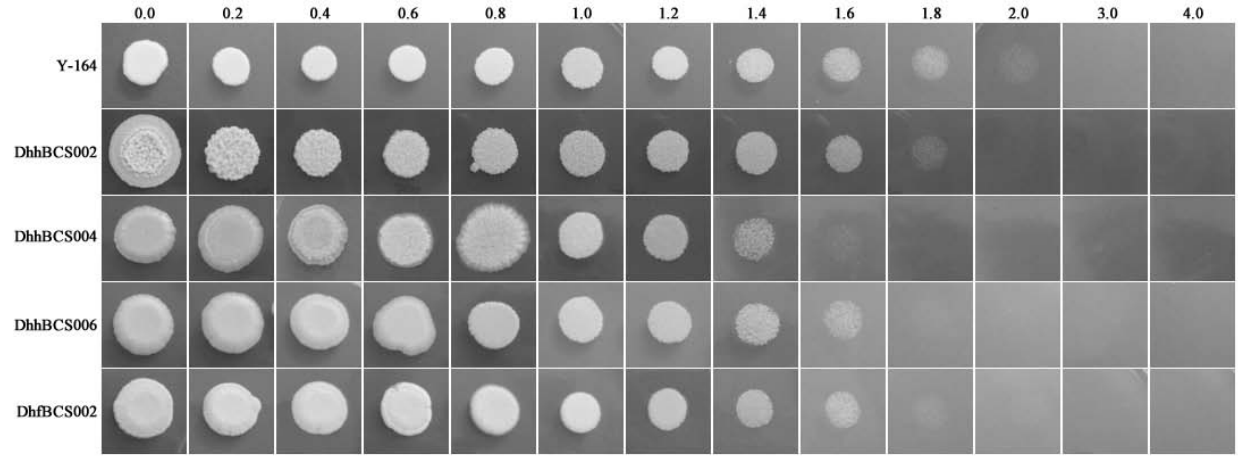

Figure 21. *Debaryomyces hansenii* strains (vertical axis) characterized as sensitive (S) based on reduced growth at concentrations less than 1.8 M NaCl (horizontal axis; M).

In brief, no direct relationship of phenotype diversity among the strains and the source of isolation was detected when cultured under either environmental condition. Table 2 summarizes the groups of tolerance levels to Co(II) and NaCl stress among *D. hansenii* strains based on the criteria established in this study. According to the Co(II) stress test, the largest group was considered T, which consisted of 12 strains (35%). The second largest group was HT with 10 strains (30%), followed by WT with 9 strains (26%). The smallest group was S, which consisted of 3 strains (9%). In addition, only 4 strains (12%) showed visible RF production. These observations suggest that most of the strains studied (65%) were able to grow at Co(II) concentrations greater than 1.3 mM (i.e., HT and T strains). However, few strains demonstrated flavinogenic activity. Based on the NaCl stress test, 23 strains were considered T (68%). The second largest group was WT with 6 strains (17%), followed by S with 5 strains (15%). No strains were grouped as HT. These results indicated that most strains in this study were capable of substantial colony growth at NaCl concentrations greater than 1.6 M (i.e., T and WT strains).

In the present study, only 3 strains (IT7, CBS 5144, and YB-327) displayed phenotypic characteristics toward greater tolerance levels for both Co(II) and NaCl stress (i.e., HT and T, respectively). On the other hand, 4 strains (DhhBCS002, DhhBCS004, DhhBCS006, and DhfBCS002) exhibited opposing phenotypes when exposed to both stress tests (i.e., HT and S in Co(II) and NaCl, respectively). Only strain YB-164, which was isolated from a meat source, displayed a highly sensitive phenotype to both Co(II) and NaCl concentrations. In general, various levels of tolerance to Co(II) stress were observed for most of the var. *hansenii* strains

(Table 2). Despite this observation, most of these strains depicted either T or WT phenotypes when exposed to NaCl stress. Among the var. *fabryi* strains (YB-327, Y-17914, DhfBCS001, and DhfBCS002), no strains were characterized as being sensitive to Co(II) stress; however, DhfBS002 was found to be sensitive to NaCl stress.

Table 2. Tolerance levels of *D. hansenii* strains to Co(II) and NaCl stress based on their ability to grow at a specific range of concentration (see Results for details). HT: highly tolerant, T: tolerant, WT: weakly tolerant, and S: sensitive.

Strain name	Source of Isolation	Level of tolerance	
		Co(II)	NaCl
IT1	Unknown	WT	T
IT2	Unknown	WT	T
IT3	Unknown	S	T
IT4	Unknown	S	T
IT5	Unknown	WT	T
IT7	Unknown	HT	T
var. *hansenii* CBS 767^{T}	Sherry (Carlsberg Research Lab, Denmark)	T	T
CBS 5144	Unknown	HT*	T
CBS 1011	Unknown	WT*	T
J6	Swedish estuary	T*	T
var. *hansenii* NRRL Y-7426	Carlsberg Research Lab, Denmark	HT	WT
var. *hansenii* NRRL Y-1448	Horse-meat sausage, France	T	WT
var. *hansenii* NRRL Y-1449	Throat of angina patient, France	T	T
var. *hansenii* NRRL Y-1454	Kentucky tobacco, Italy	T	T
var. *hansenii* NRRL YB-155	Coconut fruit salad	WT	T
var. *hansenii* NRRL YB-160	Minced ham	WT	T
var. *hansenii* NRRL YB-162	Pork	T	WT
var. *hansenii* NRRL YB-163	Meat	WT	T
var. *hansenii* NRRL YB-164	Meat	S	S
var. *hansenii* NRRL YB-165	Meat	WT	T
var. *hansenii* NRRL YB-221	Camembert cheese	T	WT
var. *hansenii* NRRL YB-306	Roka cheese spread	T	T
var. *hansenii* NRRL YB-398	High moisture corn	T	T
var. *fabryi* NRRL YB-327	Soil	HT	T
var. *fabryi* NRRL Y-17914	Interdigital mycotic lesion, Germany	WT*	T
var. *hansenii* DhhBCS001**	Surface seawater (0 m depth)	T	T
var. *hansenii* DhhBCS002**	Seawater (100 m depth)	HT	S
var. *hansenii* DhhBCS003**	Seawater (100 m depth)	HT	WT
var. *hansenii* DhhBCS004**	Surface seawater (0 m depth)	HT	S

Strain name	Source of Isolation	Level of tolerance	
		Co(II)	NaCl
var. *hansenii* DhhBCS005**	Seawater (100 m depth)	HT	WT
var. *hansenii* DhhBCS006**	Seawater (50 m depth)	HT	S
var. *hansenii* DhhBCS007**	Seawater (50 m depth)	T	T
var. *fabryi* DhfBCS001**	Seawater (100 m depth)	T	T
var. *fabryi* DhfBCS002**	Surface seawater (0 m depth)	HT	S

*Displayed visible RF production

**For locality of isolation, refer to Table 1.

3.4 Discussion

The purpose of this study was to describe and analyze the phenotypic diversity among more than 30 strains of *D. hansenii* isolated from different sources and to identify any possible correlation with environmental stress and source of isolation. To obtain this objective, these strains were exposed to oxidative (Co(II)) and osmotic (NaCl) stress at varying molar concentrations. In summary, no direct pattern of phenotypic variation among the *D. hansenii* strains was correlated to the source of isolation when cultured under either environmental condition. Similar conclusions based on the genetic and phenotypic polymorphism of *D. hansenii* isolates from cheese substrates were reached, establishing three different profiles present in the ripening process and the isolation source [16]. Shifts in the strain population of *D. hansenii* during the maturation process of fermented sausage submitted to the same conditions also suggests that a direct link between phenotypic pattern and source of isolation remains unclear [20]. Growth at 30°C, 35°C and 37°C of 55 strains of *D. hansenii* also indicated phenotypic diversity but no evident association with origin [38]. In the present study, strains isolated from marine environments (seawater and estuary) of the Pacific west coast also did not show a clear correlation with depth and locality (nearshore or offshore) associated with environmental stress, which is contrary to reports of lower number of yeast populations offshore due to higher salinities [6], and a shift from strictly aerobic to fermentative yeasts in polluted beach waters [73]. A possible explanation for this may be due to inappropriate sampling methods and possible contamination from land inputs [25,84]. Phenotype comparisons of strains from three ascomycetous yeast species, including *D. hansenii*, isolated from Moscow and Novosibirsk using physiological features was also indistinguishable without genetic intraspecific analysis [48].

Although it is well known that most isolates of *D. hansenii* have been obtained from environments with low water activity and high-salt concentrations [see 4 for a review], the results of the present study agree with the general consensus that a more thorough analysis (e.g., metabolite production, growth temperature, and response to environmental factors) that includes isolation source, phenotype, and genetic characteristics [18,50,55] is necessary to fully understand the complexity of the processes that have a direct or indirect role in the display of observable characteristics of *D. hansenii* strains.

With regard to oxidative stress by exposing *D. hansenii* strains to a range of Co(II) concentrations and thus mimicking hypoxic conditions, a qualitative analysis based on the comparison of colony growth was performed. In summary, most strains (22 out of 34 strains) were described as either highly tolerant (dense growth at 1.5 mM) or tolerant (slightly reduced growth at 1.5 mM). Very few (3) strains were categorized as being sensitive to cobalt stress. In a similar study performed by [29], growth patterns of *D. hansenii* NCYC 459 when cultured with Co(II) showed a 50% reduction in cell yield at 0.5 mM. In contrast, the present study revealed that all strains survived at 0.5 mM, while others tolerated up to concentrations that were three-fold higher. The negative growth effect of the exposure to hypoxia-mimicking agents may be counteracted by increased levels of ergosterol biosynthesis produced by greater copies of the gene *ERG25* as seen in the pathogenic yeast *Cryptococcus neoformans* [60]. Cell wall polymers may also be involved in the biosorption of cobalt ions, particularly those composed of mannans, as observed in *Debaryomyces occidentalis* after exposure to 4 M $CoCl_2$ [79]. The cellular tripeptide glutathione (GSH) has also been suggested to have critical roles in mitochondrial and membrane stability in cases of oxidative stress caused by toxic metals in several yeast species [see 74 for a review]. The response to oxidative stress in *Candida* spp. has been the expression of glutathione transferases and the formation of reactive oxygen species [1,30]. Contrary to these results, no significant role of gluthathione was detected in the accumulation of cobalt in a glutathione-deficient mutant strain of *S. cerevisiae*, but influenced the cellular uptake of other metal(loid)s [31]. Greater tolerance to cobalt ions has also been associated with the overexpression of vacuolar transporters encoded by *COT1* and *ZRC1* genes [21,22] and with components of the regulon involved in iron transport in *S. cerevisiae* [85]. Siderophore transport is yet another mechanism for iron transport that has been expressed in yeast under iron deficiency or oxidative stress [see 75 for a review]. With regard to different tolerance levels of

the strains in the present study, the observed phenotype variation among *D. hansenii* strains contributes to the mounting evidence that individual strains may express distinct mechanisms that facilitate their ability to counteract the negative effect of oxidative or hypoxic stress [18,50,55].

Given that cobalt has also been linked to the replacement of essential trace elements, such as iron [43], it has a pivotal role in the expression of the high-affinity iron transport system [85] and riboflavin (RF) production [29]. Although four different strains (CBS 1011, CBS 5144, Y-17914, and J6) of the present study coincide with reported increases of extracellular levels of RF production, this data suggests that not all strains of *D. hansenii* (otherwise known as *C. famata*) are induced by iron deficiency, caused by cobalt toxicity, to increase RF synthesis and thus affect cell survival. Therefore, it also suggested that tolerance level and RF production may not be directly correlated as previously implied by [7]. However, phenotype diversity may cause this characteristic to vary, resulting in lower cell yields with increased cobalt concentrations although RF is produced as reported by [29]. Interestingly, strains from aquatic environments did not visibly overproduce RF as seen in the marine yeast *Candida membranifaciens* subsp. *flavinogenie* W14-3 [91]. Another unexpected observation in the present study was the high tolerance level of most strains of marine origin to oxidative stress, which may be attributed to polluted marine and estuarine waters [27,40], to iron deficiency [82], or to the possible competition of iron for growth by other microorganisms [63]. These results suggest that these strains may have developed an efficient physiological system that has adapted to events of extreme oxidative stress.

With regard to osmotic stress by exposing *D. hansenii* strains to a range of NaCl concentrations, a qualitative analysis based on the comparison of colony growth was also conducted. In summary, no strains were considered highly tolerant but were mostly characterized as being tolerant (dense growth up to 2 M) to NaCl stress (23 out of 34 strains). These findings demonstrate that most of the strains analyzed are capable of surviving under rather extreme osmotic stress as compared to the model yeast *S. cerevisiae* [44-46]. Accumulating osmolytes, such as glycerol, and reducing cell volume to acclimate to osmotic stress in the form of high-salt concentrations has been elaborated in *D. hansenii* [42,77,87]. Glycerol production and accumulation has also been associated with positive turgor pressure based on intracellular solute concentrations at salinities of 1.35 M NaCl [57]. A key enzyme of glycerol production involved

in *D. hansenii* is glycerol-3-phosphate dehydrogenase and is considered NAD-dependent [2,71]. *Debaryomyces hansenii* CBS 767^T and Y-7426 accumulate glycerol and the disaccharide trehalose at much higher levels than *S. cerevisiae* when exposed to saline stress [35]. Antiporters for the expulsion of H^+/Na^+ and K^+ cations [9,10,17,89] and the P-type ATPase pump encoded by *ENA1* may play critical roles in regulating sodium toxicity [34,36,81]. Particularly in *D. hansenii* CBS 767^T, *ENA1* and hyphal cell/wall elongation gene expression has been associated with osmotic stress at 2 M NaCl [34]. Other mechanisms of coping with osmotic stress have been associated with the phosphorylation of Dhog1p in the cytoplasm during growth [83], de novo tyrosine synthesis followed by immediate oxidation to 3-nitrotyrosine [15], the presence of Ppz protein phosphastases [94], and the expression of the Na^+, K^+ and H^+ antiporter gene *DhNHX1* [66]. It has also been suggested that intracellular pH homeostasis and pKa maintenance can affect tolerance to saline stress in *D. hansenii* [68].

Based on the results of the present study, it is proposed that most of the *D. hansenii* strains analyzed are capable of tolerating high-salt stress (up to 2 M NaCl after 3 days of incubation) by the most commonly identified mechanisms such as glycerol metabolism [2, 35,71] and extrusion of cation ions by antiporters [9,10,17,89]. Interestingly, the strains isolated from aquatic environments (seawater and estuarine) were considered the least tolerant (mostly growing up to 1.6 M), suggesting that these strains may not have developed the mechanisms necessary to adapt to saline stress since the average salt content of ocean water is 35 psu or 3.5% salt [49]. These strains mostly tolerated up to the equivalent of 9.36% salt as opposed to the 11.7% tolerated by most of the other strains. Despite the possible biochemical mechanisms that may be involved in the response to osmotic stress that may be consequently observed in the form of phenotype variation, the accumulating evidence for genetic heterogeneity [55,69] should also be considered as an essential criteria for a more complete characterization of *D. hansenii* strains.

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Chapter 4: Genetic Variability among *Debaryomyces hansenii* Strains of Inducible Genes *FET3* and *ENA1* associated with Cobalt(II) and Saline Stress

4.1 Introduction

Differentiation among yeast strains of the same species has become an area of keen interest due to reported variations on a phenotypic and genotypic level [12,15,41]. Phenotype heterogeneity was reported among 50 strains of the model yeast *Saccharyomyces cerevisiae* in response to changes in the environment, exhibiting variations in stress sensitivity and gene expression [49]. This phenotypic diversity may be associated with similarities and differences in yeast habitats and in the variability of genetic expression linked to metabolic processes of industrial importance or to stress resistance [17,49,79]. In natural isolates of *S. cerevisiae* exposed to metal stress (i.e., copper sulfate), differences in gene expression pattern combined with gene function suggests distinctions in expression patterns that may be correlative or causative [35]. However, a clear relationship has not been established among differentially expressed genes and phenotype, with the exception of specific genes. In this same study [35], only one particular gene associated with freeze tolerance, *AQY2*, showed significant gene expression patterns directly linked to the predicted phenotype characteristics. In contrast, an analysis on genomic variability among natural strains of *S. cerevisiae* isolated from various sources with different environmental conditions suggested that sub-telometric regions and Ty-element insertion sites highly contributed to genome diversity and subsequent phenotype distinctions associated with the isolation source [16]. Therefore, continuing efforts to elucidate a direct correlation between gene expression (mostly referring to patterns) and phenotype by inherent mechanisms remain ardent [15].

Given that physiological traits (phenotype) of the variant strains of the hemiascomycete *Debaryomyces hansenii* (var. *hansenii* and var. *fabryii*) have been used to characterize this species, there is substantial evidence of phenotypic heterogeneity that suggests the presence of genome structure variability [see 11 for a review]. Subsequently, these observations have given rise to the analysis of genetic polymorphism in strains, revealing differences in chromosomal

length, number, and arrangement [22,63]. Approaches for species delineation of *D. hansenii* have been performed to re-examine and re-instate its taxonomy based, not only on well-established phenotype characteristics, but on phylogenetic and gene-genealogy analyses that demostrate signifcant sequence divergence among strains [22,41]. Various polymerase chain reaction (PCR)-based DNA typing methods such as the amplification of the small subunit of rRNA [40], intergenic spacer (IGS) rDNA [21,60] and interspacer (ITS) regions [64], mini- and microsatellites [41], and spliceosomal intron sequences of housekeeping genes [22] have been used to demonstrate genetic heterogeneity among strains. One particular study differentiated four patterns among 170 strains of *D. hansenii*, *Candida famata*, *D. fabryi* and *C. flareri* (*D. subglobosus*) and *D. prosopidis* using IGS *Alu*I fingerprinting, which also resulted in the description of a novel *Debaryomyces* sp. [60]. Hence, differential gene expression pattern may not be the sole explanation for phenotypic diversity among strains, and genomic variation with regard to gene structure should also be included as a potential factor when differentiating among *D. hansenii* strains.

Differences in phenotype characterization in response to cobalt and saline stress of *D. hansenii* strains studied in the previous chapter of this dissertation prompted us to analyze sequence variability of inducible genes as a causative element of phenotype diversity. Therefore, the purpose of this study was to analyze the DNA sequence heterogeneity of the multicopper oxidase gene *FET3*, which is inducible by the presence of cobalt [76] and is associated with the high-affinity iron transport system, and the P-type ATPase sodium pump gene *ENA1*, which is also upregulated by high-salt concentrations [37,39,71], from the 34 *D. hansenii* strains (Table 1) studied in Chapter 3.

The mechanisms of iron transport has been well-studied in the model yeast system *Saccharyomyces cerevisiae* and in other yeast species [see 81 for a review]; however, a complete understanding of the processes involved in the iron regulation, transport, and storage in these species continues to unfold [45,51,52,55,57]. The initial step begins with the reduction of insoluble ferric iron (Fe^{+3}) to soluble ferrous iron (Fe^{+2}) by transmembrane ferrireductases [23,42]. Through the high-affinity transport system, the reduced iron species is then oxidized and transported intracelluarly by the transmembrane multicopper oxidase and permease complex encoded by *FET3* and *FTR1*, respectively [3,26,74,77]. A genomic RNA expression profiling of *S. cerevisiae* exposed to cobalt stress (up to 2 mM $CoCl_2$) revealed upregulated gene activity of

FET3 and *FTR1*, among other genes associated with iron and siderophore transport [76]. Homology of the *FET3* gene product and its higher enzymatic activity in *Pichia pastoris* [62], and its identification in other ascomycetes [4,18,32,62] have also been reported. Based on this evidence and its essential role in iron uptake, *FET3* was identified as a suitable candidate for detecting genetic heterogeneity of an inducible gene in association with phenotype variation among *D. hansenii* strains exposed to cobalt stress in the present study.

Based on the observations of *D. hansenii* strains upon osmotic stress in Chapter 3 (see Results), the gene encoding the P-type ATPase pump, *ENA1*, was considered as an appropriate marker for genetic variability and was highly likely to reveal sequence divergence associated with phenotypic diversity due to its key role in sodium detoxification [37,69,71]. Although several studies have demonstrated the physiological mechanisms of coping with high salt stress in *D. hansenii* by means of osmolyte production, antiporters, phosphatases, amino acid synthesis for subsequent oxidation, and intracellular pH homeostasis [see Discussion in Chapter 3], few genes implicated in the regulation and metabolic pathways of osmoadaptation have been described in this species. Heterologous expression of *D. hansenii* genes homologous to *S. cerevisiae HOG1* (mitogen-activated protein kinase) [5], *PBS2* (MAP kinase kinase) [6], *GPD1* (glycerol-3-phosphate dehydrogenase) [78], *KHA1* (putative K^+/H^+ antiporter) [14], *ARO4* [13] in *S. cerevisiae* mutants have made it possible to decipher their function in osmotolerance. Genome-wide expression studies of *D. hansenii* exposed to high-salt concentration (2 mM) resulted in the upregulation of *ENA1*, *HWP* (hyphal wall protein), and mostly genes encoding for translational proteins [37]. Increased expression levels of *DhGPD1* and *DhGPP2* (DL-glycerol-3-phosphatase 2) were also correlated to high-salt stress [38], whereas *DhKHA1* [14] and *DhNHX1* (putative Na^+/H^+ exchanger) [56] showed no significant changes under similar conditions. Although attempts to understand the precise regulatory mechanisms linked to osmoadaption in this species continues, there is a lack of information regarding the structure of these genes and their possible variation among strains, which may be a contributing factor to phenotype diversity.

Therefore, the aim of this chapter was to identify heterogeneity in the open reading frame or protein coding region of the inducible genes multicopper oxidase *FET3* and the P-type ATPase sodium efflux *ENA1* of 34 *D. hansenii* strains isolated from several sources using restriction fragment length polymorphism (RFLP) [72]. Amino acid sequence comparisons of the

predicted translated products of each gene were also analyzed to identify conserved regions that may be affected by DNA sequence variations detected by RFLP.

4.2 Materials and Methods

4.2.1 Yeast strains, media, and DNA extraction. *Debaryomyces hansenii* (Zopf) Lodder & Kreger-van Rij var. *hansenii* (1985), *D. hansenii* (Zopf) Lodder & Kreger-van Rij var. *fabryi* (Ota) Nakase & M. Suzuki (1985), and *D. hansenii* strains used in this study are described in Table 1. All cell cultures were maintained in yeast-peptone-dextrose (YPD; 2% yeast, 2% peptone, 2% dextrose) plates or broth and stored as glycerol stocks in -80°C. Genomic DNA extraction of *D. hansenii* strains was performed by the methods described by [53] and using Zymo Genomic DNA extraction kit (Zymo Research).

4.2.2 PCR amplification. The first and second release of the genome sequence of *D. hansenii* CBS 767^T (Génolevures Database; http://www.genolevures.org/) was used to design primers to amplify the partial regions of the open reading frames (ORF) of *FET3* (DEHA0E13332g= DEHA2E12738g; 1,893 bp) and *ENA1* (DEHA0C02937g=DEHA2C02552g; 3,231 bp) genes from extracted genomic DNA of the *D. hansenii* strains described in Table 1. The *FET3* gene was selected specifically for analysis based on its induction upon cobalt(II) stress and its role in cobalt(II) tolerance in *Saccharyomyces cerevisiae* [76]. The *ENA1* gene was selected because of its up-regulated expression in *D. hansenii* $CBS767^T$ in response to high salt (NaCl) concentrations [37]. The primer design was optimized using the software NetPrimer (Premier Biosoft International; http://www.premierbiosoft.com/netprimer/index.html). Primers used to amplify *FET3* were forward primer F58 (5′ ACTCACACTTTCCATTTCAA 3′) and reverse primer R1860 (5′ GCCGATTGTTCACTTTCA 3′). PCR amplification was performed using a initial denaturation of 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 35-40°C for 45 s, 72°C for 2 min, and 72°C for 5 min. Primers used to amplify *ENA1* were ENA1_F2 (5′ GATCGATTGAGAACAGTA 3′) and ENA1_R2 (5′ TTCGATTTCGAGAATGAAG 3′). PCR amplification was performed with an initial denaturation of 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 30-40°C for 30 s, 72°C for 3.5 min, and 72°C for 7 min. All PCR reactions were conducted in an automatic thermal cycler. The resultant 1.8 kilobase pairs (kbp)

and 3.2 kbp PCR products for *FET3* and *ENA1*, respectively, were gel extracted using the Zymo Gel Extraction Kit with 1.5% agarose (Zymo Research). To obtain a total of ~1-2 µg of PCR product for each gene, the initial gel-extracted PCR product was then used as a DNA template for subsequent amplification with the same PCR conditions described in this study. PCR products were then gel extracted and stored in -80°C for subsequent restriction enzyme digests.

4.2.3 Genotyping by restriction fragment length polymorphism (RFLP) analysis. The partial ORFs of *FET3* and *ENA1* that were amplified successfully from *D. hansenii* strains were analyzed for DNA sequence variation using RFLP [72]. Several strains were unsucessfully amplified using the primers designed for this study (see Results). This method exposes the DNA sample to restriction enzymes (nucleases) that identify and cut the DNA fragment based on specific sequences (usually palindromes), depending on the type of enzyme used. Restriction enzymes *Eco*RI (Fisher), *Hin*dIII (Fisher), *Bam*HI (Fermentas), and *Sau*3A1 (Fisher) were used to digest the PCR product of *FET3* according to the manufacturer's instructions. The PCR product of *ENA1* was digested with *Sau*3A1 (Fisher) according to the manufacturer's instructions. Approximately 0.3-0.5 µg of each PCR product was used for each restriction enzyme digest. Digests were electrophoresed using 0.8% agarose and TAE 1X buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). All restriction profiles were run with 1-kilobase ladder (GeneRuler) as a marker. The restriction fragments were visualized by ethidium bromide staining and UV transillumination. The restriction maps of *D. hansenii* CBS 767^T *FET3* and *ENA1* genes were generated using Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/) and Restriction Mapper version 3 (http://www.restrictionmapper.org).

4.2.4 Identification of conserved regions and transmembrane helices in the amino acid sequence of *D. hansenii* CBS 767^T Fet3p and Ena1p. Translation of nucleic acid sequences to amino acid residues beginning with the first nucleotide in the ATG initiation codon were generated using ExPASy Translate Tool (Swiss Institute of Bioinformatics; http://web.expasy.org/translate/) and was confirmed by the predicted translation product of each gene (*FET3* and *ENA1*, respectively) in the Génolevures Database (http://www.genolevures.org/). To identify and compare the predicted copper and iron ligands required for enzymatic activity, and the cysteine (C) residues as reported by [62] and in the amino acid sequence of *D. hansenii* CBS 767^T Fet3p (GenBank Accession No. XP_459860), a multiple sequence alignment of *Candida albicans* Fet3p (Accession No. CAA70509), *Candida*

glabrata Fet3p (Accession No. BAB62813), *Pichia (Komagataella) pastoris* Fet3p (Accession No. CAC33177.2), *S. cerevisiae* Fet3p (Accession No. NP_013774), and *Schizosaccharomyces pombe* multicopper oxidase Fio1p (Accession No. CAA91955) was performed using default parameters of CLUSTALW version 2.1 [50; http://www.ebi.ac.uk/Tools/msa/clustalw2/]. To compare the amino acid sequence of *D. hansenii* CBS 767^T Ena1p (Accession No. AAK28385.2) with the P-type ATPases of similar yeasts, a multiple alignment of *C. albicans* Ena2p (Accession No. XP_719032), *C. glabrata* Ena1p (Accession No. XP_448738), *S. cerevisiae* Ena1p (Accession No. CAA98867.1), and *S. pombe* P-type ATPase Cta3p (Accession No. NP_595246) was performed using default parameters of ClustalW version 2.1 [50]. To predict the transmembrane helices in the amino acid sequence of Fet3p and Ena1p in *S. cerevisiae* and *D. hansenii* CBS 767^T, the software program TMHMM version 2.0 (Center for Biological Sequence Analysis, Technical University of Denmark DTU; http://www.cbs.dtu.dk/services/TMHMM/) was used.

4.3 Results

4.3.1 Genotype profiling of *D. hansenii* strains by *FET3-PCR* RFLP analysis. Initially, PCR amplification was performed to reveal possible *FET3* sequence variation in *D. hansenii* strains that demonstrated phenotypic variability from the type strain (i.e., CBS 767^T) when exposed to Co(II) stress. Two different combinations of primer sets were originally designed to amplify the partial ORF of *FET3* based on the genomic sequence of *D. hansenii* CBS 767^T. However, only one primer set was capable of generating the expected PCR product size of 1,808 bp (96% of the 1,893 bp sequence length) in most strains (see Materials and Methods). *Debaryomyces hansenii* var. *fabryi* YB-327 and Y-17914 generated only ~800 bp PCR products with the same set of primers (data not shown). No PCR products were obtained from eight strains (var. *hansenii* DhhBCS002 to var. *hansenii* DhhBCS006 and var. *fabryi* DhfBCS002; Table 1). Although no PCR products were observed, prior analyses of 28S and 26S rRNA sequences of these strains showed high similarity to *D. hansenii* var. *hansenii* and var. *fabryi* (data not shown). For most strains, the partially amplified region resulted in a single band of

about 1,800 bp, although mispriming or non-specific binding frequently occurred under the PCR conditions described in Materials and Methods (data not shown).

Discrimination of DNA sequence variation in the partially amplified ORF region of *FET3* among *D. hansenii* strains was performed using RFLP analysis. For the RFLP, PCR products of 26 strains were successfully amplified using primers designed from nucleotides (nt) 58 and 1860 of the full length sequence of *D. hansenii* CBS767^{T} *FET3*. After PCR amplification, four restriction enzymes were used to generate fragment lengths that form a restriction profile specific to the sequence produced by each strain (Table 3).

Table 3. Detailed information of the restriction enzymes used to generate *FET3* RFLP profiles among *D. hansenii* strains. The frequency of cuts, nucleotides of sites (5′→3′), and generated fragment lengths refer to the following digestions of the 1,808 bp PCR-amplified product of *D. hansenii* CBS767^{T} *FET3*. Generated fragments lengths are listed in descending order according to size.

Name	Recognition Site	Frequency	Nucleotides of Sites (nt)	Generated Fragment Lengths (bp)
*Bam*HI	G/GATCC	1	253	1555, 253
*Eco*RI	G/AATC	2	528, 877	931, 528, 349
*Hin*dIII	A/AGCTT	4	886, 898, 1485, 1753	886, 587, 268, 55, 12
*Sau*3AI	G/ATC	7	74, 120, 254, 1237, 1263, 1293, 1442	984, 366, 149, 133, 74, 46, 30, 26

Six restriction profiles were detected following *Bam*HI, *Eco*RI, *Hin*dIII, and *Sau*3AI digests of the ~1,800 bp PCR product for each strain by gel electrophoresis analysis (Figures 22-25). The restriction profile generated by CBS 767^{T} for each enzyme used is referred to as the type sequence (TS) and was the most frequent profile in all the restriction enzyme digests. Following *Bam*HI digest, two restriction profiles were observed. The restriction profile, TS, consists of two bands (1,555 and 253 bp; Table 3) (Figure 22). Strains CBS 5144, CBS 1011, J6, and YB-155 showed the restriction profile referred to as R-I (Figure 22). This profile consisted of one band at about 1,800 bp, indicating that the PCR product lacked the sequence recognition site and, thus was not digested by *Bam*HI.

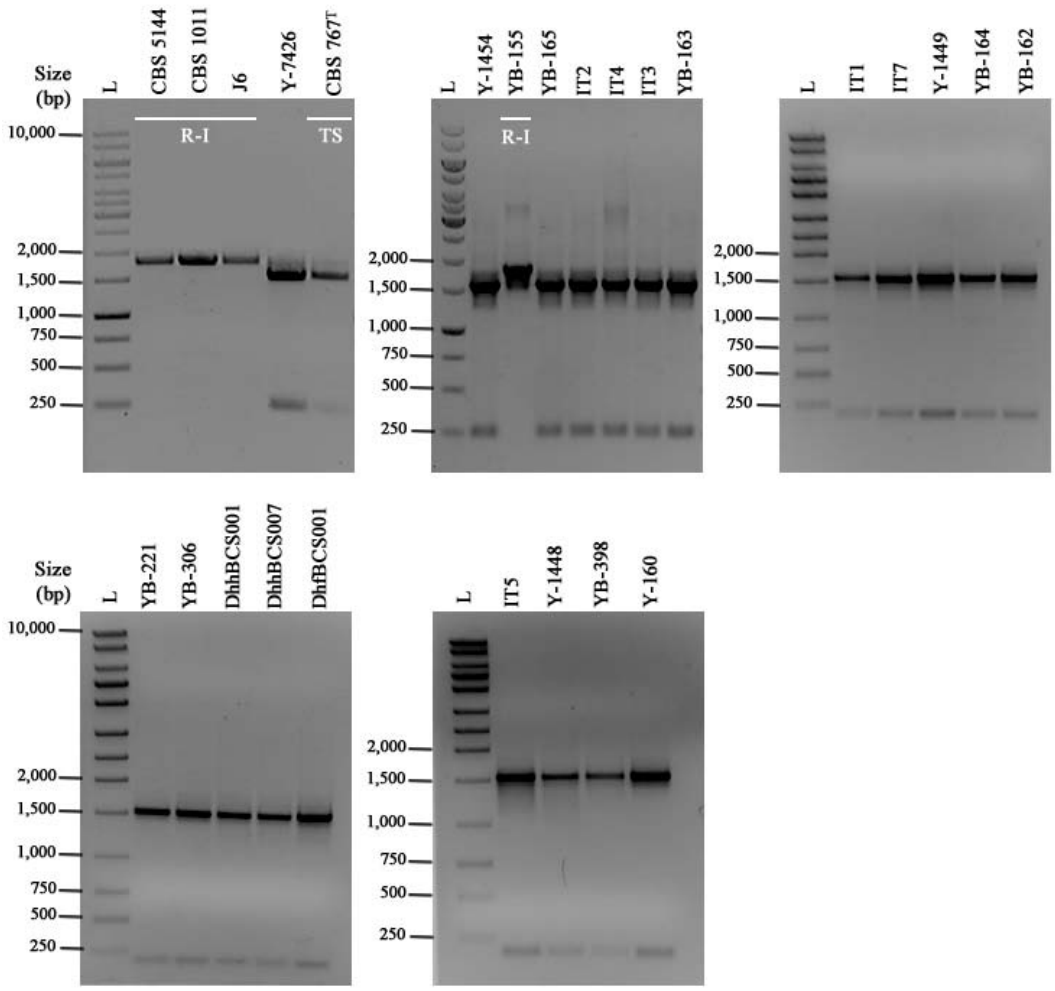

Figure 22. Separation on agarose gels of *Bam*HI restriction fragments of the partially amplified open reading frame (ORF; ~1,800 bp) of *FET3* from *D. hansenii* strains (labeled for each well). Restriction profiles TS (type sequence) and R-I are shown. L: 1-kb ladder (GeneRuler).

For the *Eco*RI digest, two restriction profiles were also observed (Figure 23). Restriction profile TS showed three bands consisting of 931, 528, and 349 bp that were generated from two recognition sites (Figure 23; Table 3). A distinct restriction profile, R-II, was revealed in CBS 5144, CBS 1011, and J6. This profile showed a thick band slightly smaller than 1,000 bp (Figure 23), suggesting that this sequence had only one *Eco*RI site that generated fragments of similar size when digested.

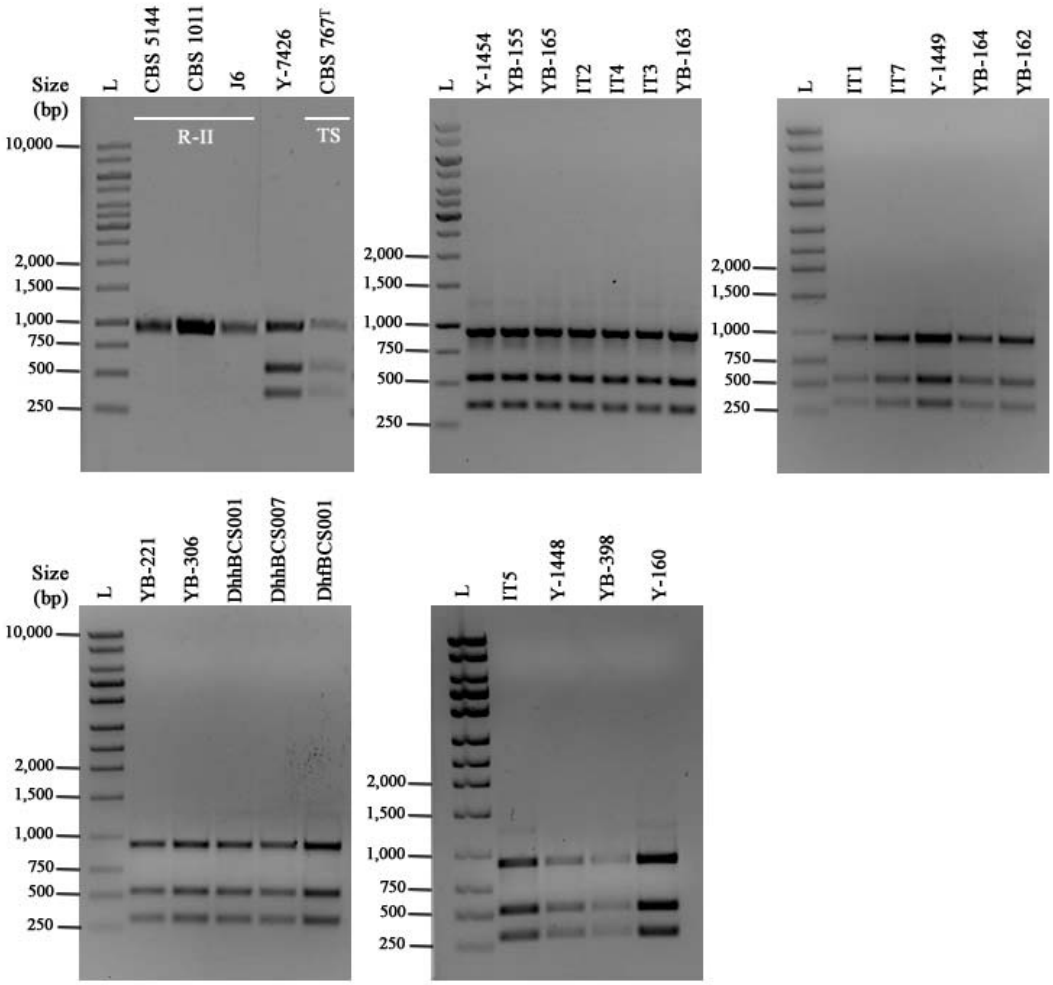

Figure 23. Separation on agarose gels of *Eco*RI restriction fragments of the partially amplified ORF (~1,800 bp) of *FET3* from *D. hansenii* strains (labeled for each well). Restriction profiles TS (type sequence) and R-II are shown. L: 1-kb ladder (GeneRuler).

Two restriction profiles were also seen following *Hin*dIII digest (Figure 24). The TS profile consists of five generated fragments (Table 3), but only three bands (886, 587, and 268 bp) were plainly visible due to the resolution of the gel electrophoresis (Figure 24). In contrast, strains CBS 5144, CBS 1011, and J6 showed the restriction profile R-III. This profile depicted a thick band slightly smaller than 1,000 bp. This observation suggests that the *Hin*dIII site 3 (nt 1485) was mutated in these strains, resulting in fewer number of fragments after digestion.

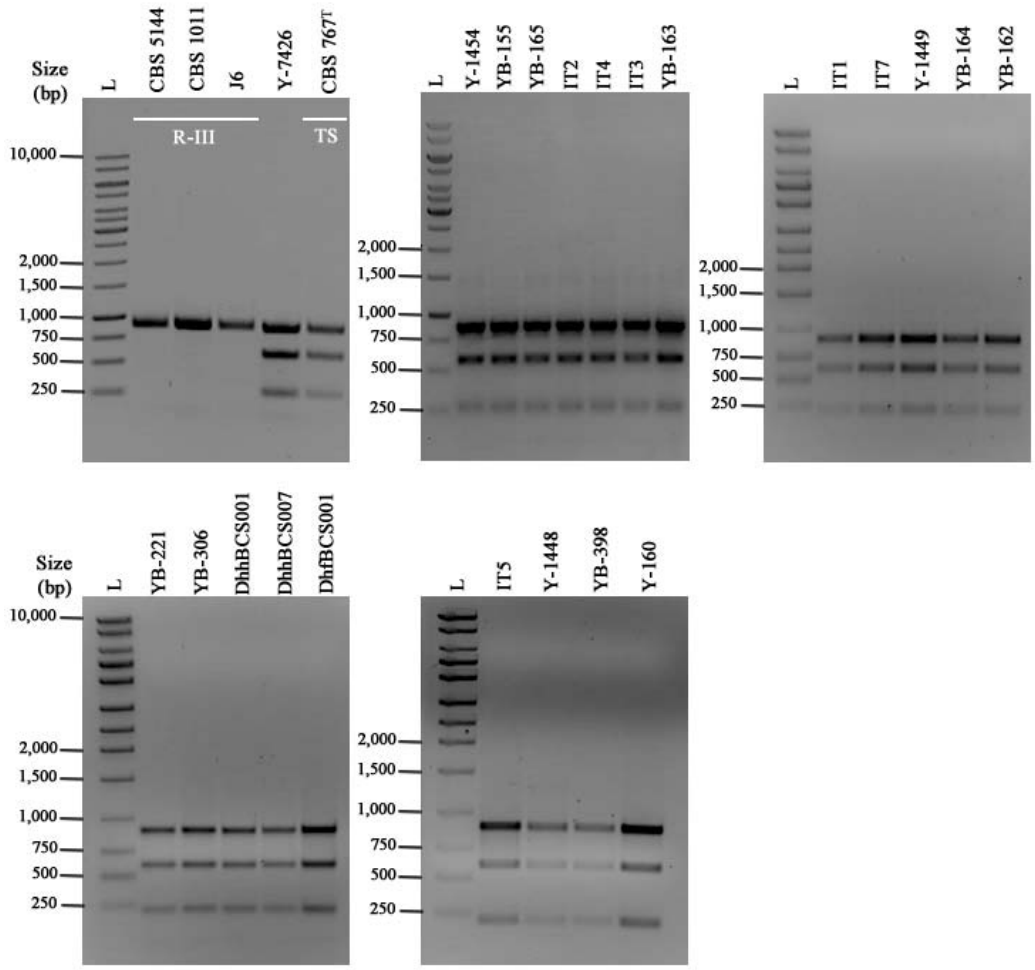

Figure 24. Separation on agarose gels of *Hin*dIII restriction fragments of the partially amplified ORF (~1,800 bp) of *FET3* from *D. hansenii* strains (labeled for each well). Restriction profiles TS (type sequence) and R-III are shown. L: 1-kb ladder (GeneRuler).

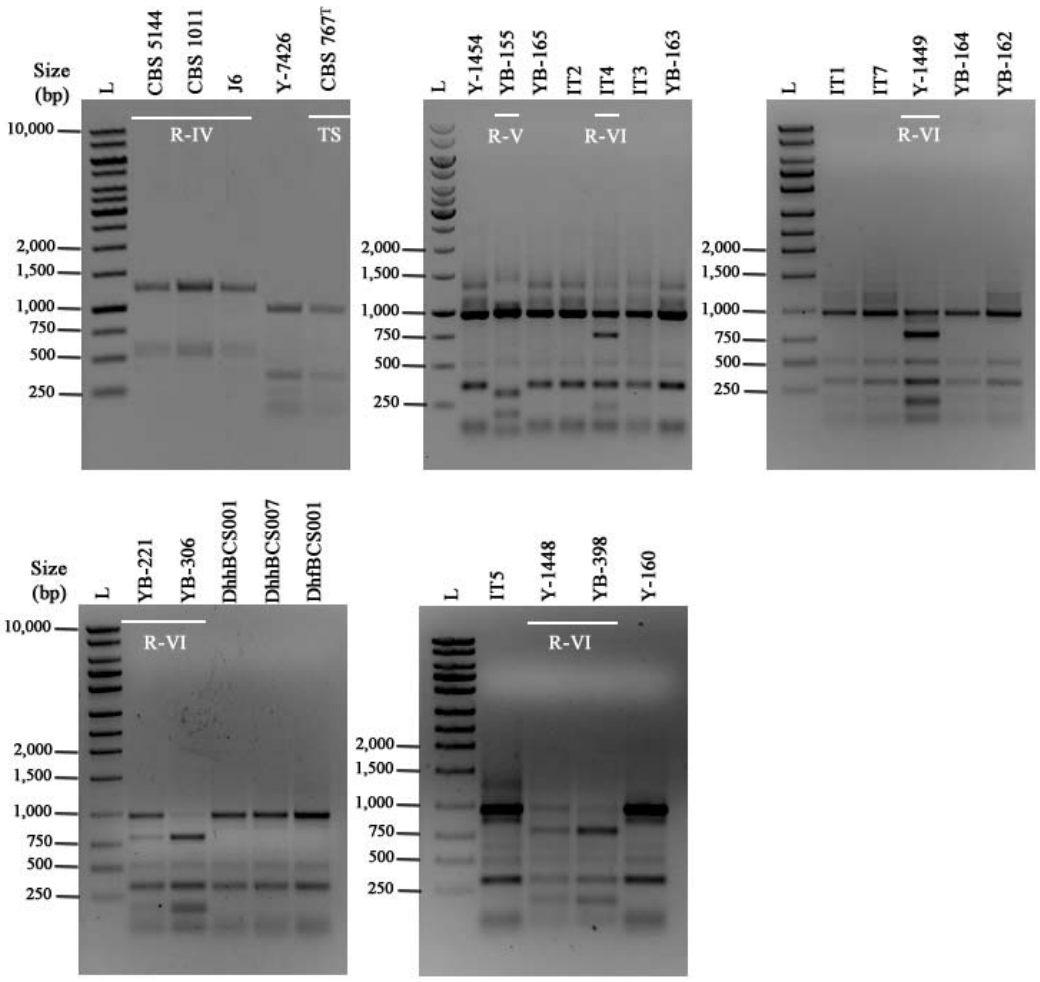

Figure 25. Separation on agarose gels of *Sau*3AI restriction fragments of the partially amplified ORF (~1,800 bp) of *FET3* from *D. hansenii* strains (labeled for each well). Restriction profiles TS (type sequence), R-IV, R-V and R-VI are shown. L: 1-kb ladder (GeneRuler).

For the *Sau*3AI digest, four restriction profiles were revealed by gel electrophoresis analysis (Figure 25). It must be noted that the sequence recognition site of *Sau*3AI is only 4 bp (G/ATC), whereas all the other enzymes have 6 bp recognition sites (Table 3). This particular characteristic allows for more discriminative power over the other enzymes used in this study due to the higher frequency of detecting these recognition sites throughout the sequence. Following the digest, the TS profile consisted of eight generated fragments, of which mostly were seen as three thick bands (984 and 366 bp, and sizes smaller than 100 bp; Figure 25). Strains CBS 5144, CBS 1011, and J6 exhibited restriction profile R-IV (Figure 25). Based on the size of these bands (both greater than 1,000 and 500 bp), this profile suggests that the first three

*Sau*3AI sites (nt 74, 120, and 254; Table 3) and the last *Sau*3AI site (nt 1442) from the TS profile were mutated in these strains. When comparing the *Sau*3A1 site 3 (nt 254) of profile R-IV with the *Bam*HI site (nt 253) of profile R-I, the results indicate that a mutation occurred within the *Sau*3A1 site that consequently eliminated the *Bam*HI recognition site of profile R-I.

The profile R-V was discovered only in strain YB-155, which generated a band slightly closer to 1,000 bp (Figure 25). This observation suggests that the *Sau*3A1 site 3 (nt 254) from the TS profile suffered a mutation that eliminates the *Bam*HI recognition site. The difference in size fragments produced by profile R-V highly supports the presence of various mutations in *Sau*3AI sites 3 through 7 of the TS profile (nt 254, 1237, 1263, 1293, and 1442, respectively; Table 3) and that other base substitutions have occurred, resulting in new *Sau*3AI recognition sites. This profile also showed a band (~200 bp) that is less than the 366 bp fragment generated by the TS profile, suggesting that this sequence has an additional *Sau*3A1 site after the last site (close to nt 1642). Another possible sequence variation that would generate this size fragment is a mutation at *Sau*3AI sites 5 and 6 (nt 1266 and 1293, respectively, Table 3). However, if these sites were mutated, fewer fragments of small size would be observed at the bottom of the gel, which is not the case (Figure 25).

Another restriction profile, R-VI, was revealed in IT4, Y-1449, YB-221, YB-306, Y-1448, and YB-398 (Figure 25). The observed generated fragments lengths suggest that all seven of the recognition sites as identified in the TS profile are present. However, the band near 750 bp suggests that an additional recognition site is located within *Sau*3AI sites 3 and 4 (nt 254 and 1237, respectively; Table 3). This additional site would generate a size fragment of about 200 bp, but less than 250 bp. Therefore, the *Sau*3AI recognition sites most probable to create that particular size fragment are proposed within nt 454 to 483 with reference to site 3 or within nt 1007 to 1037 based on the location of site 4.

In summary, the RFLP analysis discriminated 10 strains with sequences variations from the TS profile (Table 4). CBS 5144, CBS 1011, and J6 portrayed the same restriction profiles (i.e., R-I, R-II, R-III, and R-IV after *Bam*HI, *Eco*RI, *Hin*dIII, and *Sau*3AI digestion, respectively). IT4, Y-1448, YB-221, YB-306, and YB-398 revealed the TS profile for *Bam*HI, *Eco*RI, and *Hin*dIII but exhibited profile R-VI with the *Sau*3AI digestion. YB-155 depicted a combination of profiles R-I (*Bam*HI), TS (*Eco*RI and *Hin*dIII), and R-V (*Sau*3AI). Hence, these observations suggest that CBS 5144, CBS 1011, J6, and YB-155 have greater genetic variability,

although they also conserve certain recognition sites of the type sequence from CBS 767^{T}. However, the *FET3* profiles of var. *hansenii* DhhBCS002 to DhhBCS006 were not characterized because of unsuccessful PCR amplification using the same primers. These results imply that their genetic sequences are likely to vary from the TS profile and that regions that would be considered mostly conserved among *D. hansenii* may show greater heterogeneity in these particular strains. Among the var. *fabryi* strains, the *FET3* profiles of YB-327, Y-17914, and DhfBCS002 are also absent due to mispriming or for the lack of PCR amplification. In contrast, only DhfBCS001 *FET3* was successfully amplified and depicted a TS profile. These observations indicate that a substantial genotypic variation exists in *D. hansenii* strains, including unidentified variants analyzed in this study. Overall, strains that showed greater genetic sequence variation strongly suggests the possibility of cryptic species among this complex of *D. hansenii* species.

Table 4. Comparison of *FET3* RFLP profiles of *D. hansenii* strains with the partially amplified type sequence (TS) of CBS767^{T}. R-I: no *Bam*HI site at nucleotide (nt) 253; R-II: no *Eco*RI site at nt 528; R-III: no *Hin*dIII site at nt 1485; R-IV: no *Sau*3AI sites at nt 74, 120, 254, and 1442; R-V: potential recognition sites within *Sau*3AI sites 3–7 (nt 254 to 1442) and *Sau*3AI near nt 1642; and R-VI: additional *Sau*3AI site between nt 254 and 1237; NA: PCR product not amplified.

Strain name	*FET3* RFLP Profile			
	***Bam*HI**	***Eco*RI**	***Hind*III**	***Sau*3AI**
IT1	TS	TS	TS	TS
IT2	TS	TS	TS	TS
IT3	TS	TS	TS	TS
IT4	TS	TS	TS	R-VI
IT5	TS	TS	TS	TS
IT7	TS	TS	TS	TS
var. *hansenii* CBS 767^{T}	TS	TS	TS	TS
CBS 5144*	R-I	R-II	R-III	R-IV
CBS 1011*	R-I	R-II	R-III	R-IV
J6*	R-I	R-II	R-III	R-IV
var. *hansenii* NRRL Y-7426	TS	TS	TS	TS
var. *hansenii* NRRL Y-1448	TS	TS	TS	R-VI
var. *hansenii* NRRL Y-1449	TS	TS	TS	R-VI
var. *hansenii* NRRL Y-1454	TS	TS	TS	TS
var. *hansenii* NRRL YB-155	R-I	TS	TS	R-V

Strain name	*FET3* RFLP Profile			
	***Bam*HI**	***Eco*RI**	***Hind*III**	***Sau*3AI**
var. *hansenii* NRRL YB-160	TS	TS	TS	TS
var. *hansenii* NRRL YB-162	TS	TS	TS	TS
var. *hansenii* NRRL YB-163	TS	TS	TS	TS
var. *hansenii* NRRL YB-164	TS	TS	TS	TS
var. *hansenii* NRRL YB-165	TS	TS	TS	TS
var. *hansenii* NRRL YB-221	TS	TS	TS	R-VI
var. *hansenii* NRRL YB-306	TS	TS	TS	R-VI
var. *hansenii* NRRL YB-398	TS	TS	TS	R-VI
var. *fabryi* NRRL YB-327	NA	NA	NA	NA
var. *fabryi* NRRL Y-17914*	NA	NA	NA	NA
var. *hansenii* DhhBCS001	TS	TS	TS	TS
var. *hansenii* DhhBCS002	NA	NA	NA	NA
var. *hansenii* DhhBCS003	NA	NA	NA	NA
var. *hansenii* DhhBCS004	NA	NA	NA	NA
var. *hansenii* DhhBCS005	NA	NA	NA	NA
var. *hansenii* DhhBCS006	NA	NA	NA	NA
var. *hansenii* DhhBCS007	TS	TS	TS	TS
var. *fabryi* DhfBCS001	TS	TS	TS	TS
var. *fabryi* DhfBCS002	NA	NA	NA	NA

*Strains with visible riboflavin production upon cobalt stress (see Results in Chapter 3).

4.3.2 Comprehensive analysis of variation in nucleotide and translational sequences of *FET3* in *D. hansenii* CBS 767^{T} and in similar yeasts. To identify the predicted copper and iron ligands required for catalytic activity as reported in [62], the cysteine residues (C) commonly found in the development of a disulphide bridge in multicopper (blue) oxidases, laccase, ascorbate oxidase, and ceruloplasmin [54] of *D. hansenii* CBS767^{T} Fet3p, a comparative amino acid sequence analysis was perfomed with *C. glabrata* Fet3p, *S. cerevisiae* Fet3p, *C. albicans* Fet3p, *P. pastoris* Fet3p, and *S. pombe* Fio1p using ClustalW. All residues necessary for enzymatic activity were consistent in *D. hansenii* CBS767^{T} (Figures 26 and 27). The nucleotide sequence variations from the TS profile of CBS767^{T} following restriction enzyme digests are depicted based on profiles R-I, R-II, R-III, and R-IV in accordance with its predicted translated product (Figure 28). In the first 124 bp of the partially amplified ORF of *FET3*, profile R-IV revealed two mutated *Sau*3AI sites (Figure 28). These recognition sites are not markedly

close to any of the potential ligand sites of the enzyme, suggesting that these particular variations may not affect catalytic activity. However, a *Bam*HI and *Sau*3AI mutations at nt 253 to 258, which are near the first C residue (Cys99), were observed in profiles R-I and R-IV, respectively (Figures 26 and 28). Strains depicting these profiles may alter the conformational structure of the protein at this particular site. Similarily, an *Eco*RI mutation is positioned at nt 258 as revealed by profile R-II and is near the potential iron ligand or the glutamic acid residue (E) of the amino acid sequence N-P-T-G-A-E-P-I-P-D that is highly conserved among all the yeasts analyzed, except for two residues in *S. pombe* Fio1p (Figures 26 and 28). A proposed *Sau*3AI site within nt 454 to 483 (residues 171 to 180) or nt 1007 to 1037 (residues 352 to 365) based on profile R-VI (regions not shown in Figure 28) may be found near a potential iron ligand (E or Y, respectively), indicating potential changes in catalytic activity in the strains portraying this profile. However, no apparent mutations were detected in nt 877 to 1297 of the TS recognition sites *Eco*RI, *Hin*dIII, and *Sau*3AI, except for those mutations suggested by profile R-V (regions not shown). Subsequently, changes were detected from nt 1442 to 1490 at two recognition sites revealed by profiles R-III and R-IV (one *Hin*dIII site and one *Sau*3AI site, respectively) (Figure 28). The mutated *Sau*3AI site at nt 1442 is near three potential copper ligands, which include the H-C-H at residues 488-490, His494 (H), and Lys499 (L). The mutated recognition site is specifically found near the last ligand (Lys499). These results suggest that a base substitution close to this region of the ligands may indirectly affect the catalytic activity of the protein. The mutated *Hin*dIII site at nt 1485 is also located close to the second C residue (Cys524) (Figure 28). The conserved domains of Fet3p were not assessed in this study. In summary, these observations suggest that strains portraying these profiles may show slight alterations in its enzymatic activity to oxidize Fe^{+2} to Fe^{+3} and in the conformational structure of Fet3p at these particular sites. However, the enzymatic activity of Fet3p for each strain was not assayed in this particular study.

A multiple sequence alignment was used to compare the putative transmembrane helices (TMH) the predicted translated products of Fet3p in the model yeast organism *S. cerevisiae* and *D. hansenii* CBS 767^{T} amongst other similar ascomycetes using ClustalW and TMHMM (Figure 27). A potential *Sau*3AI site near nt 1642 based on profile R-V (region not shown) suggests a mutation close to the only predicted TMH of *D. hansenii* Fet3p (residues 567 to 586; Figure 28). The amino acid sequences of the TMH were more similar between *C. glabrata* and *S. cerevisiae*

as well as those between *C. albicans* and *P. pastoris*. However, the alignment revealed that the TMH of *D. hansenii* CBS767^{T} (residues 567 to 586) highly diverges from that identified in *S. cerevisiae* (residues 560 to 582). Twenty residues were identified in *D. hansenii* CBS767^{T}, whereas 23 residues were predicted in *S. cerevisiae* (Figure 27). Of the residues that coincided between these two yeasts (i.e., the first 20 residues), seven residues (35%) are identical, four residues (20%) are considered strongly similar in chemical properties, and nine residues (45%) differ entirely (Figure 27). These results indicate that most of the residues identified in the only predicted TMH of *D. hansenii* CBS767^{T} depict a greater divergence from *S. cerevisiae*, establishing its large genetic variation and, consequently, its establishment as a model organism for yeasts that differ substantially from the conventionally known baker's yeast.

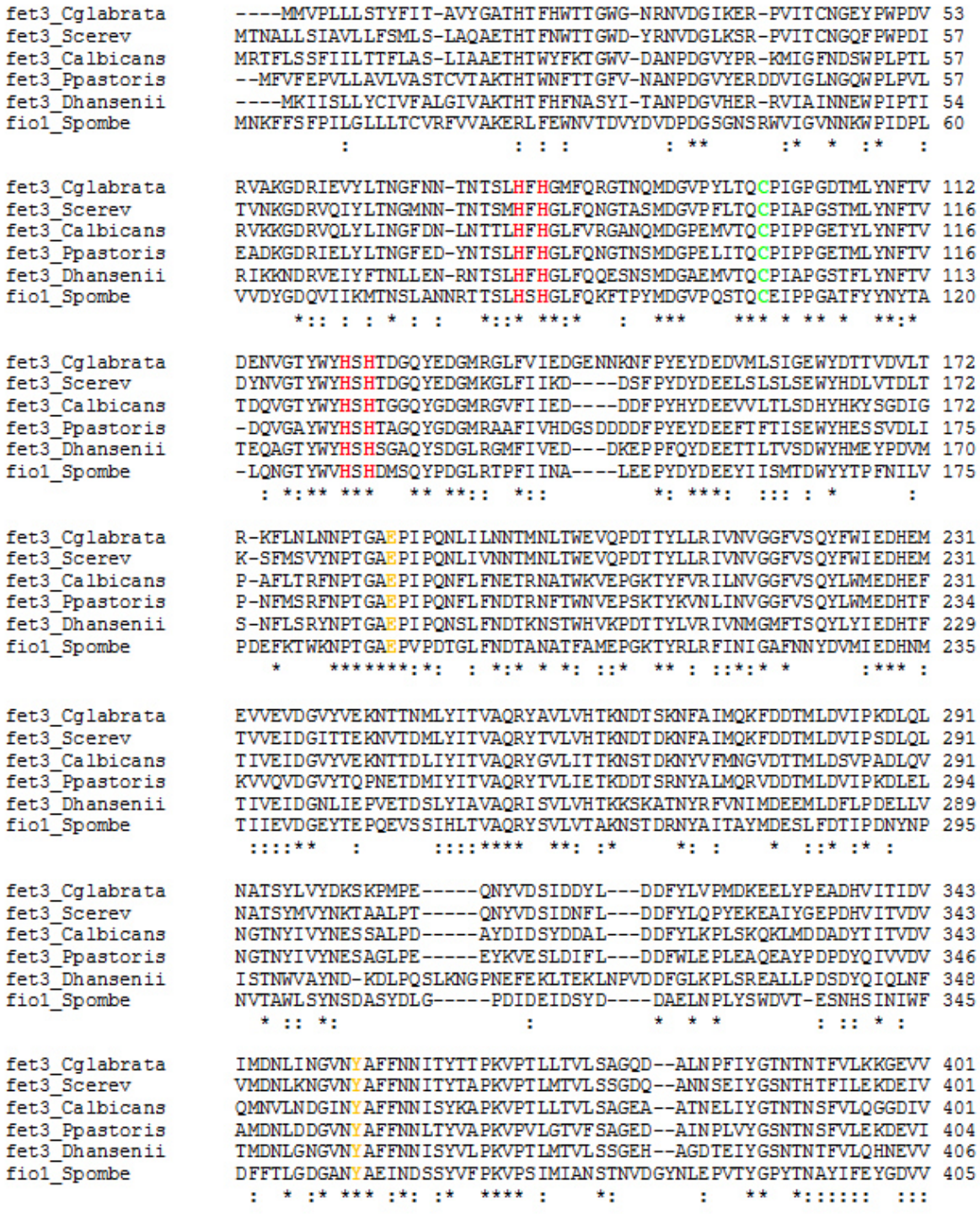

Figure 26. Sequence comparison of the predicted translated product of *Candida glabrata* Fet3p (fet3_glabrata), *Saccharomyces cerevisiae* Fet3p (fet3_Scerev), *Candida albicans* Fet3p (fet3_Calbicans), *Pichia pastoris* Fet3p (fet3_Ppastoris), *Debaryomyces hansenii* Fet3p (fet3_Dhansenii), and *Schizosaccharomyces pombe* Fio1p (fio1_Spombe) from residues 0 to 406. Sequences were obtained from GenBank and aligned using ClustalW v2.1. Predicted copper ligands are highlighted in red. Predicted iron ligands are highlighted in orange. Residues identical in all sequences are marked with an "*" and ":" indicates strongly similar residues.

Figure 27. Sequence comparison of the predicted translated product of *Candida glabrata* Fet3p (fet3_Cglabrata), *Saccharomyces cerevisiae* Fet3p (fet3_Scerev), *Candida albicans* Fet3p (fet3_Calbicans), *Pichia pastoris* Fet3p (fet3_Ppastoris), *Debaryomyces hansenii* Fet3p (fet3_Dhansenii), and *Schizosaccharomyces pombe* Fio1p (fio1_Spombe) from residues 461 to 636. Sequences were obtained from GenBank (see Materials and Methods) and aligned using ClustalW 2.1 [50]. Predicted copper ligands are highlighted in red. Predicted iron ligands are highlighted in orange. The cysteines involved in the development of the disulphide bridge are highlighted in green. Residues identical in all sequences are marked with an "*" and ":" indicates strongly similar residues. White capital letters on black background indicate the putative transmembrane helices in *S. cerevisiae* Fet3p. Black capital letters on gray background indicate the sequence variation in *D. hansenii* CBS 767^{T}.

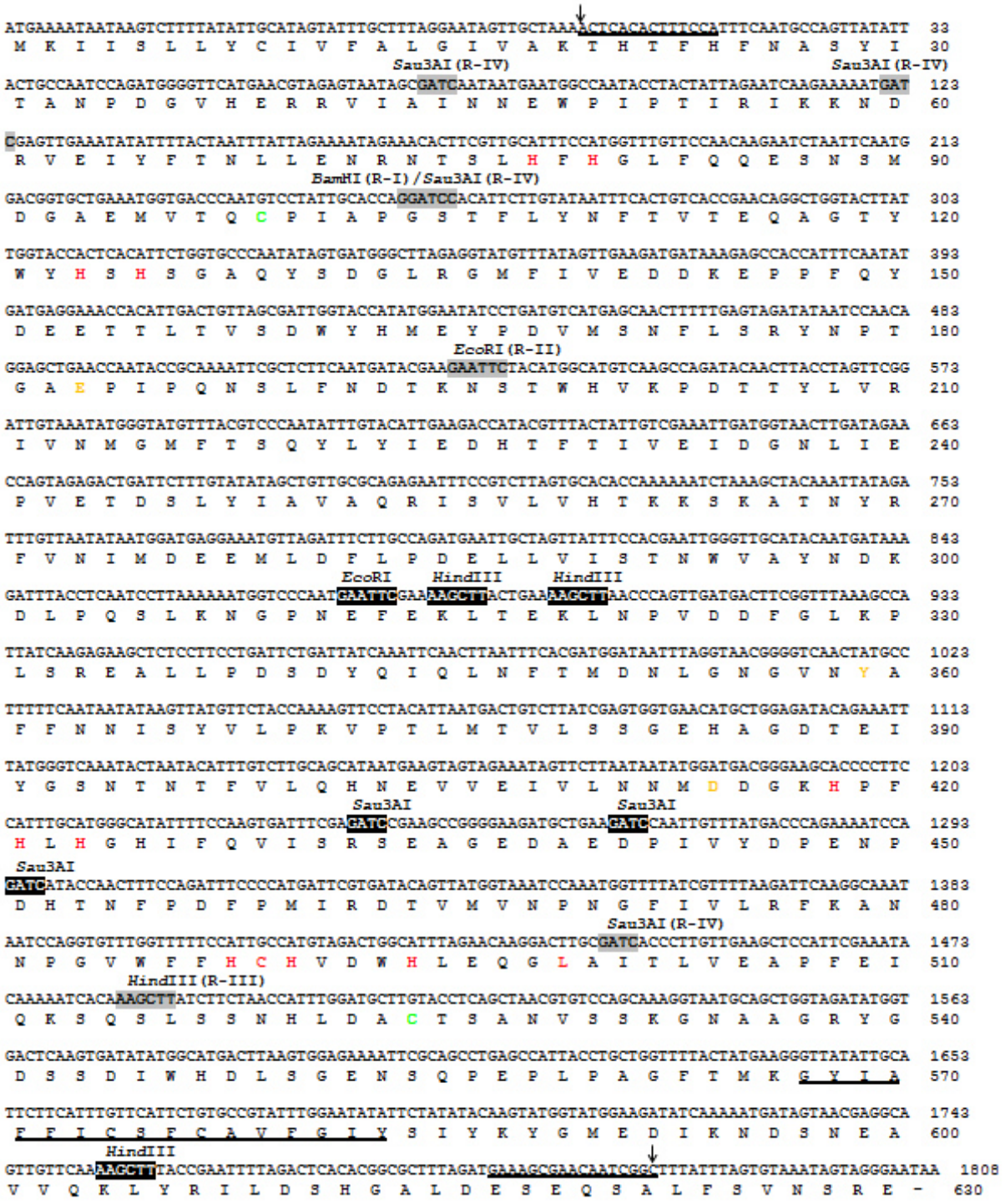

Figure 28. Nucleotide sequence of *D. hansenii* CBS767^{T} *FET3* (GenBank Accession No. XM_459860) and the predicted translated product beginning with the first nucleotide in the ATG initiation codon (0 to 630 residues). Arrows indicate the start (0 bp) and end points (1,808 bp) of the amplified region. Primer sequences used to amplify the gene are underlined. Recognition sites of the CBS767^{T} type sequence (TS) profile are identified with white capital letters on black background. Black letters on gray background indicate the sequence variations associated with strains showing restriction profiles R-I to R-IV. Predicted

copper ligands, iron ligands, and cysteine residues are identified by red, yellow, and green letters, respectively. The predicted transmembrane helice is underlined in the amino acid sequence.

4.3.3 Genotype profiling of *D. hansenii* strains by *ENA1-PCR* RFLP analysis. Initially, the *ENA1* gene was expected to exhibit less sequence variation based on similar phenotypic patterns of the strains analyzed in this study (see Chapter 3) and of its well known association with salt stress in *D. hansenii*, *S. cerevisiae*, and *Hortaea werneckii* [37,39,71]. However, four distinct combinations of primer sets using the genomic sequence of *D. hansenii* CBS 767^T were designed to amplify the partial ORF of *ENA1*. The only primer set capable of generating the expected PCR product size (3,193 bp) in most strains was used in this study (see Materials and Methods). No PCR products of the expected size were obtained from strains CBS 5144, CBS 1011, J6, YB-163, YB-165, YB-327, Y-17914, DhhBCS002 to DhhBCS006, and DhfBCS002 (a total of 13 strains). Of the remaining 21 strains, amplification resulted in a single fragment of about 3,200 bp. Mispriming was not frequently observed with the primer set under the PCR conditions described in this study.

The discriminative power of RFLP was used to detect DNA sequence variation in the partially amplified ORF region of *ENA1* among *D. hansenii* strains. For the RFLP, the PCR products of 21 strains were successfully amplified using the primer set designed from nt 20 and 3211 of the 3,231 bp full length sequence of *D. hansenii* CBS767^T *ENA1* (see Materials and Methods). Only one restriction enzyme, *Sau*3AI, was used to generate fragment lengths that formed a restriction profile specific to the PCR product generated from each strain. Given that *Sau*3AI is a 4-base cutter, it was used to detect greater incidences of sequence variation in *ENA1* as opposed to using 6-base cutters due to its frequency in encountering the 4-base recognition site (Table 5). A virtual digest (Webcutter 2.0) of the expected PCR product of CBS767^T *ENA1* revealed that no recognition sites were found for restriction enzymes *Bam*HI and *Eco*RI and only one *Hin*dIII site was located at nt 608 (data not shown).

Table 5. Detailed information of the restriction enzyme *Sau*3AI used to generate *ENA1* RFLP profiles among *D. hansenii* strains. The frequency of cuts, nucleotides of sites (5′→3′), and generated fragment lengths refer to the digested 3,193 bp PCR-amplified product of *D. hansenii* CBS767^T *ENA1*. Generated fragments lengths are listed in descending order according to size.

Name	Recognition Site	Frequency	Nucleotides of Sites (nt)	Generated Fragment Lengths (bp)
*Sau*3AI	G/ATC	15	0, 420, 429, 760, 786, 1301, 1445, 1907, 1985, 2185, 2455, 2781, 3014, 3121, 3155	515, 462, 420, 331, 326, 270, 233, 200, 144, 107, 78, 38, 34, 26, 9

Three restriction profiles were observed following *Sau*3AI digests among those strains that successfully amplified the PCR product (Figure 29). The restriction profile of CBS 767^T is referred to as the type-sequence (TS) and was the most frequent profile observed. This profile consisted of 15 recognition sites (or cuts), which generated 16 fragments that were visible as a series of bands of different sizes (Figure 29).

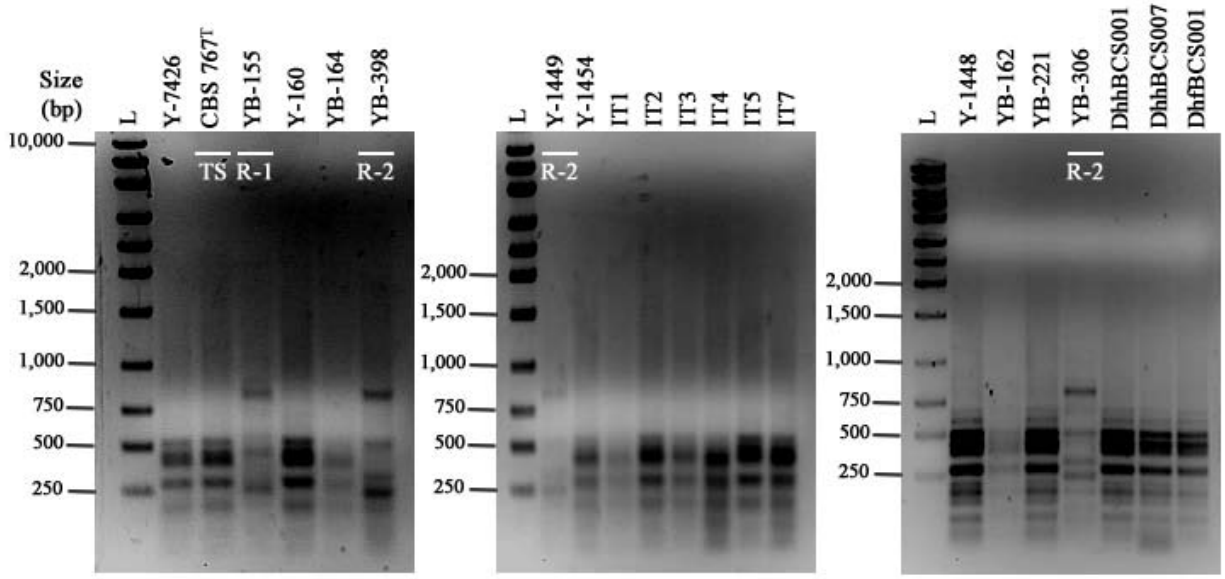

Figure 29. Separation on agarose gels of *Sau*3AI restriction fragments of the partially amplified ORF (~3,200 bp) of *ENA1* from *D. hansenii* strains (labeled for each well). Restriction profiles TS (type sequence), R-1, and R-2 are shown. L: 1-kb ladder (GeneRuler).

Two distinct restriction profiles, R-1 and R-2, were detected in YB-155, YB-398, Y-1449, and YB-306 (Figure 29). Both profiles generated a band greater than 750 bp, which was not observed in the TS profile. This fragment suggests that both profiles exhibit a mutation at *Sau*3AI sites 10 and 11 (nt 2185 and 2455, respectively) and, thus produced a band of ~796 bp. Profile R-1 (i.e., YB-155) did not show the 515 bp fragment generated by the TS profile, which indicates that a mutation is present as a new recognition site between *Sau*3AI sites 5 and 6 (nt 786 and 1301, respectively). This same profile also showed fragment sizes unlike those from profiles TS and R-2, suggesting that the ORF of YB-155 *ENA1* has greater sequence variation compared to the other strains. Strains from profile R-2 (YB-398, Y-1449, and YB-306) did not

generate the ~400 bp fragments (420 and 462 bp) that *Sau*3AI sites 1 and 2 (nt 0 and 420, respectively), and sites 7 and 8 (nt 1445 and 1907, respectively) produce based on the TS profile (Table 5). Therefore, these results suggest that additional recognition sites are located between these sites, thus generating smaller fragments than those seen in the TS profile.

In general, the RFLP analysis discriminated only four strains with sequences variations from the *ENA1* TS profile of the 21 strains that successfully generated PCR products of the expected size (Table 6). Strain YB-155 depicted profile R-1, whereas Y-1449, YB-306, and YB-398 revealed profile R-2. YB-155 exhibited the most diverse sequence variation when compared to profile R-2 due to potential base substitutions in other regions of the gene that generated fragments that were not observed in the other strains. It must be noted that the *ENA1* restriction profiles of 13 strains (from both variants of *hansenii* and *fabryii*, Table 6) were not generated using the primer set designed in this study (see Material and Methods). Therefore, these results suggest that most of the strains analyzed in this study possess large genetic heterogeneity in the ORF of *ENA1* and, hence, demonstrate greater probabilities of having DNA sequence variation in other essential functional genes.

Table 6. Comparison of *ENA1* RFLP profiles of *D. hansenii* strains with the partially amplified type sequence (TS) of CBS767^T. R-1: loss of *Sau*3A sites at nucleotides (nt) 2185 and 2455, new recognition sequences between nt 786 and 1301, and other potential *Sau*3AI sites unresolved by gel electrophoresis; R-2: loss of *Sau*3A sites at nt 2185 and 2455, a potential recognition site between nt 0 and 420, and an additional site between nt 1445 and 1907; NA: PCR product not amplified.

Strain name	***ENA1*** **RFLP Profile**
IT1	TS
IT2	TS
IT3	TS
IT4	TS
IT5	TS
IT7	TS
CBS 767^T	TS
CBS 5144	NA
CBS 1011	NA
J6	NA
var. *hansenii* NRRL Y-7426	TS

Strain name	*ENA1* RFLP Profile
var. *hansenii* NRRL Y-1448	TS
var. *hansenii* NRRL Y-1449	R-2
var. *hansenii* NRRL Y-1454	TS
var. *hansenii* NRRL YB-155	R-1
var. *hansenii* NRRL YB-160	TS
var. *hansenii* NRRL YB-162	TS
var. *hansenii* NRRL YB-163	NA
var. *hansenii* NRRL YB-164	TS
var. *hansenii* NRRL YB-165	NA
var. *hansenii* NRRL YB-221	TS
var. *hansenii* NRRL YB-306	R-2
var. *hansenii* NRRL YB-398	R-2
var. *fabryi* NRRL YB-327	NA
var. *fabryi* NRRL Y-17914	NA
var. *hansenii* DhhBCS001	TS
var. *hansenii* DhhBCS002	NA
var. *hansenii* DhhBCS003	NA
var. *hansenii* DhhBCS004	NA
var. *hansenii* DhhBCS005	NA
var. *hansenii* DhhBCS006	NA
var. *hansenii* DhhBCS007	TS
var. *fabryi* DhfBCS001	TS
var. *fabryi* DhfBCS002	NA

4.3.4 Comprehensive analysis of variation in nucleotide and translational sequences of *ENA1* in *D. hansenii* CBS 767^{T} and in similar yeasts. The conserved domains of the Ena1p P-type ATPase were not assessed in this study. To identify the putative transmembrane helices (TMH) in the predicted translated product of *D. hansenii* CBS 767^{T} Ena1p, a comparative amino acid sequence analysis was performed with *C. albicans* Ena2p, *S. cerevisiae* Ena1p, *C. glabrata* Ena1p, and *S. pombe* Cta3p using ClustalW and TMHMM (Figures 30 and 31). Similar to that reported in the model yeast organism *S. cerevisiae* [70,71], ten potential TMH were identified in CBS767^{T}. However, only the third TMH was highly conserved (96%, residues 347 to 368), whereas the sixth TMH differed the most in the number of residues (17%) (residues 836 to 858)

and the seventh TMH varied mostly in the type of residues (25%) (residues 889 to 911) when compared to the predicted TMH in *S. cerevisiae* (Figures 30 and 31). However, several TMH sequences were mostly analogous between *C. albicans* and *D. hansenii* (regions not emphasized for analysis). In general, substantial amino acid sequence variations were observed in the TMH predicted for *D. hansenii* CBS767^{T} Ena1p compared to those *S. cerevisiae*, suggesting that these regions may play a potential role in the stability of the protein within the cellular membrane and may provide physiological advantages to changes in osmotic stress.

The nucleotide sequence variations from the TS profile of CBS767^{T} *ENA1* following restriction *Sau*3AI enzyme digest are shown based on profiles R-1 and R-2 with its predicted translated product (Figures 32 and 33). Profile R-2 revealed a potential *Sau*3AI sites that are predicted between the first 425 nt (sites 1 and 2, respectively), which includes the first two putative TMH of the protein (residues 92 to 137) (Figure 32). A second new *Sau*3AI site is predicted between nt 786 and 1301 (sites 5 and 6, respectively) based on profile R-1. This particular sequence change may be within the third and fourth TMH (residues 317 to 369) (Figure 32). Sequence variations unresolved by gel electrophoresis are also proposed for profile R-1 and should be assessed in conjunction with Ena1p activity for strains that protray this particular profile. Profile R-2 revealed another potential *Sau*3AI site between nt 1445 and 1907 (sites 7 and 8, respectively), but no predicted TMH were identified in this region. Both profiles R-1 and R-2 showed a potential mutation at nt 2185 and 2455 (sites 10 and 11, respectively), the latter located specifically within the fifth TMH (residues 809 to 831) (Figure 33). In summary, at least 3 out of 10 TMH may be affected in conformational structure by mutations or substitutions found in strains possessing *ENA1* profiles R-1 or R-2 as identified by RFLP analysis in this study.

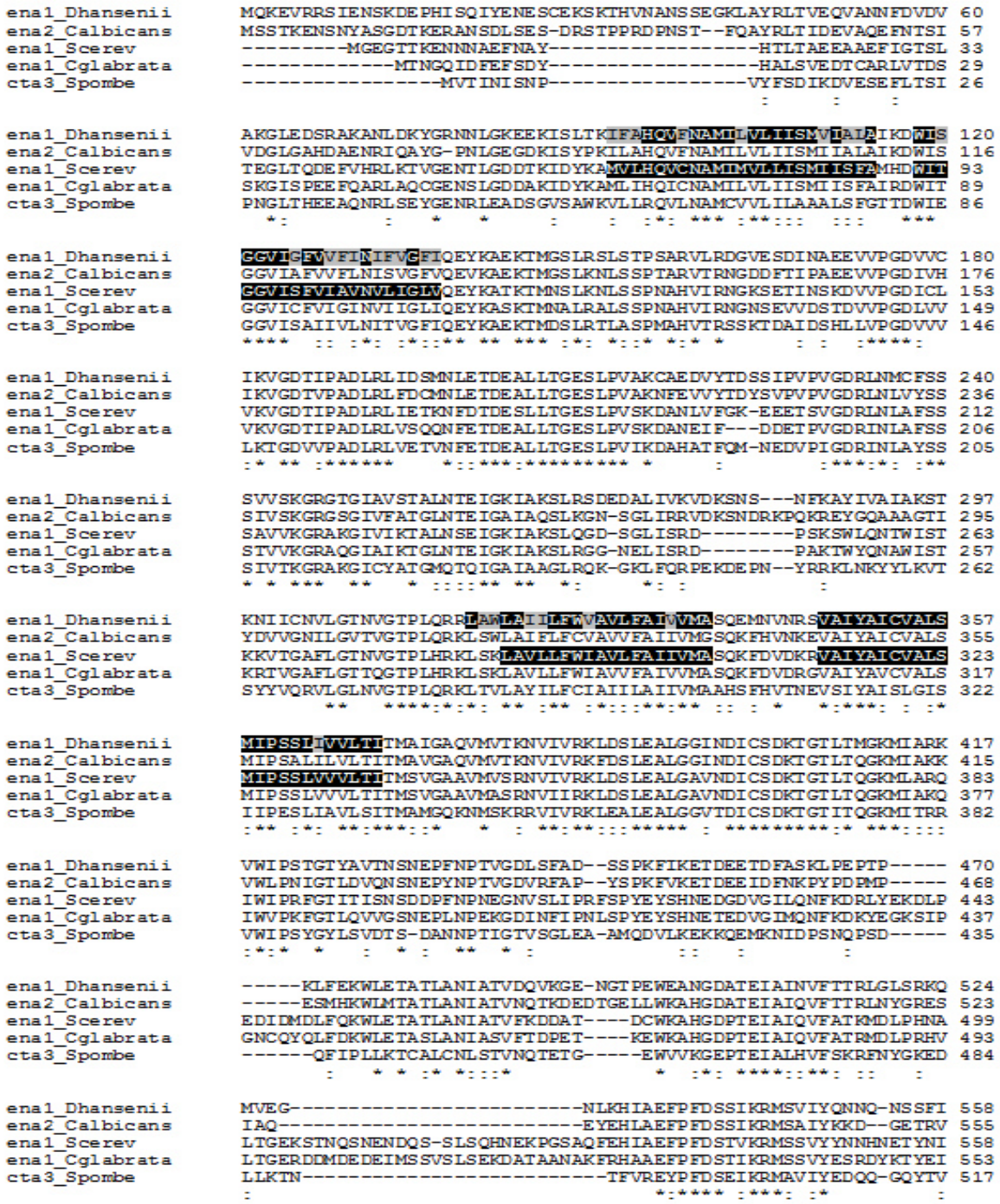

Figure 30. Sequence comparison of the predicted translated product of *Debaryomyces hansenii* Ena1p (ena1_Dhansenii), *Candida albicans* Ena2p (ena2_Calbicans), *Saccharomyces cerevisiae* Ena1p (ena1_Scerev), *Candida glabrata* Ena1p (ena1_Cglabrata), and *Schizosaccharomyces pombe* Cta3p from residues 0 to 558. Sequences were obtained from GenBank (see Materials and Methods) and aligned using ClustalW v2.1 [50]. White capital letters on black background indicate the putative transmembrane helices in *S. cerevisiae* Ena1p. Black capital letters on gray background indicate the sequence variation in *D. hansenii* CBS 767^{T} from these regions. Residues identical in all sequences are marked with an "*" and ":" indicates strongly similar residues among yeast species.

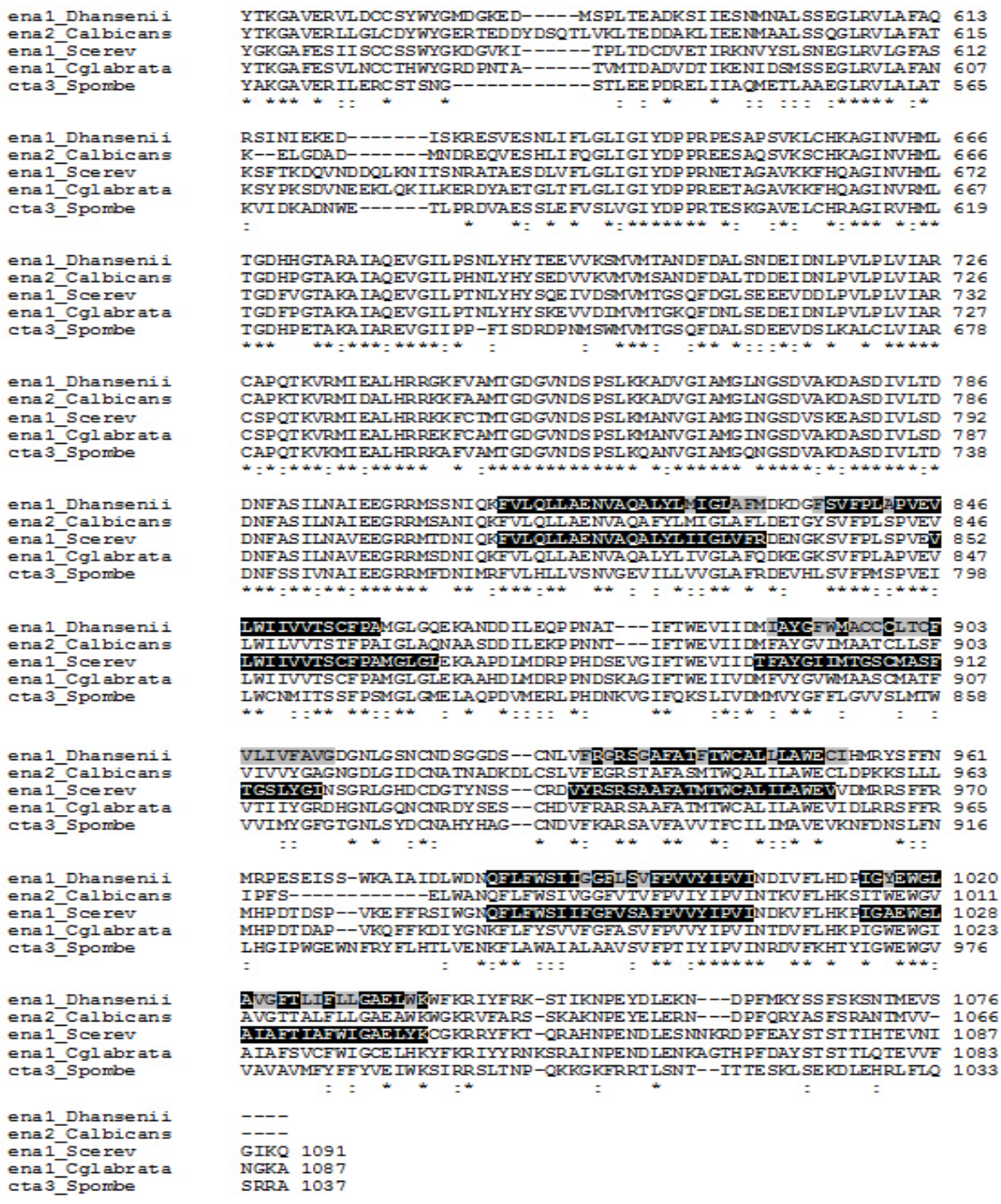

Figure 31. Sequence comparison of the predicted translated product of *Debaryomyces hansenii* Ena1p (ena1_Dhansenii), *Candida albicans* Ena2p (ena2_Calbicans), *Saccharomyces cerevisiae* Ena1p (ena1_Scerev), *Candida glabrata* Ena1p (ena1_Cglabrata), and *Schizosaccharomyces pombe* Cta3p from residues 613 to 1091. Sequences were obtained from GenBank (see Materials and Methods) and aligned using ClustalW v2.1. White capital letters on black background indicate putative transmembrane domains in *S. cerevisiae* Ena1p. Black capital letters on gray background indicate sequence divergence associated with these domains in *D. hansenii* Ena1p. Residues identical in all sequences are marked with an "*" and ":" indicates strongly similar residues.

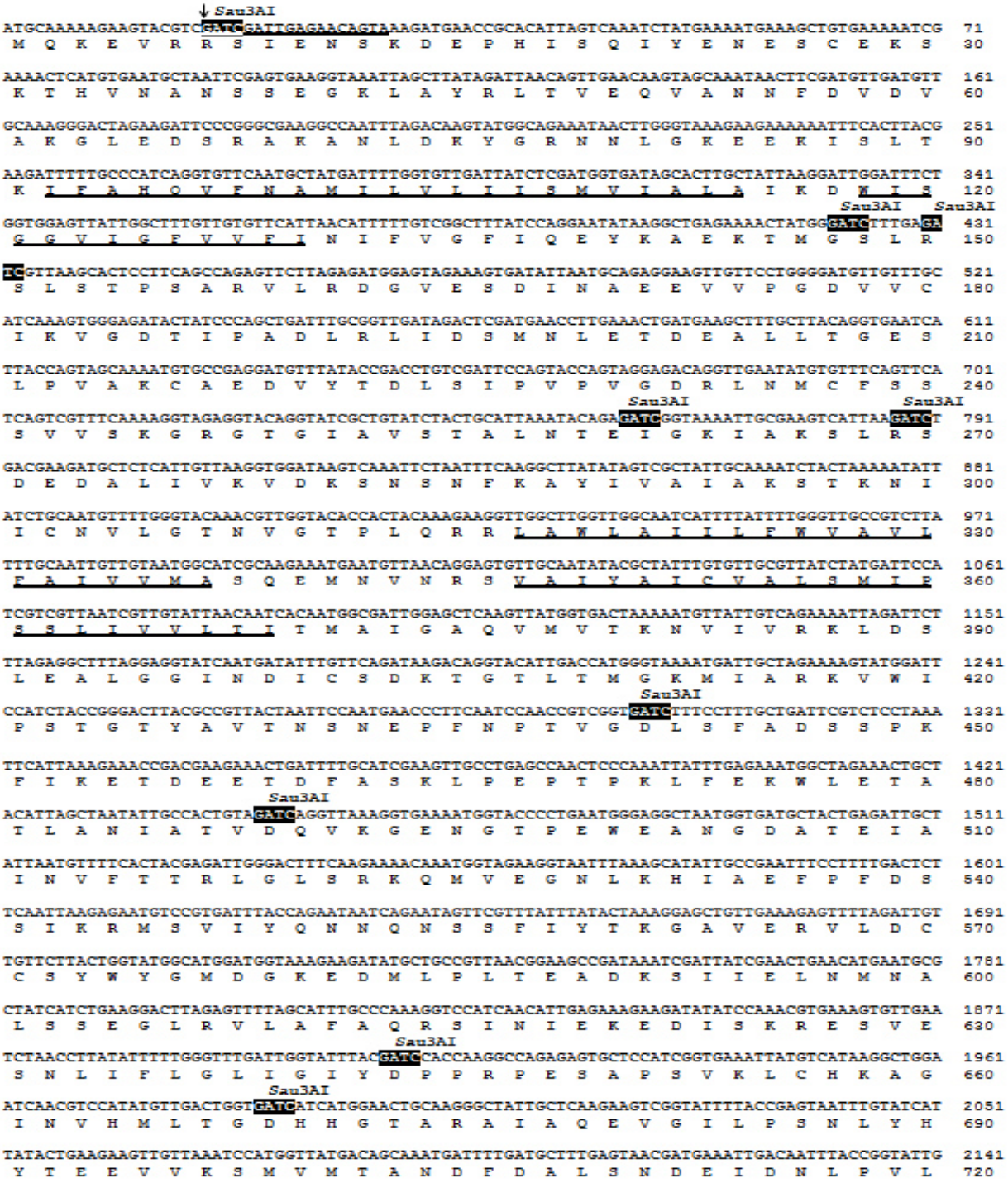

Figure 32. Nucleotide sequence of *D. hansenii* CBS767^{T} *ENA1* (GenBank Accession No. XM_457790) and the predicted amino acid sequence of its protein product beginning with the first nucleotide in the ATG initiation codon (0 to 720 residues). Primer sequence used to amplify PCR product is underlined. Arrow indicates the starting point (0 bp) of the amplified region (3,193 nt; see Figure 33 for the remaining sequence). Restriction enzymes sites are identified with white capital letters on black background. Black letters on gray background indicate the sequence variation associated with strains showing the restriction profiles R-1 and R-2 (see Fig. 29). Predicted transmembrane helices in the amino acid sequence are underlined.

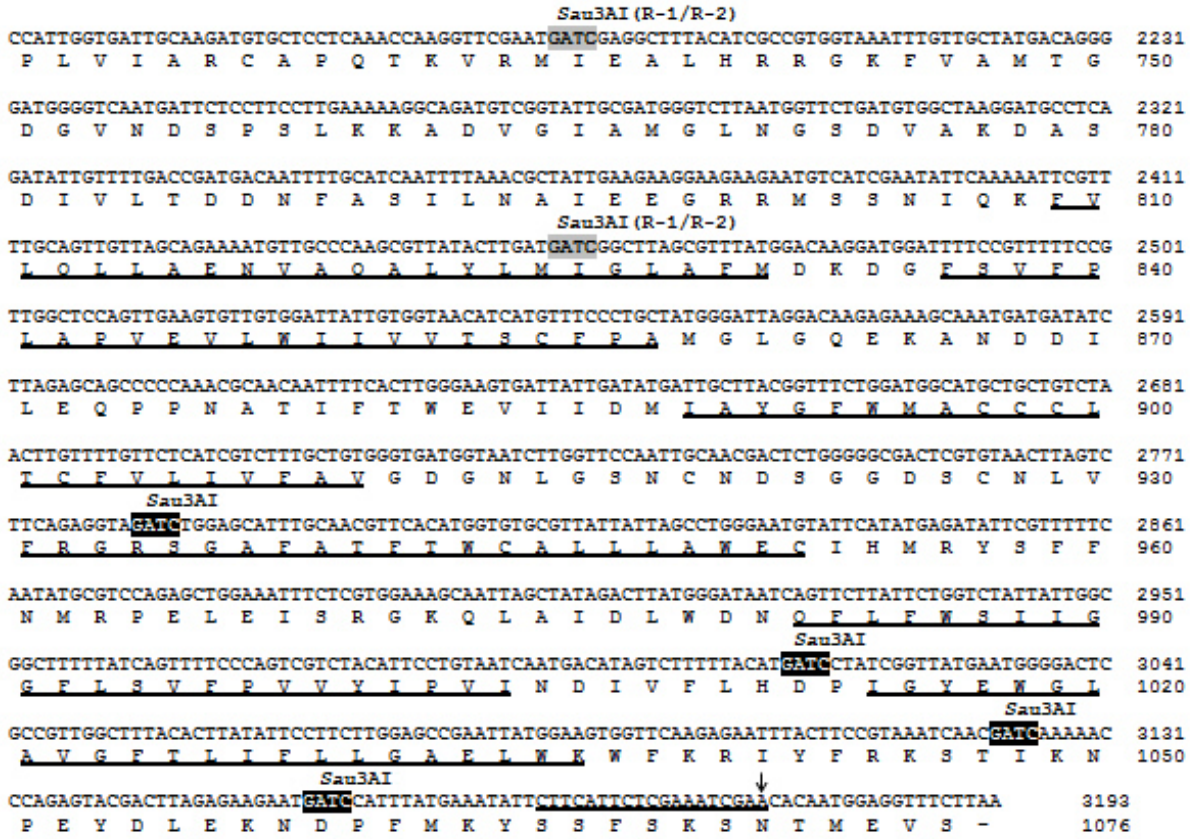

Figure 33. Continuation of the nucleotide sequence of *D. hansenii* CBS767^{T} *ENA1* (GenBank Accession No. XM_457790) and the predicted amino acid sequence of its protein product (721 to 1076 residues). Primer sequence used to amplify PCR product is underlined. Arrow indicates the ending point (3,193 nt) of the amplified region. Restriction enzymes sites are identified with white capital letters on black background. Black letters on gray background indicate the sequence variation associated with strains showing the restriction profiles R-1 and R-2. Predicted transmembrane helices in the amino acid sequence are underlined.

4.5 Discussion

The purpose of this study was to detect sequence heterogeneity in the gene structure (or ORF) of inducible genes, explicitly multicopper oxidase *FET3* and the P-type ATPase sodium pump *ENA1* among *D. hansenii* strains isolated from different sources. To accomplish this objective, variation was identified using sequence differentiation by RFLP. This analysis also included a sequence comparison of the predicted translated products of each gene to identify regions or domains that may be conserved but that may be affected by DNA sequence substitutions.

To summarize the results of the RFLP performed on *FET3* PCR products, six restriction profiles were detected after four distinct restriction enzyme digests (*Bam*HI, *Eco*RI, *Hin*dIII, and *Sau*3AI). Overall, 10 strains (or ~30%) of those studied were differentially discriminated from the most frequently detected restriction profile TS, which was the *FET3* RFLP pattern revealed by *D. hansenii* CBS767^{T}. DNA sequence comparisons of either highly conserved regions [21,41,60,64] or housekeeping genes [22] have also produced comparable results on the occurence of high sequence variability among strains of *D. hansenii*, which also has included analyses on its anamorph *C. famata* [60]. Nguyen et al. [60] reported 83-88% similarity levels of riboflavin synthesis genes *RIB1*, *RIB6*, and *RIB7* from *C. famata* VKM Y-9 [82] (later described as *C. flareri*) and *D. hansenii* CBS767^{T}. Similarity levels of 90-94% were also stated in the same study [60], but after comparison with sequences obtained from *D. fabryi* CBS 789^{T}.

In this dissertation, an initial intent to conduct a genome-wide analysis of gene expression by means of DNA microarray suggested sequence variations between the genome of *D. hansenii* CBS767^{T} and the strain used for the experiment that exceeded the limitations of the technique based on the probes used for hybridization [31], and thus required the use of consensus sequences to determine RNA expression patterns. Incidences of mispriming after PCR amplification and the need to design primers that would amplify more than 80% of the gene of interest were also reported in [60], which supports the results obtained among several strains in this study. Mispriming during PCR-based methods is frequently encountered and has suggested the presence of single nucleotide polymorphisms [48], hybridization of non-specific regions [80], and may be indicative of sequence variants in populations with gene polymorphisms [10]. Frequent mispriming in gene amplification may indicate strong primer homology to other

regions of the genome, although this was minimized in the present study using the generated PCR product of *FET3* (1.8 kbp) as a template to ensure less mispriming and increasing the annealing temperature (35-40°C) depending on the strain being analyzed (see Materials and Methods). The second release of the *D. hansenii* CBS 767 genome revealed a second sequence annotated as *FET3* (DEHA2G05082g). However, a nucleotide sequence comparison between the two versions using discontiguous megablast parameters of BLASTN 2.2.26+ [87], revealing 68% identity, suggests that the second release of this sequence may be a pseudogene (data not shown). Therefore, universal primers to amplify protein-coding genes involved in important metabolic pathways are suggested to identify strains with favorable phenotypes, particularly for industrial applications. However, it should be noted that an appropriate evaluation of the gene of interest with regard to phenotypic or protein expression under certain conditions be equally considered when developing these primers to detect sequence variation. Other methods, such as PCR-duplex-RAPD (random-amplified polymorphic DNA) binding patterns [27], AFLP (amplified fragment length polymorphism) [25], singleplex PCR [33] and pulse-field gel electrophoresis [44,58] have been employed to discriminate genotypic variation among several yeast species. Various protein-coding genes, such as *COX2* (the mitochondrial cytochrome-c oxidase subunit), *ACT1* (actin), *TUB2* (beta-tublin), ribosomal proteins *RPL31* and *RPL33*, and *GPD1* (glycerol phosphate dehydrogenase gene) [24,43,60], have been used to delineate the genetic diversity of the genus *Debaryomyces* and increasing evidence supports the unanticipated large sequence variation in this species complex.

Interestingly, three strains that demonstrated flavinogenic activity upon cobalt stress (CBS 1011, CBS 5144, and J6) in Chapter 3 differed from the restriction profile of *D. hansenii* CBS 767^{T}. Riboflavin-producing strain Y-17914 also suggested large sequence variation due to mispriming and, consequently, the absence of the expected PCR product. However, other strains depicting restriction profiles distinct from the TS profile did not necessarily show flavinogenic properties. In particular, most strains of marine origin did not show any visible signs of riboflavin synthesis despite their high tolerance to oxidative stress and lack of PCR amplification using the primers in this study. Nonetheless, *RIB5* (riboflavin synthase) was the only gene of the riboflavin synthesis pathway that was upregulated upon cobalt stress in *S. cerevsiae* [76]. In contrast, riboflavin activity was reported in the marine yeast *C. membranifaciens* subsp. *flavinogenie* W14-3 isolated from seawaters of the China Eastern Sea and revealed high identity

with partial protein sequences of GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone-4-phosphate synthase of the riboflavin synthesis pathway when compared with other yeasts [83]. Although DNA and amino acid sequence analysis of several genes of the riboflavin synthesis pathway have suggested possible divergence of species [60,83,90] and their regulation may occur due to various types of stress factors [1], the results in the present study indicate that these genes may not be the most appropriate to imply sequence variation associated with flavinogenic properties as a measure of phenotypic prediction.

In the present study, *FET3* was selected due to its upregulation upon cobalt stress and its role in the iron regulon as reported by [76]; however, it is recommended that other inducible genes, such as *COT1* [76,20,19], *SOD1* (superoxide dismutase) [30,59,76], *YFH1* (fraxatin homologue) [57], the *FIT* gene family [67], and the *ARN* family of siderophore transporters [65,86] may be considered as promising candidates for detecting sequence diversity among strains of the same species based on their specific role in oxidative stress or anoxic conditions and possibly link their expression to phenotype upon environmental stress. In addition, genes involved in the role of iron-sulfur cluster synthesis associated with the iron transport system (e.g., *FRA1, FRA2*, and *ISU2*) should also be considered when elucidating a possible correlation between DNA sequence variation and phenotypic expression [36,47].

In addition to analyzing *FET3*, the P-type ATPase pump *ENA1* was used to indicate sequence divergence due to its involvement in the function and regulation of sodium toxicity in yeast [71,37,39]. To summarize, the RFLP analysis discriminated only four patterns that varied from the *ENA1* TS profile. Mispriming and absence of the expected PCR product (~3.2 kbp) occurred in several (38%) strains in the present study, which was greater than what was anticipated due to the its frequent association with the regulation of sodium or potassium stress [69]. However, several copies of *ENA* (also referred to as *PMR2* or *HOR6*) genes have been found in *S. cerevisiae* strains [84] and have shown homology and similar function of *ENA* genes in *Candida sp.* [34,46], in *H. werkenii* [39], and in *D. hansenii* [2]. Although two genes encoding for Na^+-ATPases were reported in *D. hansenii* PYCC 2968, namely *DhENA1* and *DhENA2*, the upregulated *ENA1* gene reported by [37] was used to detect sequence polymorphism. A nucleotide sequence comparison between the two released versions of the *D. hansenii* CBS 767 genome using megablast parameters of BLASTN 2.2.26+ [87] revealed 99% identity, implicating the absence of a pseudogene for *ENA1* as not seen in the case with *FET3* (data not

shown). Despite the importance of *ENA1* expression in osmotic stress, it is proposed that other genes known for their upregulation or association with a physiological response upon high-salt stress should be considered when delineating *D. hansenii* species or identifying possible cryptic species such as *GPP1* (*RHR2*, isoform of DL-glycerol-3-phosphatase) [37,61], *HWP1* (hyphal cell wall protein) [37,75], *PRT1* (translation initiation factor involved in ribosomal protein synthesis) [37,66], mitochondrial genes [37], and genes that encode for Ppz protein phosphatases [85]. Based on our observations, the possiblilty of sequence divergence in *ENA1* among *D. hansenii* strains is very likely [84] and may be related to yeast habitat [69].

Regarding the substitutions or mutations in the open reading frame (ORF) of *FET3* as revealed in the six RLFP profiles and their possible effect on the predicted translated product (Fet3p) of strain CBS 767^T, it is proposed that most of the ligands necessary for complete protein function have not been drastically changed and would not result in its impairment. However, possible mutations near the potential iron and copper ligands identified for the multicopper oxidase may affect its catalytic activity. Substitutions in the amino acid sequence of *Pichia pastoris* Fet3p and higher levels of catalytic efficiency were reported, but were unable to be fully elucidated without an accurate description of the protein structure [62]. A three-dimensional homology model of *S. cerevisiae* Fet3p compared with the structural template of ascorbate oxidase provided insight into the underlying mechanism of iron transport [29]. In a later study by [8] revealed substitutions generated by site-mutagenesis of two residues (Glu-185 and Tyr-354) that may alter its percent efficiency as an ferrioxidase and oxidase. Specific mutations of six aspartate residues found in *S. cerevisiae* Fet3p reduced growth ability under iron-limiting conditions and susceptiblity to proteolytic degradation when unprotected by the plasma membrane [9]. More recent studies have described the stability, maturation, glycosylation, and unfolding of the Fet3p protein to better understand its complexity [73,88,89]. Based on the potential substitutions identified near potential ligand sites and in the putative transmembrane helices in the present study, it is proposed that these changes are may affect catalytic activity or efficiency in *D. hansenii* strains, but not in its overall function.

The comparison of putative transmembrane helices of *D. hansenii* Ena1p with several yeast species, particularly to the model yeast *S. cerevisiae*, revealed substantial differences in the residues of the ten potential helices predicted in *D. hansenii* CBS 767^T. Nine to twelve transmembrane domains have been predicted for Ena1p in *S. cerevisiae* and *Schwanniomyces*

occidentalis [7,84]. Given that successful PCR amplification of *ENA1* of only 21 (~61%) of the strains studied revealed large sequence variation, jointly these results suggest that sequence divergence in the *ENA1* gene and, consequently, protein structure are strongly favored and may affect its conformational structure and, hence, efficiency as a sodium ATPase pump in yeast upon osmotic stress.

In general, no direct correlation between genetic variation and phenotypic expression was detected based on the genes selected in this study. However, it is recommended that several inducible genes, such as those previously mentioned, should be considered for the delineation of *D. hansenii* species and its variants due to their ability to cause phenotypic changes when expressed. In addition, high-throughput genome sequencing of coding and non-coding genes is strongly recommended to compare sequence variation in strains from different populations and evolutionary history [68], such as in the case of the strains of marine origin. Although this study provides a first report on the genetic variability of the protein coding region of two inducible genes associated with environmental stress, further research is needed to fully elucidate the effect of this variation on protein structure and function. With this additional information, a clearer correlation between phenotypic and genetic variation may be established. Thus, studies on the proteomic diversity with regards to protein structure and enzymatic activity among strains of *D. hansenii* must continue if we intend to achieve an integrative approach to understanding the complexity of these model organisms.

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Chapter 5: Overall Conclusion

To summarize the overall aim of this dissertation, *Debaryomyces hansenii* was described and analyzed by phenotype and genotype profiling due to its remarkable ability to tolerate factors that would otherwise cause severe cellular distress or death. The results of this dissertation affirm that *D. hansenii* is a promising candidate as a model organism to study various physiological and metabolic processes that occur in eukaryotic organisms due to its versatility, its abundance of biochemical pathways, and its potential for biotechnological applications. Yeasts are becoming regarded as model organisms for their capabilities to survive under physiologically demanding conditions [15,54], for their homology with genes that are implicated in molecular processes of higher level organisms [28], for similarities in pathways associated with human diseases [4,37], and for genetic differentiation related to population genetics [34,39]. With this increasing trend, *D. hansenii* is gaining favor as an ideal organism for further study on the implicit network of mechanisms that impart its physiological and genetic characteristics [7,10, 27,29,56,57]. Therefore, the general purpose of this study consisted of profiling various strains of *D. hansenii* upon exposure to two types of stress factors, oxidative (cobalt) and osmotic (NaCl). Based on the main objectives of each chapter, the overall outcome of the evidence presented demonstrates that *D. hansenii* is an exemplar organism capable of surviving environmental stress and of depicting substantial phenotypic and genetic heterogeneity among strains, which may imply proteomic diversity (Table 7). The proposed explanations, as detailed in the following pages, for understanding the implicit mechanisms involved in the phenotype and genotype diversity among *D. hansenii* strains analyzed in this study consist of: (i) variation in transcriptional or gene regulation (ii) genomic organization, (iii) phenotype plasticity, (iv) the presence of prion proteins, (v) and selective pressure from enviromental conditions.

Table 7. *Debaryomyces hansenii* strains identified by phenotype and genotype based on tolerance levels to Co(II) and NaCl stress in accordance with *FET3* and *ENA1* RFLP profiles. Levels of tolerance – HT: Highly Tolerant, T: Tolerant, WT: Weakly tolerant, S: Sensitive. *FET3* RFLP profiles – TS (Type Sequence): profile based on the DNA sequence of CBS767^T; R-I: no *Bam*HI site at nucleotide (nt) 253; R-II: no *Eco*RI site at nt 528; R-III: no *Hin*dIII site at nt 1485; R-IV: no *Sau*3AI sites at nt 74, 120, 254, and 1442, R-V: potential recognition sites within *Sau*3AI sites 3–7 (nt 254 to 1442) and *Sau*3AI near nt 1642, R-VI: additional *Sau*3AI site between nt 254 and 1237. *ENA1* RFLP profile - TS: profile based on the DNA sequence of CBS767^T; R-1: loss of *Sau*3A sites at nt 2185 and 2455, new recognition sequences between nt 786 and 1301, and other potential *Sau*3AI sites unresolved by gel electrophoresis; R-2: loss of *Sau*3A sites at nt 2185 and 2455, a potential recognition site between nt 0 and 420, and an additional site between nt 1445 and 1907; NA: PCR product not amplified.

Strain name	Level of tolerance		RFLP Profile				
			FET3				*ENA1*
	Co(II)	NaCl	*Bam*HI	*Eco*RI	*Hind*III	*Sau*3AI	*Sau*3AI
IT1	WT	T	TS	TS	TS	TS	TS
IT2	WT	T	TS	TS	TS	TS	TS
IT3	S	T	TS	TS	TS	TS	TS
IT4	S	T	TS	TS	TS	R-VI	TS
IT5	WT	T	TS	TS	TS	TS	TS
IT7	HT	T	TS	TS	TS	TS	TS
var. *hansenii* CBS 767^T	T	T	TS	TS	TS	TS	TS
CBS 5144	HT*	T	R-I	R-II	R-III	R-IV	NA
CBS 1011	WT*	T	R-I	R-II	R-III	R-IV	NA
J6	T*	T	R-I	R-II	R-III	R-IV	NA
var. *hansenii* NRRL Y-7426	HT	WT	TS	TS	TS	TS	TS
var. *hansenii* NRRL Y-1448	T	WT	TS	TS	TS	R-VI	TS
var. *hansenii* NRRL Y-1449	T	T	TS	TS	TS	R-VI	R-2
var. *hansenii* NRRL Y-1454	T	T	TS	TS	TS	TS	TS
var. *hansenii* NRRL YB-155	WT	T	R-I	TS	TS	R-V	R-1
var. *hansenii* NRRL YB-160	WT	T	TS	TS	TS	TS	TS
var. *hansenii* NRRL YB-162	T	WT	TS	TS	TS	TS	NA
var. *hansenii* NRRL YB-163	WT	T	TS	TS	TS	TS	TS
var. *hansenii* NRRL YB-164	S	S	TS	TS	TS	TS	TS
var. *hansenii* NRRL YB-165	WT	T	TS	TS	TS	TS	NA
var. *hansenii* NRRL YB-221	T	WT	TS	TS	TS	R-VI	TS
var. *hansenii* NRRL YB-306	T	T	TS	TS	TS	R-VI	R-2
var. *hansenii* NRRL YB-398	T	T	TS	TS	TS	R-VI	R-2
var. *fabryi* NRRL YB-327	HT	T	NA	NA	NA	NA	NA
var. *fabryi* NRRL Y-17914	WT*	T	NA	NA	NA	NA	NA

Strain name	Level of tolerance		RFLP Profile				
			FET3				*ENA1*
	Co(II)	NaCl	*Bam*HI	*Eco*RI	*Hind*III	*Sau*3AI	*Sau*3AI
var. *hansenii* DhhBCS001	T	T	TS	TS	TS	TS	TS
var. *hansenii* DhhBCS002	HT	S	NA	NA	NA	NA	NA
var. *hansenii* DhhBCS003	HT	WT	NA	NA	NA	NA	NA
var. *hansenii* DhhBCS004	HT	S	NA	NA	NA	NA	NA
var. *hansenii* DhhBCS005	HT	WT	NA	NA	NA	NA	NA
var. *hansenii* DhhBCS006	HT	S	NA	NA	NA	NA	NA
var. *hansenii* DhhBCS007	T	T	TS	TS	TS	TS	TS
var. *fabryi* DhfBCS001	T	T	TS	TS	TS	TS	TS
var. *fabryi* DhfBCS002	HT	S	NA	NA	NA	NA	NA

*****Visible riboflavin production.

Variability in the form of differential gene expression and genetic differences in transcriptional regulators have been reported as factors that may contribute to the distinct responses of yeast species and, thus may be correlated with environmental stresses and growth phases [8,21,27,43]. In environmental isolates of *Saccharyomyces cerevisiae* exposed to minimal requirements for growth, gene transcripts involved in stress response sustained low levels of abundance, except for significant variation for those related to nitrogen metabolism [8]. The presence of a TATA box in the promoters of genes, regardless of their function, have also shown an increased tendency for interspecies variability in expression, suggesting that TATA-dependent regulation may be involved in expression divergence [55]. In addition, analyses in the variability of transcription binding sites have revealed sufficient diversity to affect the expression of genes such as by the binding of the transcription factor Ste12 (activated by mitogen-activated protein kinase signalling pathway) [3,23]. In contrast, regulatory processes associated with biological function have shown tendencies towards post-transcriptional regulatory mechanisms instead of transcriptional regulation by network patterns, or motifs, of interactions between transcription factors and their targets, including protein-protein interactions [41]. Genetic pleiotropy, multiple phenotypic outcomes due to a mutation in a single gene, may be more commonly found in yeast genes than previously stated and may contribute to gene variations in signal transduction and transcription [22]. Divergence in gene expression has also been proposed as a result of the evolution of DNA-encoded nucleosome organization of promoters that may be dependent on the presence of nucleosomes on chromatin structure [25], whereas chromatin

silencing as a regulatory mechanism of genomic response to environmental stress has also been contemplated [13]. Transcriptional studies of genes associated with cobalt stress (or genes of the iron regulon) and high salt conditions among strains of *D. hansenii* should be undertaken to better understand the importance of their expression when exposed to these conditions, whether the end product is being produced, and how it may affect phenotype trait.

With regard to genomic organization, the genus *Debaryomyces* is well known for its chromosomal length polymorphism, varying from 4 to 10 chromosomal bands ranging from 0.7 to 4.2 Mb each as revealed by pulse-gel electrophoresis of 41 strains [14]. Another study of 56 *D. hansenii* isolates discovered an average of 6 chromosomal bands for a total of genome size of 12.6 Mb [45]. The type strain CBS 767, whose genome was released on two separate occasions (2004 [19] and 2008 (http://www.genolevures.org/download.html#deha), consists of 7 chromosomal bands varying from 1.25 Mb to 2.33 Mb for a total of 12.2 Mb, not including ribosomal DNA (http://www.genolevures.org/deha.htm). These size differences may be attributed to repeated sequences, duplicated genes, and chromosomal rearrangement [14,38, 42,45]. The highest number of nuclear mitochondrial DNA (NUMT) insertions has been reported in haploid and diploid *D. hansenii* strains [33], although it has been found in other hemiascomycetes [49]. The generation of aneuploid (genome contents with abnormal chromosomal number) and euploid budding yeast strains have suggested that aneuploidy can directly affect gene expression on transcriptional and protemic levels, resulting in significant phenotypic variations dependent on environmental conditions [44] and that aneuploidy induced by proteotoxic stress brought about by the temporary inhibition of the Hsp90 protein or heat shock may contribute to chromosome instability and increased karyotype diversity [11]. The insertion of Ty elements in yeast species has also been contemplated as an significant factor for genomic diversity [2,9,38,51] as well as chromosomal translocation and gene conversion events [51]. The heterogeneity observed in the genotype profiling using *FET3* and *ENA1* highly suggests greater possibility of chromosomal sequence variation among these strains. Karyotype analysis, particularly chromosomal length polymorphism, using pulse-field gel electrophoresis (PFGE) should be considered when elucidating the genomic organization of *D. hansenii* strains.

Phenotype plasticity was also contemplated as a plausible explanation for phenotype variation among the strains studied upon different environmental stresses. Several definitions of phenotype plasticity have been developed and modified throughout the literature [5,26,50]. As

reviewed by [26], phenotype plasticity may be referred to as a particular genotype that is able to present various observable characteristics, such as morphological, physiological, and behavioral, as a direct reaction to changes in the surrounding environment. As mentioned in this dissertation, no single genotype, based on the inducible genes *FET3* and *ENA1* analyzed, was directly linked to phenotype upon cobalt and saline stress. Various strains exhibited distinct levels of tolerance (Table 7), varying from sensitivity to cobalt stress to tolerance to salt stress. Strains of marine origin mostly depicted this type of plasticity, revealing an ability to grow under conditions of high oxidative stress while those same strains demonstrated limited growth upon high osmotic stress (Table 2). One possible method of studying this phenomenon is by analyzing gene regulatory networks in combination with differential gene expression of yeast strains exposed to different environments, which may show a positive corrrelation with the number of transcription factors regulating a specific gene [47]. In strains of *S. cerevisiae*, transcriptional plasticity, or significant variation in gene regulation, associated with rich and poor natural conditions corresponded to phenotypic plasticity and tended towards the presence of paralogous genes [36]. However, studies on the transcriptional profiles of yeast mutants of MAPK pathway (which is involved in pheromone and filamentous growth), despite changes generated at the same level of the pathway, revealed differences in gene expression and phenotype [6].

An alternate explanation that has been proposed for the phenotype variation is the presence of prions. In yeast, prions have been identified as amyloids of prolonged existence in the cytoplasm or nucleus, are mostly modulated by proteins or are altered states of normal proteins, and are associated with non-Mendelian inheritance [32,35,60]. Several studies have revealed that prions are more common in yeast than previously speculated, may be an important driving force for phenotypic diversity in yeast under environmental stress, and may be influenced by heat shock chaperone proteins [12,30]. Interestingly, upregulation of heat shock proteins (Hsp10, Hsp12, and Hsp26) were detected in *S. cerevisiae* upon cobalt stress [53], which may have also occurred in the strains analyzed in this dissertation. The presence of prions, particularly [PSI] and [MOT3], and the induction of heat shock proteins should be considered when studying phenotypic traits and heterogeneity that may be heritable among budding yeasts upon environmental stress, especially in wild type strains [1,31,58].

As previously mentioned in Chapter 3, *D. hansenii* has been isolated from different habitats that exhibit profiles of stressful environments (e.g., low-water activity, high salt

concentrations, poor nutrients) that require resilient metabolic pathways that are able to acclimate or eventually adapt to such conditions. In *S. cerevisiae*, a complex genotype-environment interaction contributes to phenotypic variation mostly by transcriptional regulation and mostly during transitions in the environment or nutrient sources [20,52]. Industrial processes, such as the ripening process of surface ripened cheeses, have shown that more than one strain of *D. hansenii* was involved and suggested a microbial succession during the process [46]. Other strains of *D. hansenii* have also presented high variation on a molecular and metabolic level despite genetic homogeneity, suggesting an indirect correlation to the source of isolation [16]. Population history has also been considered as a possible explanation for phenotypic trait diversity, of which domesticated laboratory strain *S. cerevisiae* 288C showed atypical characteristics compared to its nearest relatives from the natural environment [59]. Based on the diverse sources from which this yeast has been isolated, it can be proposed that the habitat/environment and positive selection can be contributing factors to the phenotype and genotype diversity that was observed in this dissertation although no direct relationship was found using the established criteria.

In conclusion, the main objectives of this dissertation support evidence that *D. hansenii* (i) has the potential to be used as a biosensor of metal contaminated environments (which has been reported in the Caribbean [18,24,40,48]) using riboflavin production as an indicator, (ii) exhibits large phenotypic diversity among strains that undergo cobalt (oxidative) and saline (osmotic) stress, and (iii) revealed high heterogeneity among strains using genes *FET3* and *ENA1* that have been shown to be actively transcribed under stressful conditions. Although no direct phenotype prediction could be made based on the genotype profiles of the strains analyzed in this dissertation, the evidence suggests that an extensive effort to integrate information from genetic sequences, phenotypic characteristics and habitat sources is critical to understanding the complexity of the genetic and molecular networks involved in the intrinsic physiological and metabolic processes of strains from *D. hansenii*. To accomplish this, comparative genome analyses jointly with transcriptomic and proteomic studies are necessary to help address our inquiries with regard to predicting phenotypic characteristics of organisms when exposed to environmental stress factors.

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