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NITRORREDUTASES: UM ESTUDO DAS POSSÍVEIS FUNÇÕES NA RESPOSTA AO ESTRESSE OXIDATIVO

TESE DE DOUTORADO

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Tese submetida ao Programa de Pós-graduação em Biologia Celular e Molecular da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do grau de Doutor em Ciências

Orientador: Prof. Dr. João Antonio Pêgas Henriques

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"Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas também não sou o que era antes".

(Marthin Luther King)

A meus pais, família e amigos

APRESENTAÇÃO

Esse trabalho foi desenvolvido no Laboratório de Reparação de DNA de Eucariotos do Departamento de Biofísica da Universidade Federal do Rio Grande do Sul. O projeto foi subsidiado pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento e Formação de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) -PRONEX/FAPERGS/CNPq (nº 10/0044-3) e pelo – Instituto de Educação para Pesquisa, Desenvolvimento e Inovação Tecnológica Royal, Unidade GENOTOX-ROYAL, Centro de Biotecnologia/Departamento de Biofísica da Universidade Federal do Rio Grande do Sul. A redação da tese fundamentou-se nas recomendações do Programa de Pós-Graduação em Biologia Celular e Molecular, desta Universidade.

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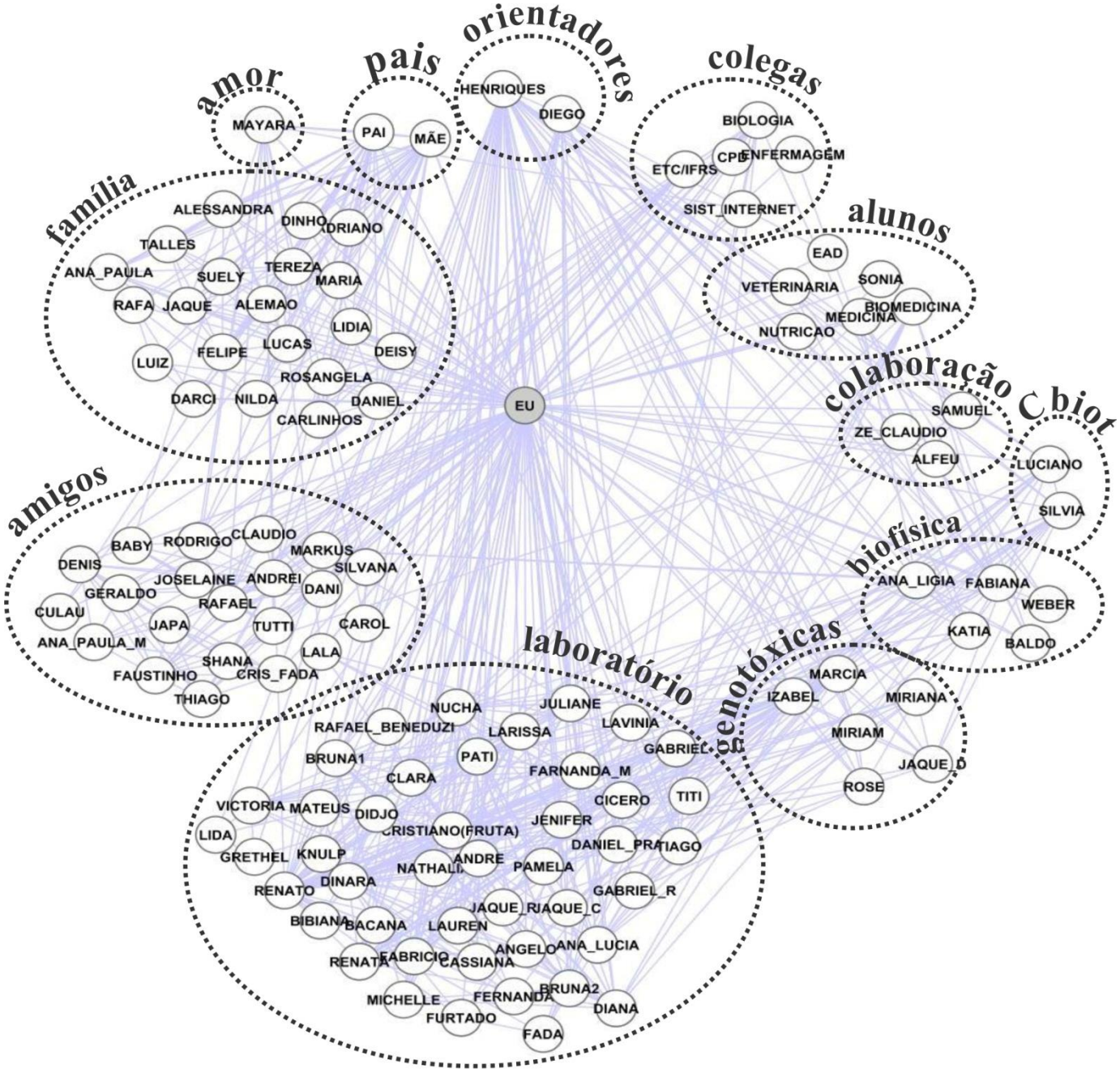

Figura I: Cada nó (pessoa) da rede e cada linha (interação) é uma história que se somam para formar a minha história. Entenda a presença do nó com seu nome como um singelo agradecimento por, de alguma forma, ter contribuído para que eu chegasse até este momento.

Neste espaço para agradecimentos tentei representar um pouco da rede de interações da minha vida, mesmo sabendo que é dificil fazer isso sabendo que muitas interações ficariam de fora. Peço desculpas a quem eu tenha esquecido no momento da construção, não por dar menos importância, mas pelo banco de dados (memória) ser falho, mas se sinta pertencente a mais esta etapa que está sendo concluída.

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Agradeço ao Professor Diego Bonatto, meu co-orientador, pela colaboração ao longo desses anos, podemos resumir tudo dizendo que sem ele este projeto nem sequer existiria. O Diego é uma pessoa que pode ser definida em apenas uma palavra: Gênio.

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contribuido na formação de vocês, mas acredito que eu tenha aprendido muito mais do que ensinado.

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Aos meus grandes amigos. Poderia escrever muitas páginas falando de cada um, mas infelizmente só citarei os nomes na figura, mas isso não diminui em nada a importância que possuem na minha vida.

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À toda minha família, que sempre me deu base para prosseguir. Muito especialmente oos meus pais ANTÔNIO CASTRO DE OLIVEIRA e ONEIDE RODRIGUES MARQUES, pelo amor e apoio que sempre me deram, por me possibilitarem tudo na vida e sem eles eu não estaria aqui (inclusive literalmente).

ESTRUTURA DA TESE

A presente tese está estruturada da seguinte maneira: uma introdução geral, os objetivos (gerais e específicos), os quatros capítulos principais escritos na forma de artigos científicos, uma discussão geral, conclusões (gerais e específicas) e perspectivas.

A introdução apresenta uma visão geral sobre nitrocompostos e as enzimas nitrorredutases, com ênfase nas nitrorredutases Frm2p e Hbn1p de *Saccharomyces cerevisiae*. Nessa seção são abordados conceitos importantes para o entendimento do estresse oxidativo, como as principais espécies reativas e danos em biomoléculas. Também são mostrados os principais mecanismos de defesa antioxidante, com ênfase em bactérias e leveduras, eas principais características e vantagens do modelo biológico utilizado no estudo, a levedura *S. cerevisiae*. Ao final da introdução, são abordados conceitos de biologia de sistemas discorrendo a respeito das redes de interações e parâmetros utilizados na sua análise.

O Capítulo I se trata de uma revisão que apresenta uma breve visão sobre nitrocompostos e características gerais das nitrorredutases, sendo focada na importância ambiental e para saúde humana, bem como nas aplicações biotecnológicas e clínicas dessas enzimas. Este capítulo foi publicado no livro *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology.*

O Capítulo II consiste de uma revisão, em forma de manuscrito, dos aspectos intrínsecos das nitrorredutases, como mecanismos de redução, classificação filogenética, estrutura e possíveis funções fisiológicas. É importante salientar que esta é a primeira revisão a abordar as nitrorredutases presentes em eucariotos.

No Capítulo III foram investigadas possíveis funções das nitrorredutases NfsA e NfsB no metabolismo de *E. coli*, com ênfase na reposta ao estresse oxidativo. Essa

análise foi realizada pela construção da rede de interações dessas enzimas e identificação dos processos biológicos associados.

No Capítulo IV é relatado o estudo da influência das nitrorredutases Frm2p e Hbn1p na resposta ao estresse oxidativo em *S. cerevisiae*. Nesta seção, é mostrado que as nitrorredutases de levedura são capazes de modular as defesas antioxidantes de *S. cerevisiae*. Este capítulo foi publicado no periódico *Yeast*.

O Capítulo V é um complemento do Capítulo anterior, já que nele se busca elucidar os possíveis mecanismos pelos quais as nitrorredutases Frm2p e Hbn1p modulam as defesas antioxidantes em *S. cerevisiae*. Neste capítulo, fica evidenciado que interações das nitrorredutases Frm2p e Hbn1p com outras proteínas podem afetar a resposta ao estresse oxidativo.

Na sequência apresenta-se uma discussão geral dividida em tópicos inter-relacionando as informações descritas nos capítulos acima, as conclusões e perspectivas geradas por esta tese.

No item "Anexos" encontram-se: (1) um artigo contendo a primeira identificação, por análises filogenéticas, das nitrorredutases Frm2p e Hbn1p, publicado no periódico *Biochemical and Biophysical Research Communications* (BBRC); (2) Resultados referentes à exposição de linhagens de *S. cerevisiae* proficientes e deficientes nas proteínas Frm2 e Hbn1 expostas a agentes oxidantes e mutagênicos. (3) uma tabela contendo a comparação dos resultados da exposição de linhagens de *S. cerevisiae* proficientes e deficientes nas proteínas Frm2 e Hbn1 a agentes oxidantes em fase estacionária ou exponencial de crescimento (4) uma tabela contendo a comparação dos resultados obtidos na exposição em metabolismo fermentativo ou respiratório

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ADEPT	*antibody-directed enzyme prodrug therapy*
CAT	catalase
CDNB	1-cloro-2,4-dinitrobenzeno
DCFH-DA	diacetato de 2′,7 diclorodihidrofluoresceina
DNA	ácido desoxirribonucleico
dAMP	deoxiadenosina monofosfato
EROs	espécies reativas de oxigênio
FAD	flavina adenina dinucleotídeo oxidado
FMN	flavina mononucleotideo
Fapy	formamidopirimidinas
GDEPT	*gene-directed enzyme prodrug therapy*
GPx	glutationa peroxidase
GR	glutationa redutase
GSH	glutationa reduzida
GSSG	glutationa oxidada
HCA	análise de agrupamentos hidrofóbicos
HNE	4-hidroxi-nonenal
MDA	malondialdeído
MMS	metil metano sulfonato
NADH	nicotinamida adenina dinucleotídeo reduzido
NADPH	nicotinamida adenina dinucleotídeo fosfato reduzido
NBT	*nitroblue tetrazolium*
NDEA	N-nitrosodietilamina
PCP	pentaclorofenol
RNA	ácido ribonucleico
ROH	forma alcoólica após redução de algum hidroperóxido orgânico
ROOH	hidroperóxido orgânico
SOD	superóxido dismutase
STRE	elemento de resposta a estresse

RESUMO

As nitrorredutases compreendem uma família de enzimas dependentes de flavina adenina mononucleotideo (FMN) capazes de metabolizar nitrocompostos usando nicotinamida adenina dinucleotídeo (NAD(P)H) como fonte de elétrons. Essas enzimas desempenham um papel central na metabolização de nitrocompostos recebendo grande atenção devido a sua habilidade em mediar a toxicidade desses compostos, tendo aplicações biotecnológicas e importância clínica. Essas enzimas podem ser encontradas em bactérias e em menor escala em fungos, protozoários e mamíferos. Em relação ao seu papel fisiológico, algumas hipóteses, como a participação na bioluminescência, homeostase metálica, biossíntese de cobalamina e resposta a estresse oxidativo têm sido propostas. Entretanto, não se tem conhecimento exato sobre a sua real função biológica. Neste cenário, este estudo tem como objetivo investigar possíveis funções das nitrorredutases no metabolismo de *Escherichia coli* e *Saccharomyces cerevisiae*, com ênfase na possível participação dessas enzimas na resposta ao estresse oxidativo. Para tanto, foi construída a rede de interações proteína-proteína das nitrorredutases NfsA e NfsB de *E. coli* e identificadas cinco sub-redes representando diferentes processos biológicos. Os resultados permitiram a elaboração de modelos sugerindo que as nitrorredutases de *E. coli* podem participar do metabolismo de ferro, manutenção do conteúdo de NADPH, metabolismo de compostos aromáticos e síntese de glicogênio. Estas vias podem contribuir nas respostas a estresse oxidativo e a limitação de nutrientes. Na levedura *S. cerevisiae*, foi determinada a influência das nitrorredutases Frm2p e Hbn1p na resposta a estresse oxidativo. Os resultados mostraram uma menor atividade basal de superóxido dismutase (SOD) e elevada sensibilidade a óxido de 4-nitroquinolina (4-NQO) e N-nitrosodietilamina (NDEA), indução de mutantes citoplasmáticos (petites), produção intracelular de ERO e peroxidação lipídica nas linhagens *frm2*Δ, *hbn1*Δ e *frm2*Δ *hbn1*Δ quando expostas a estes agentes geradores de superóxido. Ainda foi observada elevada atividade basal de catalase (CAT), glutationa peroxidase (GPx) e conteúdo de glutationa (GSH) nas linhagens *frm2*Δ e *frm2*Δ *hbn1*Δ. Estas linhagens possuem menor produção de espécies reativas de oxigênio (ERO) e peroxidação lipídica quando expostas aos peróxidos H_2O_2 e *t*-BOOH. Para elucidar os mecanismos pelos quais as nitrorredutases Frm2p e Hbn1p podem regular as defesas antioxidantes, foram identificadas, por biologia de sistemas, as interações dessas proteínas, tendo cinco sub-redes representando diferentes processos biológicos. Esta análise foi seguida por uma avaliação de índices de centralidade, que identificaram importantes proteínas da rede. Uma triagem dos fenótipos de sensibilidade a oxidantes foi realizada com linhagens proficientes e deficientes nestas proteínas identificadas e foram selecionadas as proteínas envolvidas nas respostas mais evidentes ao estresse oxidativo: as proteínas Ski8 (contribui na degradação do RNAm no sentido 3'-5') e Cad1 (um ativador transcricional). Esta informação foi usada para a construção de linhagens duplo e triplo mutantes deficientes em Frm2p, Hbn1p, Ski8p ou Cad1p, seguindo a determinação da sensibilidade, acúmulo intracelular de ERO e nível de peroxidação lipídica na exposição a oxidantes e ainda a atividade basal de enzimas antioxidantes. Com base nos resultados obtidos, foi construído um modelo considerando que Cad1p ativa a expressão do gene *FRM2* e a interação Frm2p-Ski8p regula as atividades das enzimas antioxidantes pela degradação do RNAm ou pela modulação da degradação dos transcritos do gene *OLE1* (Ole1p atua na síntese de ácidos graxos insaturados) modificando a composição de ácidos graxos da membrana plasmática. A interação Hbn1p-Nab2p (Nab2p é necessária para a exportação do RNAm do núcleo para o citoplasma) controla a atividade de SOD pela exportação do RNAm.

ABSTRACT

The nitroreductase family comprises a group of flavine mononucleotide (FMN)-dependent enzymes able to metabolize nitrosubstituted compounds using the reducing potential of nicotinamide adenine dinucleotide (NAD(P)H). Nitroreductase proteins play a central role in the activation of nitrocompounds and have received attention in recent decades based on their role in mediating nitrosubstituted compound toxicity, by its biotechnological application for bioremediation biocatalysis, and clinical importance in chemotherapeutic tumor treatment, ablation of specific cells and antibiotic resistance. Due to its relevance, different bacterial nitroreductases have been purified, and their biochemical, kinetic parameters and structure have been determined. Nitroreductases can be found within bacterial species and, in a less extend, in eukaryotes, such as fungi, protozoan and mammalian. A feature of the nitroreductase family is our lack of knowledge about its biological function. Therefore, new hypotheses have been proposed to solve the physiological role of nitroreductases, such as bioluminescence, metal homeostasis, vitamin B_{12} biosynthesis and oxidative stress response. In this context, this study aims to investigate possible functions of nitroreductases in *Escherichia coli* and *Saccharomyces cerevisiae* metabolism, with emphasis on possible role of these enzymes in oxidative stress response. Thus, a systems biology study was performed by generating protein-protein interactions (PPI) for NfsA and NfsB nitroreductases of *E. coli*. The results obtained from these systems biology analyses allow us to draw some models suggesting that *E. coli* nitroreductases can participate in iron metabolism, NADPH pool maintenance, aromatic compound metabolism, methionine and glycogen synthesis. In the yeast *S. cerevisiae*, the influence of Frm2p and Hbn1p nitroreductases in oxidative stress response was determined. The results showed a weaker basal activity of superoxide dismutase (SOD) and higher sensitivity for 4-nitroquinoline-oxide (4-NQO) and N-nitrosodiethylamine (NDEA), induction of *petites*, production of reactive oxygen species (ROS) and lipid peroxidation when exposed the these superoxide-generating agents. The results showed a higher basal activity of catalase (CAT), glutathione peroxidase (GPx) and reduced glutathione (GSH) content in the single and double mutant strains *frm2*Δ and *frm2*Δ *hbn1*Δ. These strains were less ROS-producing and lipid peroxidation when exposed to peroxides-generating agents such as H_2O_2 and *t*-BOOH. To elucidate the mechanisms how nitroreductases regulate antioxidant defenses, we undertook a systems biology approach to identify Frm2p and Hbn1p interactions. A protein-protein interaction (PPI) network was obtained and biological processes related to RNA metabolism were observed. Thus, network centrality analysis was performed, which allows for selection of important proteins of network. A sensitivity screening of yeast strains proficient and deficient in these proteins to oxidants was performed and selected Ski8p (mediates 3'-5' RNA degradation) and Cad1p (transcriptional activator). This information was used to construct double and triple mutants defective for Frm2p, Hbn1p, Cad1p or Ski8p followed by determination of sensitivity, ROS accumulation, lipid peroxidation following oxidants exposure and basal antioxidant-enzyme activities. The results obtained allow us to draw model suggesting that Cad1p activate *FRM2* following Frm2p-Ski8p interaction influences to oxidative stress response by regulates mRNA degradation of antioxidant-enzyme following their activities or *OLE1* (Ole1p act in unsaturated fatty acid synthesis) transcripts degradation modifying the plasma membrane fatty acid composition. The Hbn1p-Nab2p (Nab2p act in mRNA export) interaction controls SOD activity by mRNA export.

Introdução

Introdução

1. Os nitrocompostos

Os nitrocompostos são agentes químicos que se caracterizam pela presença de um ou mais grupos nitro (NO_2^-) (Spain, 1995). Poucos compostos nitroaromáticos são de origem natural, tal como cloranfenicol (Figura 1) (AOUICHE *et al.*, 2012), e algumas plantas do gênero *Astragalus* sintetizam nitroglicosídeos como mecanismo de defesa (ANDERSON *et al.*, 1993). Entretanto, a grande maioria dos compostos nitroaromáticos é de origem antropogênica, sobretudo como consequência de processos industriais (SPAIN, 1995; DEMARINI *et al.*, 1996). Essas substâncias são utilizadas como matéria-prima na produção de vários produtos, tais como plásticos, explosivos, quimioterápicos, agentes microbianos, aditivos alimentares e defensivos agrícolas (EBRINGER *et al.*, 1982; SPAIN, 1995; RYAN *et al.*, 2011; MIR *et al.*, 2012). Alguns nitrocompostos são resultado de processos de combustão incompleta provenientes de combustíveis fósseis (MORI *et al.*, 2003; SHEN *et al.*, 2012). Também alguns nitrocompostos são recalcitrantes no ambiente por serem absorvidos e retidos no solo pela matéria húmica. Um exemplo disso é o 2,4,6 – trinitrotolueno (TNT) (Figura 1), encontrado sobretudo em solos de áreas militares por deposição de explosivos (SMETS *et al.*, 2007; PANZ & MIKSCH, 2012). Nitrocompostos também estão envolvidos na poluição da água por solventes e pesticidas, principalmente por erosão de solos contaminados (PERES & AGATHOS, 2000; PANZ & MIKSCH, 2012). São largamente encontrados em cigarros, exaustores a diesel e partículas aéreas urbanas (TOPINKA *et al.*, 2000; SCIPIONI *et al.*, 2012). Assim, os compostos nitroaromáticos e nitroheterocíclicos são considerados um importante grupo de poluentes ambientais (JU & PARALES, 2010; ARORA *et al.*, 2012).

A metabolização de nitrocompostos pode levar à formação de intermediários tóxicos, genotóxicos, mutagênicos e/ou carcinogênicos (PUROHIT & BASU, 2000; MAEDA *et al.*, 2007). Muitos nitrocompostos podem gerar espécies reativas de oxigênio e de nitrogênio (ERO e ERNs, respectivamente) que podem reagir com biomoléculas (MASUDA *et al.*, 2000; BOELSTERLI *et al.*, 2006). Desta forma, esses compostos têm atraído considerável atenção devido ao seu risco potencial à saúde humana (TCHOUNWOU *et al.*, 2003; BOELSTERLI *et al.*, 2006). Entretanto, também são moléculas com um interessante potencial farmacológico, podendo alguns nitroderivados ser utilizados como antibióticos e em terapias antitumorais (BHAUMIK, 2011; IWANCZAK & IWANCZAK, 2012).

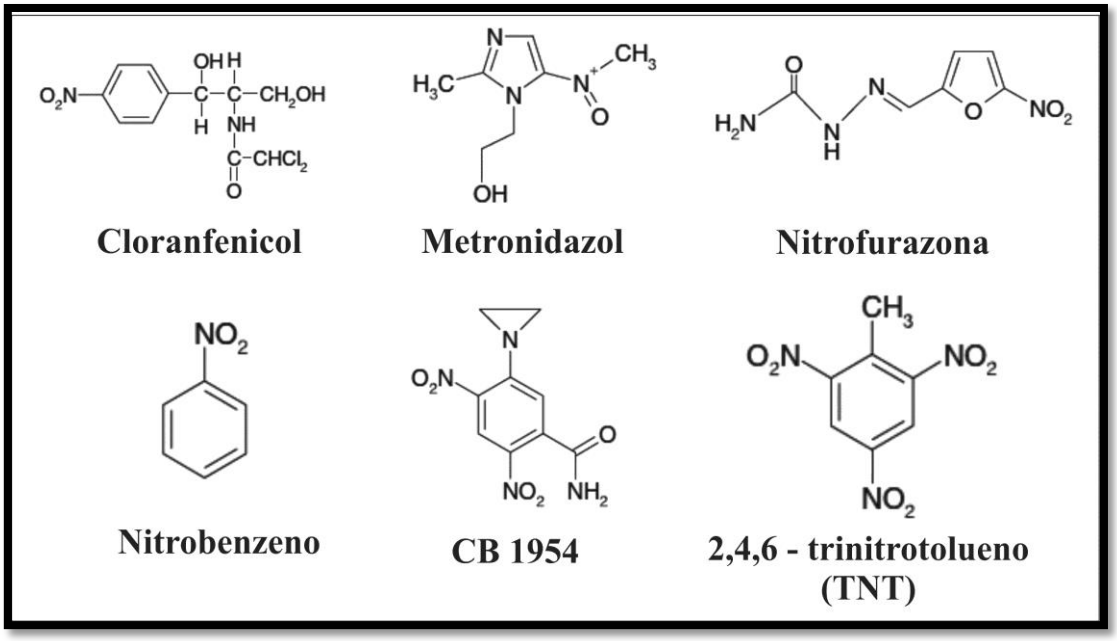


Figura 1: Estruturas químicas de alguns nitrocompostos bem conhecidos

Os nitrocompostos podem ser metabolizados e degradados por microorganismos, como bactérias e fungos, que os utilizando até mesmo como fonte de nitrogênio e carbono (SPAIN, 1995; JU & PARALES, 2010). As vias metabólicas que resultam na ativação desses nitrocompostos são complexas. A nitrorredução é o passo

inicial no catabolismo de uma variedade de compostos nitroaromáticos (SPAIN, 1995; ROLDAN *et al.*, 2008). Normalmente, são requeridas transformações enzimáticas que geralmente resultam em espécies mais reativas, responsáveis por sua toxicidade (ROLDAN *et al.*, 2008). Neste sentido, as nitrorredutases desempenham um papel central na ativação de nitrocompostos (WHITEWAY *et al.*, 1998; ROLDAN *et al.*, 2008; CHRISTOFFERSON & WILKIE, 2009).

2. Nitrorredutases

As nitrorredutases são enzimas capazes de catalisar a redução do grupo nitro e utilizam flavina mononucleotideo (FMN) ou flavina adenina dinucleotídeo (FAD) como grupo prostético e nicotinamida adenina dinucleotídeo (NAD(P)H) como agente redutor (BRYANT & DELUCA, 1991; SKELLY *et al.*, 1994; KOBORI *et al.*, 1999). Estruturalmente, as nitrorredutases são proteínas homodiméricas, com subunidades constituídas por α-hélices e estruturas β, sendo que as subunidades se unem formando uma cavidade hidrofóbica onde ficam localizados o grupo prostético e o sítio ativo da enzima (ver Capítulo II) (KOBORI *et al.*, 1999; RACE *et al.*, 2005).

Decorrente da capacidade de metabolização e ativação de nitrocompostos, essas proteínas vêm atraindo um grande interesse, tendo importância ambiental, para saúde humana, bem como aplicações biotecnológicas e clínicas (ver Capítulo I). Essas enzimas podem ser empregadas na biorremediação e na biocatálise (HANNINK *et al.*, 2001; KADIYALA *et al.*, 2003). As nitrorredutases apresentam aplicação na terapia antiproliferativa devido à sua capacidade de converter pró-drogas não tóxicas, como a 5-aziridina-1-il-2,4-dinitrobenzamida (CB1954) (Figura 1) em agentes citotóxicos (CHRISTOFFERSON & WILKIE, 2009; PROSSER *et al.*, 2013). Muitos estudos têm associado nitrorredutases às infecções hospitalares, já que bactérias deficientes nessas

enzimas (como *Helicobacter pylori*) são mais resistentes ao antibiótico Metronidazol (Figura 1), que necessita ser ativado por redução (VAN DER WOUDEN *et al.*, 2000; KARGAR *et al.*, 2010).

Dois grupos de nitrorredutases têm sido bioquimicamente caracterizados: as nitrorredutases insensíveis ao oxigênio (tipo I) que realizam uma redução sequencial do nitrocomposto transferindo dois elétrons do NADPH ou NADH para o grupo NO_2^-, levando à formação de intermediários nitroso, hidroxilamina e finalmente aminas primárias (ZENNO *et al.*, 1996b; RACE *et al.*, 2005); e as nitrorredutases sensíveis ao oxigênio (tipo II), que catalisam a redução sequencial do nitrocomposto transferindo um elétron dos agentes redutores para o grupo NO_2^-, produzindo um radical nitro-ânion o qual reage com oxigênio e origina superóxido, regenerando o nitrocomposto original. Este "ciclo fútil" pode causar estresse oxidativo por produzir grandes quantidades de superóxido (ver Capítulos I e II) (HOMMA-TAKEDA *et al.*, 2002; SARLAUSKAS *et al.*, 2004). As nitrorredutases do tipo I participam da redução de uma variedade de nitrocompostos, incluindo nitrofuranos, nitrobenzenos, nitrofenóis, nitrobenzoatos, nitrotoluenos, nitroimidazóis, entre outros (ROLDAN *et al.*, 2008). Por sua importância, muitos estudos sobre clonagem, isolamento de genes, análise estrutural das proteínas e caracterização funcional têm sido realizados em relação à nitrorredutases de vários organismos (BRYANT *et al.*, 1991; ZENNO *et al.*, 1996b; KIM & SONG, 2005; MANINA *et al.*, 2010). Entretanto, os genes para nitrorredutases do tipo II não foram clonados até o momento, assim como não foi realizada a caracterização estrutural dessas proteínas.

As nitrorredutases compreendem uma família de proteínas conservadas evolutivamente e originalmente identificadas em eubactérias (ROLDAN *et al.*, 2008). Com base na similaridade da estrutura primária das proteínas, as nitrorredutases

bacterianas do tipo I foram divididas em dois grupos principais: NfsA/Frp (Grupo A) e NfsB/FRaseI (Grupo B), sendo que estes grupos possuem baixa similaridade entre si (ver Capítulo II) (CABALLERO *et al.*, 2005; ROLDAN *et al.*, 2008). Diferentemente, os registros de nitrorredutases em eucariotos são bastante restritos. Em mamíferos existem algumas enzimas funcionalmente similares às nitrorredutases do tipo I, como a NAD(P)H-quinona oxidorredutase (DT – diaforase) e a xantina desidrogenase. Entretanto, ambas as enzimas não são filogeneticamente relacionadas e não apresentam o domínio característico da família (DJURIC *et al.*, 1986; FRIEDLOS *et al.*, 1992). Contudo, uma proteína com domínio de nitrorredutases foi descrita em humanos, a iodotirosina deiodinase, envolvida na produção do hormônio da tireoide (Tabela 1 e ver Capítulos I e II) (GNIDEHOU *et al.*, 2006; THOMAS *et al.*, 2009). Na Tabela 1 podem ser observadas algumas das nitrorredutases já identificadas.

Tabela 1 – Principais nitrorredutases caracterizadas até o momento

	Nitrorredutase	Organismo	Ref.
Procariotos			
Grupo A	Frp	*Vibrio harveyi*	(LEI *et al.*, 1994)
	NfrA1 (YwcG)	*Bacillus subtilis*	(ZENNO *et al.*, 1998)
	NfsA	*Escherichia coli*	(ZENNO *et al.*, 1996a)
	NfrA	*Staphylococcus aureus*	(STREKER *et al.*, 2005)
	NitA e NitB	*Clostridium acetobutylicum*	(KUTTY & BENNETT, 2005)
	PnrA	*Pseudomonas putida*	(CABALLERO *et al.*, 2005)
	SrnA	*Salmonella typhimurium*	(WATANABE *et al.*, 1990)
Grupo B	Cnr	*Salmonella typhimurium*	(WATANABE *et al.*, 1989)
	DrgA	*Synechocystis* sp	(ELANSKAYA *et al.*, 1998)
	FRase I	*Vibrio fischeri*	(ZENNO *et al.*, 1994)
	NbzA	*Pseudomonas pseudoalcaligenes*	(SOMERVILLE *et al.*, 1995)
	NfsB	*Escherichia coli*	(ZENNO *et al.*, 1996b)
	NR	*Enterobacter cloacae*	(BRYANT & DELUCA, 1991)
	PnrB	*Pseudomonas putida*	(CABALLERO *et al.*, 2005)
	RdxA	*Helicobacter pylori*	(GOODWIN *et al.*, 1998)
Eucariotos			
	Frm2p e Hbn1p	*Saccharomyces cerevisiae*	(DE OLIVEIRA *et al.*, 2007)
	Iodotirosina deiodinase	*Homo sapiens*	(GNIDEHOU *et al.*, 2004)

Em *Saccharomyces cerevisiae* há dois genes chamados *FRM2* (YCL026c-A) e *HBN1* (YCL026c-B) que codificam duas nitrorredutases, Frm2p e Hbn1p, respectivamente (Tabela I). Essa descrição foi realizada em trabalhos anteriores pelo nosso grupo de pesquisas utilizando, para tanto, abordagens de análises filogenéticas (ver Anexo I).

3. Nitrorredutases e seu papel fisiológico

Um aspecto marcante da família das nitrorredutases é a falta de conhecimento sobre a função biológica dos seus membros. A primeira hipótese a este respeito foi que as nitrorredutases poderiam apresentar a capacidade de reduzir 3-nitrotirosina em proteínas. Entretanto, estudos realizados por Lightfoot e colaboradores (2000) indicaram que NfsA e NfsB de *Escherichia coli* não reduzem 3-nitrotirosina.

Outras possibilidades de atuação fisiológica são também consideradas, como a possível participação na homeostase metálica, bioluminescência, metabolismo de cobalamina (vitamina B_{12}) e resposta a estresse oxidativo são consideradas (LEI & TU, 1998; PATERSON *et al.*, 2002; CAMPBELL *et al.*, 2006; TAKEDA *et al.*, 2010). A vasta maioria dos estudos tem mostrado que as nitrorredutases podem estar realmente envolvidas na resposta a estresse oxidativo (LIOCHEV *et al.*, 1999; PATERSON *et al.*, 2002; ROLDAN *et al.*, 2008) (ver Capítulo II). Neste contexto, o primeiro trabalho a este respeito foi realizado por Liochev e colaboradores (1999), mostrando que o gene *nfsA* de *E. coli*, o qual codifica a nitrorredutase NfsA, é altamente induzido por paraquat, que gera superóxido por ciclo redox (SHEN *et al.*, 2013). O gene *nfsA* está sob o controle do regulon *SoxRS*, o qual é induzido por este radical (FUJIKAWA *et al.*, 2012). O gene *nprA*, que codifica a nitrorredutase NprA em *R. capsulatos*, é também induzido por paraquat (PEREZ-REINADO *et al.*, 2005).

Outras nitrorredutases também se apresentaram envolvidas com resposta a estresse oxidativo, como as nitrorredutases NfrA1 de *B. subtilis* e NfrA de *S. aureus* podem estar envolvidas no metabolismo redox desses organimos, por possivelmente estarem atuando no balanço tiólico celular. Esses fatos reforçam o envolvimento das nitrorredutases na resposta ao estresse oxidativo (TAVARES *et al.*, 2009; CORTIAL *et al.*, 2010).

4. Nitrorredutases Frm2p e Hbn1p de *S. cerevisiae*

Em trabalhos prévios do nosso grupo de pesquisa, foram identificadas por análise filogenética as proteínas Frm2 e Hbn1 como duas nitrorredutases (ver Anexo I). Os nossos resultados indicaram que essas proteínas pertencem a uma nova família de nitrorredutases ainda não caracterizada, apresentando ocorrência apenas em bactérias, fungos e em algumas espécies de protozoários (ver Anexo I). Recentemente, essas proteínas foram purificadas e caracterizadas quanto à capacidade de redução de nitrocompostos, mais especificamente do óxido de 4-nitroquinolina (4-NQO), confirmando-as funcionalmente como novas nitrorredutases (BANG *et al.*, 2012). Entretanto, a proteína Frm2p foi primeiramente identificada por McHale e colaboradores (1996) em uma triagem de mutantes que, na presença de ácido araquidônico, eram deficientes na repressão da expressão do gene *OLE1*, o qual codifica uma Δ9-ácido graxo desaturase, responsável pela insaturação dos ácidos graxos oléico e palmitoléico (VIEGAS *et al.*, 2005).

O gene *FRM2* apresenta elementos de resposta a glicose e a ácidos graxos, sugerindo que Frm2p tenha possível função na via de sinalização de lipídeos. Ainda há a presença no gene *FRM2* de um sítio de ligação a Yap2p, um fator de transcrição associado com resposta a cádmio e resistência e a outros agentes químicos e confirmou-

se que sua expressão é altamente dependente deste fator (AZEVEDO *et al.*, 2007; BANG *et al.*, 2013). Consistentemente, foi constatada a presença sequências STRE (*stress response element*) na região promotora de *FRM2*, as quais são alvos da ligação de proteínas, como Yap2p, responsáveis pela regulação da transcrição em resposta a estresse (MCHALE *et al.*, 1996).

Em relação à proteína Hbn1 a única evidência registrada quanto à possível função fisiológica, foi no estudo realizado por Kim e colaboradores (2007 que identificou por eletroforese bidimensional (2-D) o aumento de expressão de diversas proteínas em resposta a menadiona, um conhecido gerador de superóxido. Entre as proteínas identificadas estava presente a Hbn1p, sugerindo uma possível participação na resposta a estresse oxidativo provocado por este oxidante.

5. Espécies Reativas de Oxigênio

Todos os organismos vivos aeróbios utilizam o oxigênio como aceptor final de elétrons na cadeia transportadora de elétrons. A redução do oxigênio à água (Figura 2), entretanto, gera subprodutos altamente reativos, conhecidos como espécies reativas de oxigênio (ERO) (HALLIWELL & GUTTERIDGE, 2007).

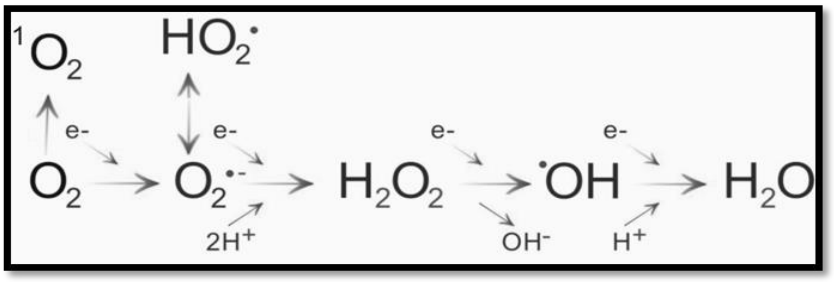


Figura 2: Formação de espécies reativas de oxigênio. Adaptado de Imlay (2008).

Um radical livre pode ser definido como uma espécie química qualquer que contenha um ou mais elétrons desemparelhados (HALLIWELL & GUTTERIDGE, 2007). Uma molécula pode tornar-se um radical livre, tanto ganhando como perdendo um elétron em uma reação química, bem como por fissão homogênea de uma ligação covalente. Geralmente estas espécies são instáveis, possuindo uma meia vida curta, por poder reagir rapidamente com diversas substâncias (HALLIWELL & GUTTERIDGE, 2007; CAPUTO *et al.*, 2012; CHATGILIALOGLU *et al.*, 2012).

Muitos termos são empregados para descrever os radicais livres e espécies reativas de oxigênio. O termo ERO inclui todas as moléculas quimicamente reativas derivadas de oxigênio, abrangendo radicais livres como o radical hidroxil ($HO^{\bullet}$) e o ânion superóxido ($O_2^{\bullet-}$), assim como não radicais, como o peróxido de hidrogênio (H_2O_2) e o oxigênio *singlet* ($^1O_2$). Estes últimos não são considerados radicais livres pois não possuem elétrons desemparelhados (HALLIWELL & GUTTERIDGE, 2007). Nos organismos as ERO podem ser produzidas como subprodutos normais do metabolismo, por consequência de diversas reações de transferências de elétrons, ou por ação de agentes físicos ou químicos (VALKO *et al.*, 2006; HALLIWELL & GUTTERIDGE, 2007; FREINBICHLER *et al.*, 2011).

5.1. Ânion superóxido ($O_2^{\bullet-}$)

O ânion superóxido é formado a partir do oxigênio pela adição de um elétron e, apesar de ser um radical livre, não é altamente reativo (FRIDOVICH, 1997). A formação de $O_2^{\bullet-}$ acontece especialmente em ambientes aeróbios ricos em elétrons, como na mitocôndria (FRIDOVICH, 2004). O $O_2^{\bullet-}$ também pode ser gerado por algumas enzimas, como a xantina oxidase (HALLIWELL & GUTTERIDGE, 2007; CHEN *et al.*, 2013) ou por diferentes compostos químicos como paraquat, 4-NQO e N-

nitrosodietilamina (NDEA), que podem sofrer ciclo redox via redução enzimática (FANN *et al.*, 1999; RANA & SONI, 2008; IANNONE *et al.*, 2012).

Um mecanismo proposto de toxicidade do $O_2^{\bullet-}$ é baseado na observação de que este radical pode oxidar especificamente o sítio [4Fe-4S] de proteínas e levar a inativação de enzimas, sobretudo pertencentes ao metabolismo energético e de aminoácidos (WALLACE *et al.*, 2004; HALLIWELL & GUTTERIDGE, 2007). Esse processo pode causar a liberação do íon Fe^{2+} das proteínas e levar a um dano oxidativo adicional de outros componentes celulares, uma vez que o ferro livre pode promover, via reação de Haber Weiss/Fenton, a formação do radical hidroxil ($HO^{\bullet}$) (Figura 3) (NEYENS & BAEYENS, 2003; CORNELIS *et al.*, 2011). O $O_2^{\bullet-}$ pode gerar H_2O_2 e oxigênio espontaneamente ou pela ação da enzima superóxido dismutase (SOD) (FRIDOVICH, 1986; LIOCHEV & FRIDOVICH, 2007). A forma protonada do $O_2^{\bullet-}$, o radical hidroperoxil ($HO_2^{\bullet}$) é mais reativo do que o $O_2^{\bullet-}$, podendo atacar ácidos graxos insaturados, iniciando a cascata de peroxidação lipídica e ocasionar destruição das membranas biológicas (TEMPLE *et al.*, 2005; HALLIWELL & GUTTERIDGE, 2007).

5.2. Peróxido de hidrogênio (H_2O_2)

O H_2O_2 não é um radical livre, mas pode originar formas mais reativas, como $OH^{\bullet}$, via reação com metais de transição (Figura 3) (TOYOKUNI, 2002; NEYENS & BAEYENS, 2003). O H_2O_2 é formado principalmente na matriz mitocondrial durante o processo de redução do O_2, ou pela dismutação do $O_2^{\bullet-}$, pela enzima SOD (AFONSO *et al.*, 2007; LIOCHEV & FRIDOVICH, 2007). Pode ainda ser produzido endogenamente pela β-oxidação no metabolismo de lipídeos no peroxissomo (HILTUNEN, 1991; RAO & REDDY, 2001; ELSNER *et al.*, 2011). Apesar de poder causar danos em

biomolécular, o H_2O_2 tem importância em processos fisiológicos, por exemplo como molécula sinalizadora (YU *et al.*, 2012; BINDOLI & RIGOBELLO, 2013).

Reação de Waber-Weiss

$$Cu^{2+}/Fe^{3+} + O_2^{\bullet -} \rightarrow Cu^{+}/Fe^{2+} + O_2$$

Reação de Fenton

$$H_2O_2 + Cu^{+}/Fe^{2+} \rightarrow OH^{\bullet} + OH^{-} + Cu^{2+}/Fe^{3+}$$

Reação de Harber-Weiss/Fenton

$$H_2O_2 + O_2^{\bullet -} \rightarrow OH^{\bullet} + OH^{-} + O_2$$

Figura 3: Representação da formação do radical HO• pelas reações de Haber Weiss/Fenton. Adaptado de Nordberg & Arner (2001).

5.3. Radical hidroxil ($HO^{\bullet}$)

O radical $HO^{\bullet}$ é formado pelo H_2O_2 na reação catalisada por íons metálicos, como Fe^{2+} e Cu^{+}, muitas vezes ligados em complexos com diferentes proteínas ou outras moléculas (NEYENS & BAEYENS, 2003). Esta reação é conhecida como Reação de Haber Weiss/Fenton (Figura 3) (KOPPENOL, 2001).

Este radical pode formar outros radicais com reatividade menor ou reagir com lipídeos, proteínas e ácidos nucléicos, por reações de adição, abstração de átomos de hidrogênio ou transferência de elétrons (HALLIWELL & GUTTERIDGE, 2007). A capacidade deste radical em lesar as biomoléculas é superior às demais ERO devido a sua alta reatividade e pelo fato do organismo não dispor de um sistema enzimático de

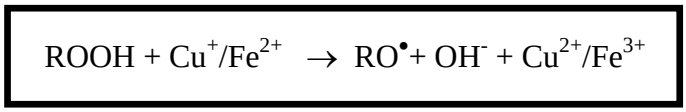
defesa contra esta ERO (WINTERBOURN, 1995; HALLIWELL & GUTTERIDGE, 2007). A melhor defesa que a célula possui contra este radical é evitar que o mesmo seja gerado. Alguns sistemas podem contribuir neste sentido, como por exemplo, o controle sobre a homeostase metálica (FRIDOVICH, 1998; CORNELIS *et al.*, 2011).

5.4. Oxigênio *singlet* ($^1O_2$)

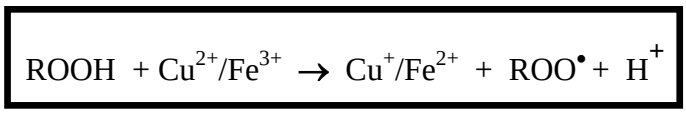
O $^1O_2$ é formado pela transferência de energia, como por fotoexcitação, com consequente inversão do spin de um elétron do oxigênio molecular, completando uma camada quântica, de modo a converter de *triplete* para *singlete* (HALLIWELL & GUTTERIDGE, 2007; OGILBY, 2010; FISCHER *et al.*, 2013). Esta ERO é a forma mais reativa do oxigênio (HALLIWELL & GUTTERIDGE, 2007). Pode reagir diretamente com lipídeos, proteínas e com o DNA. (GIROTTI, 1998; DAVIES, 2003; RAVANAT *et al.*, 2012). Entretanto, fisiologicamente pode atuar, em alguns casos, na sinalização celular (KIM *et al.*, 2008; FOYER & NOCTOR, 2013).

5.5. Radical Peroxil ($RO_2^{\bullet}$)

O radical $RO_2^{\bullet}$ pode ser formado pelo ataque de ERO, como o $HO^{\bullet}$, $^1O_2$ ou $HO_2^{\bullet}$, a lipídeos ou por decomposição de peróxidos orgânicos (ROOH) (SCHNEIDER, 2009). A reatividade do $RO_2^{\bullet}$ está relacionada com a parte orgânica do radical, sendo os radicais aromáticos menos reativos pela possibilidade de rearranjo dos elétrons. Além disso, ainda podem participar da reação de Fenton (Figura 4) (VALKO *et al.*, 2006).



$$ROOH + Cu^{+}/Fe^{2+} \rightarrow RO^{\bullet} + OH^{-} + Cu^{2+}/Fe^{3+}$$



$$ROOH + Cu^{2+}/Fe^{3+} \rightarrow Cu^{+}/Fe^{2+} + ROO^{\bullet} + H^{+}$$

Figura 4: Representação da formação do radical OH• pela reação de Fenton com o radical peroxil. Adaptado de Halliwell & Gutteridge (2007).

6. Estresse oxidativo e danos nas biomoléculas

As ERO são mantidas em certas concentrações no organismo, e não degradadas completamente, já que são importantes em determinadas funções celulares, como na sinalização celular (HALLIWELL & GUTTERIDGE, 2007; BURHANS & HEINTZ, 2009). Já foi observado que a produção de ERO leva à alterações na expressão gênica via fosforilação de fatores de transcrição (LIU *et al.*, 2005; SUNDAR *et al.*, 2010). No entanto, as ERO podem ser produzidas em quantidades excessivas, provocando um desequilíbrio entre geração de ERO e defesas antioxidantes, estado este denominado **estresse oxidativo** (HALLIWELL & GUTTERIDGE, 2007). As ERO podem danificar diferentes biomoléculas, tais como lipídeos, proteínas, carboidratos e ácidos nucléicos (FRIDOVICH, 1998; VALKO *et al.*, 2006; CADET *et al.*, 2010). Desta forma podem comprometer a integridade celular por causar danos à membrana, perda de função de organelas, redução na eficiência metabólica, danos na cromatina e mutações, levando à morte celular (HALLIWELL & GUTTERIDGE, 2007; GRUNING *et al.*, 2010; KRYSTON *et al.*, 2011).

6.1. Danos oxidativos no DNA

As ERO podem produzir uma série de lesões no DNA, danificando as bases nitrogenadas, criando sítios apurínicos e apirimidínicos (sítios AP), modificações nas bases e gerando ligações cruzadas entre DNA e proteínas ou lipídeos ou atacando desoxirribose, causando quebras simples e duplas na cadeia, **(**HALLIWELL & GUTTERIDGE, 2007; CADET *et al.*, 2010; RAI, 2010). Portanto, os danos oxidativos no DNA podem provocar bloqueio na replicação, mutações, anormalidades cromossômicas e consequentemente citotoxicidade **(**BOITEUX *et al.*, 2002; FRIEDBERG, 2006; CADET *et al.*, 2010).

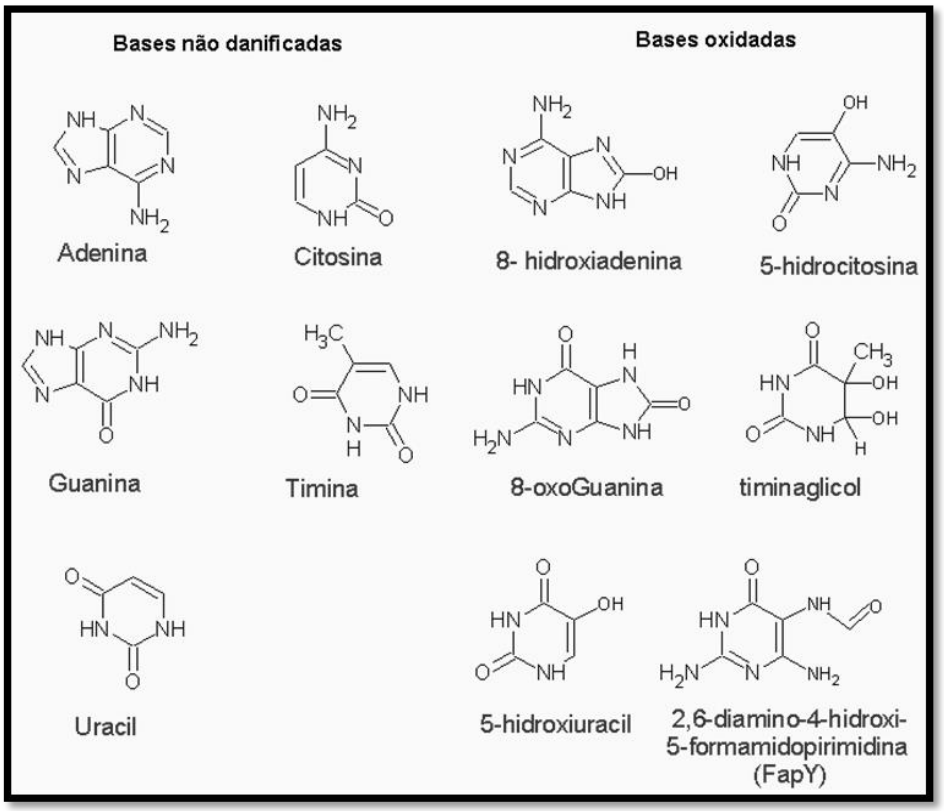


Figura 5: Principais modificações nas bases do DNA ocasionadas por lesões oxidativas. Adaptado de Svilar e colaboradores (2011).

O ataque a purinas provoca também a formação de formamidopirimidinas (Fapy) (Figura 5), produtos da abertura do anel imidazólico (FRIEDBERG, 2006). Em

relação às pirimidinas, o radical $HO^{\bullet}$ reage preferencialmente com ligações duplas C5-C6, formando glicóis de timina e citosina (CHATGILIALOGLU & O'NEILL, 2001; FRIEDBERG, 2006). A base oxidada 5'hidróxi-2'-desoxicitidina causa transições do tipo C→T e a timina glicol causa transições do tipo T→C, sendo altamente mutagênicas (SVILAR *et al.*, 2011). A ribose é um alvo menos frequente, porém o ataque do radical $HO^{\bullet}$ pode abstrair um dos átomos de hidrogênio da ribose, formando radicais que podem levar a quebra simples de cadeia, sítios abásicos e sítios alcalilábeis (BROZMANOVA *et al.*, 2001; FRIEDBERG, 2006).

É bem conhecida a pouca reatividade $O_2^{\bullet-}$ e do H_2O_2 com biomoléculas. Entretanto, o radical $HO^{\bullet}$ e o $^1O_2$ são altamente reativos (HALLIWELL & GUTTERIDGE, 2007). O $HO^{\bullet}$ pode reagir com bases ou com a desoxirribose, produzindo danos nas bases e quebras nas fitas do DNA (CHATGILIALOGLU & O'NEILL, 2001; DIZDAROGLU & JARUGA, 2012; RODRIGUEZ-MUNIZ *et al.*, 2012). As mais frequentes modificações de bases geradas pelo radical $HO^{\bullet}$ é a 7,8-diidro-8-oxo-2'-desoxiguanina (8-oxo-G) (Figura 5). A 8-oxo-G, embora não cause bloqueio na síntese de DNA, é fortemente mutagênico, pois pode causar transversões do tipo G→T (FRIEDBERG, 2006; SVILAR *et al.*, 2011).

O $^1O_2$ pode retirar elétrons ou pode causar ciclo-adição aos carbonos de ligação dupla do anel imidazol (YUN *et al.*, 2007). A guanina é o alvo preferencial das lesões e o produto final mais frequente é a 8-hidroxiguanina (8-HG). Mas, raramente, pode gerar sítios AP, alcalilábeis e quebras de cadeia simples em posições adjacentes à guanina (DIZDAROGLU & JARUGA, 2012). O $RO_2^{\bullet}$ está envolvido na clivagem do DNA (BARTSCH & NAIR, 2000). Já o $O_2^{\bullet-}$ não ataca diretamente o DNA, mas pode reagir com os centros [4Fe-4S] de proteínas liberando Fe^{+2}, contribuindo na geração do radical $HO^{\bullet}$ ou causando a inativação de enzimas envolvidas na reparação do DNA,

como a DNA glicosilase MutY, podendo levar a uma instabilidade genômica (KEYER *et al.*, 1995; KOPPENOL, 2001; LIN *et al.*, 2008).

6.2. Danos oxidativos em lipídeos

As membranas celulares são muito suscetíveis a danos oxidativos, já que os fosfolipídeos são sensíveis à oxidação por ERO, sendo capazes de gerar reações em cadeia (VALKO *et al.*, 2006; SCHNEIDER, 2009). Os ácidos graxos insaturados contêm um ou mais grupos metileno posicionados entre as duplas ligações *cis*. Esses grupamentos são altamente reativos em relação a agentes oxidantes, atuando como doadores de hidrogênios para radicais centrados em carbono, os quais reagem com o oxigênio formando radicais peroxil, cujo destino pode ser a conversão a hidroperóxidos (HALLIWELL & GUTTERIDGE, 2007). Na presença de complexos metálicos e metaloproteínas, ocorre redução dos hidroperóxidos de ácidos graxos, produzindo radicais alcoxil e iniciando uma reação em cadeia responsável pela produção de diversos epóxidos, hidroperóxidos e compostos carbonilados, a denominada peroxidação lipídica (HALLIWELL & GUTTERIDGE, 2007; GUERAUD *et al.*, 2010; REPETTO *et al.*, 2010).

O processo de peroxidação lipídica consiste de três estágios: iniciação, propagação e terminação (HALLIWELL & GUTTERIDGE, 2007). O produto inicial da oxidação de ácidos graxos insaturados são os hidroperóxidos lipídicos, mas são formados diversos intermediários. Os produtos mais conhecidos formados na peroxidação lipídica são o malondialdeído (MDA) e o 4-hidroxi-noneal (4-HNE) (DEL RIO *et al.*, 2005; HALLIWELL & GUTTERIDGE, 2007). É bem estabelecido que o MDA tem ação mutagênica em bactérias e mamíferos, podendo reagir com o as bases nitrogenadas, formando adutos (LUCZAJ & SKRZYDLEWSKA, 2003;

VOULGARIDOU *et al.*, 2011). Diferentemente, o 4-HNE é pouco mutagênico, mas possui grande efeito na transdução de sinal, sendo considerado o produto mais tóxico formado na peroxidação lipídica (ZARKOVIC, 2003; HIGDON *et al.*, 2012; WINCZURA *et al.*, 2012).

O $HO^{\bullet}$ ataca as membranas lipídicas e pode causar uma série de reações que podem levar a danos celulares, sendo inquestionável a sua capacidade de iniciar a peroxidação lipídica (GIROTTI, 2008; THOTALA *et al.*, 2009). Os radicais $RO_2^{\bullet}$ também são conhecidos por causar peroxidação lipídica. Esses radicais podem abstrair hidrogênio de uma molécula de lipídio e assim propagar o processo nas demais moléculas adjacentes (GIROTTI, 2008). Entretanto, o $O_2^{\bullet-}$ não pode iniciar a peroxidação lipídica, mas a sua forma protonada tem-se mostrado muito eficiente neste tipo de reação, bem como o $^1O_2$ (GIROTTI, 1998; TEMPLE *et al.*, 2005).

6.3. Danos oxidativos em proteínas

As reações de aminoácidos com $HO^{\bullet}$ podem levar a abstração de hidrogênio do aminoácido, que pode formar diversos produtos em determinadas condições, como radicais peroxil (LEVINE, 2002; MENG & ZHANG, 2013). O $HO^{\bullet}$ pode causar inativação da proteína por reagir com resíduos de aminoácidos, como ácido glutâmico, triptofano, metionina e prolina, formando diversos produtos ou ainda oxidar seus grupos sulfidrila (-SH) a pontes de dissulfeto (-S-S) (STADTMAN & BERLETT, 1991; STADTMAN, 1993). O $^1O_2$ pode provocar inativação de proteínas reagindo principalmente com os aminoácidos histidina, tirosina, metionina, cisteína e triptofano (STADTMAN, 2006; NAM *et al.*, 2013). O $O_2^{\bullet-}$ pode oxidar o sítio [4Fe-4S] de enzimas e levar a sua inativação (VALKO *et al.*, 2006; HALLIWELL & GUTTERIDGE, 2007).

7. Agentes indutores de danos oxidativos utilizados neste estudo

Diversos agentes são capazes de gerar espécies reativas, entre eles estão os agentes utilizados neste estudo: 4-NQO, NDEA, H_2O_2 e *tert*-butil-hidroperóxido (*t*-BOOH).

7.1. Óxido de 4-nitroquinolina (4-NQO)

O 4-NQO é um conhecido agente carcinogênico, sendo considerado um agente UV-mimético (NUNOSHIBA & DEMPLE, 1993). A ação tóxica deste composto é iniciada por redução enzimática do grupo nitro. Resumidamente, o 4-NQO é convertido em óxido de 4-hidroxiaminolina (4HAQO) e, finalmente, em óxido de 4-aminoquinolina (4AQO) que serve como aceptor final de hidrogênio. Este intermediário pode formar monoadutos de quinolina estáveis com o DNA como 3-(N^6-desoxiadenosinil)-4AQO e N^4-(7-guanosinil-4AQO) (WILLIAMS *et al.,* 2010). O 4-NQO pode ainda ser metabolizado por nitrorredução gerando um ciclo redox, produzindo grande quantidade de ERO, principalmente $O_2^{\bullet -}$, e espécies derivadas deste, como H_2O_2 e radicais $HO^{\bullet}$ (NUNOSHIBA & DEMPLE, 1993; FANN *et al.*, 1999). Este composto também é capaz de formar lesões do tipo 8-hidroxi-2-deoxiguanosina (8-HG) e outros danos oxidativos como a peroxidação lipídica (ARIMA *et al.*, 2006; SRINIVASAN *et al.*, 2007; MIRANDA *et al.*, 2011).

7.2. N-nitrosodietilamina (NDEA)

A NDEA é um agente carcinogênico capaz de produzir danos no DNA (principalmente O^4-etiltimina), induzir lesões oxidativas (8-hidroxiguanina) e peroxidação lipídica (AIUB *et al.*, 2003). Os agentes N-nitroso não interagem

diretamente com biomoléculas como os ácidos nucléicos, sendo que necessitam de metabolização por redução do grupo nitro, para exercer o seu efeito tóxico, formando espécies que podem interagir com o DNA ou pela produção de ERO, como $O_2^{\bullet-}$, por ciclo redox (MASUDA *et al.*, 2000; ZHANG *et al.*, 2012).

7.3. Peróxido de hidrogênio (H_2O_2)

O H_2O_2 pode formar $HO^{\bullet}$ pela reação de Haber Weiss/Fenton, levando assim à oxidação de lipídeos, DNA e proteínas (HALLIWELL & GUTTERIDGE, 2007). Alguns danos celulares podem ser mediados pelo H_2O_2 diretamente, como a inativação da enzima gliceraldeído-3-fosfato desidrogenase (HALLIWELL & GUTTERIDGE, 2007; CYRNE *et al.*, 2010).

7.4. *tert*-butil-hidroperóxido (*t*-BOOH)

O *t*-BOOH é um análogo de cadeia curta de hidroperóxidos lipídicos, amplamente utilizado para induzir estresse oxidativo em variados tipos de células (HALLIWELL & GUTTERIDGE, 2007; O'SULLIVAN *et al.*, 2012; OIDOVSAMBUU *et al.*, 2013). A toxicidade do *t*-BOOH é atribuída a sua capacidade de gerar radicais $HO^{\bullet}$ e butoxil devido à reação de Fenton (GARCIA-ALONSO *et al.*, 2007). Este composto é capaz de induzir diversas disfunções celulares, como peroxidação de lipídeos da membrana, redução dos níveis de GSH, oxidação de resíduos tiólicos, bem como quebras simples no DNA e danos na mitocôndria (BAKER & HE, 1991; KANUPRIYA *et al.*, 2007; OH *et al.*, 2012).

8. Resposta ao Estresse Oxidativo

A resposta ao estresse oxidativo é designada como o fenômeno em que a célula reage a alterações no estado redox (HALLIWELL & GUTTERIDGE, 2007). Portanto, todas as células devem ser capazes de perceber, responder e inativar as ERO produzidas e repararem ou substituirem macromoléculas danificadas (HALLIWELL & GUTTERIDGE, 2007; POLJSAK & MILISAV, 2012). O controle da resposta ao estresse oxidativo pode se dar por mecanismos transcricionais, pós-transcricionais ou pós-traducionais ou até mesmo alterações no fluxo metabólico (TEMPLE *et al.*, 2005; MORANO *et al.*, 2012; SIEDENBURG *et al.*, 2012).

8.1. Principais Defesas Antioxidantes

Para proteger o organismo do ataque de ERO, existe uma série de sistemas de defesas antioxidantes que podem ser enzimáticos ou não enzimáticos. Segundo Halliwell e Gutteridge (2007) as estratégias de defesa compreendem três etapas, que são: prevenção (evitar a formação de ERO), interceptação (neutralização de ERO gerada) e reparação (reparar os danos ocasionados por ERO).

A prevenção contra a formação de ERO é representada, por exemplo, pelos sistemas de homeostase metálica. A interceptação pode ser representada pelos sistemas enzimáticos ou não enzimáticos (Tabela 2), que consistem em evitar que as ERO provoquem danos aos componentes celulares. Por último, quando o dano já estiver ocorrido, existem sistemas capazes de repará-lo, como os sistemas de reparação de DNA (VALKO *et al.*, 2006; HALLIWELL & GUTTERIDGE, 2007; POLJSAK, 2011).

Tabela 2: Principais defesas antioxidantes

Defesa	Função
Superóxido dismutase (SOD)	
CuZnSOD	Dismutação do $O_2^{\bullet-}$ a H_2O_2 no citoplasma
MnSOD	Dismutação do $O_2^{\bullet-}$ a H_2O_2 na mitocôndria
Catalase (CAT)	Distribuição intra e extracelular; dismuta o H_2O_2 a H_2O e O_2
Glutationa peroxidase (GPx)	Família de enzimas que contém selênio envolvida na redução de peróxidos especialmente peróxidos lipídicos
Glutationa redutase	Enzima responsável pela regeneração de glutationa oxidada (GSSG) a glutationa reduzida (GSH) utilizando o NADPH doador de elétrons
Peroxirredoxina	Peroxidase que reduz H_2O_2 e alquil-hidroperóxidos com o uso de equivalentes redutores fornecidos pela tiorredoxina
Tiorredoxina	Oxidorredutase de dissulfetos em proteínas além de fornecer elétrons para as reações catalisadas especificamente pelas peroxirredoxinas
Glutationa (GSH)	doador de elétrons em várias reações enzimáticas antioxidantes além de um "sequestrador" de ERO

8.1.1. Superóxido dismutase (SOD)

A SOD está presente tanto em eucariotos como em procariotos. São metaloproteínas que catalisam a dismutação de $O_2^{\bullet-}$ a H_2O_2 e oxigênio (Figura 6) (FRIDOVICH, 1998b). Há várias isoenzimas do tipo SOD, sendo classificadas de acordo com seu grupo prostético, podendo conter zinco, manganês ou ferro em seus sítios ativos e geralmente distribuídas em compartimentos celulares distintos (HALLIWELL & GUTTERIDGE, 2007; MILLER, 2012). A enzima CuZnSOD é homodimérica e possui Cu^{+2} e Zn^{+2}, estando presente principalmente no citosol de células eucarióticas, mas também pode ser encontrada nos lisossomos, no núcleo ou nos espaços entre as membranas mitocondriais interna e externa (PERRY *et al.*, 2010). A MnSOD é homotetramérica e contém um íon de Mn^{+3} em cada uma das suas

subunidades, estando localizada primariamente na mitocôndria (ZHENG *et al.*, 2007). A FeSOD é expressa principalmente em procariotos e plantas, possuindo um íon Fe^{+2} em seu sítio ativo (MUNOZ *et al.*, 2005).

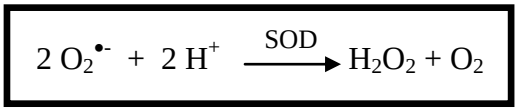


Figura 6: Reação de dismutação do $O2^{\bullet-}$ pela SOD

Na mitocôndria, a MnSOD é essencial para detoxificar o $O_2^{\bullet-}$ que é formado relativamente em altas concentrações, devido à dispersão de elétrons da cadeia respiratória (HOLLEY *et al.*, 2012).

A função principal das enzimas SOD seria proteger as proteínas contendo [4Fe-4S] da ação do $O_2^{\bullet-}$, prevenindo o acúmulo de ferro intracelular (IMLAY, 2006), o que é bem característicos em bactérias e leveduras deficientes nesta enzimas (DE FREITAS *et al.*, 2000). É sugerido também um possível papel regulatório para estas enzimas, uma vez que estudos mostram que a redução na atividade de SOD leva a uma diminuição na atividade nas vias de reparação (SLUPPHAUG *et al.*, 2003). Neste sentido, análises de biologia de sistemas sugerem que as SODs de *S. cerevisiae* podem atuar como um sensor de $O_2^{\bullet-}$, ativando e controlando mecanismos específicos de reparação de DNA, como o reparo recombinacional e por excisão, bem como o remodelamento da cromatina e a síntese de dNTPs (BONATTO, 2007).

8.1.2. Catalase (CAT)

A CAT é uma hemeproteína que catalisa a dismutação de H_2O_2 em água e oxigênio (Figura 7) (HALLIWELL & GUTTERIDGE, 2007; ZAMOCKY *et al.*, 2008). Como a CAT tem o H_2O_2 como único substrato, a sua atividade está intimamente

relacionada com a concentração desta espécie (DOMINGUEZ *et al.*, 2010). A ação desta enzima envolve oxidação divalente do ferro heme (IV), acompanhada pela redução divalente do H_2O_2. Possuem NADPH fortemente ligado, o qual pode prevenir a acumulação da forma ferro (IV) da enzima, a qual é inativa (MATE *et al.*, 1999; NICHOLLS, 2012). Em células eucariotas, há catalases citosólicas e peroxissomais (HANSBERG *et al.*, 2012). A catalase peroxissomal é importante para destoxificar o H_2O_2 produzido nas reações da β-oxidação (HILTUNEN, 1991; HALLIWELL & GUTTERIDGE, 2007).

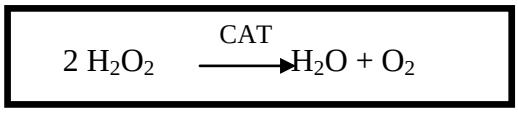


Figura 7: Reação de dismutação do H_2O_2 pela catalase

8.1.3. Glutationa Peroxidase (GPx)

A GPx clássica é multimérica e importante para a proteção contra peróxidos inorgânicos e orgânicos. Para sua atividade, a GPx necessita da presença de GSH e converte esta para a forma oxidada oxidada (GSSG) (Figura 8) (HALLIWELL & GUTTERIDGE, 2007; STEINBRENNER & SIES, 2009).

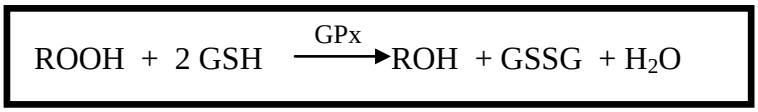


Figura 8: Mecanismo catalítico da GPx

8.1.5. Glutationa (GSH)

A GSH é um tripeptídeo (γ-glutamil-cisteinil-glicina) que desempenha função fundamental na proteção das células contra danos oxidativos causados por oxidantes, atuando como "sequestradora" de radicais (PASTORE *et al.*, 2003; NOCTOR *et al.*, 2011). Está envolvida na detoxificação de peróxidos orgânicos e inorgânicos, bem como pode interagir com outras espécies reativas como $^1O_2$, $O_2^{\bullet-}$ e $HO^{\bullet}$ (LAFLEUR *et al.*, 1994; NOCTOR *et al.*, 2011). É utilizada por uma série de enzimas como GPx, glutationa-S-transferase e glutaredoxina (MEISTER, 1995; HALLIWELL & GUTTERIDGE, 2007). A GSH também tem importância em vários processos fisiológicos, como na homeostasia tiólica, metabolismo de aminoácidos, sinalização celular e defesa contra agentes eletrofilicos, como xenobióticos (DIAZ VIVANCOS *et al.*, 2010; CIRCU & AW, 2012; GEENEN *et al.*, 2012; ZHANG & FORMAN, 2012).

Os mecanismos de sistemas antioxidantes atuam cooperativamente (Figura 9), onde a ausência de uma das defesas acarreta um desequilíbrio no estado redox da célula podendo levá-la a um aumento na sensibilidade a agentes oxidantes intra ou extracelular (DROGE, 2002).

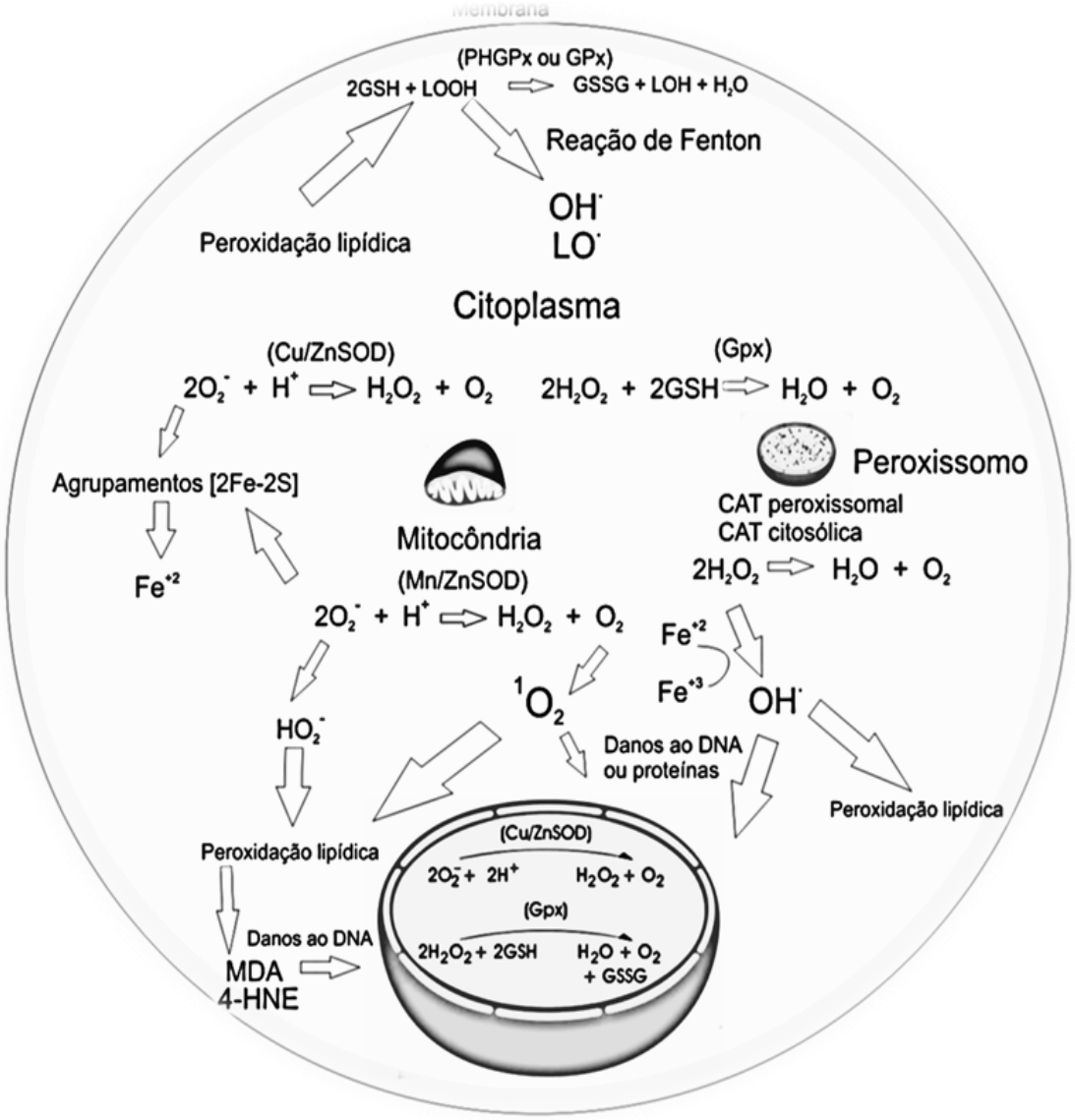


Figura 9: Representação da interação dos mecanismos oxidantes e antioxidantes, na qual são mostradas as principais vias endógenas antioxidantes e os principais efeitos das ERO**.** Adaptado de Engelhardt (1999).

8.2. Resposta a estresse oxidativo em *E. coli*

A resposta antioxidante em bactérias é coordenada principalmente por dois sistemas: os regulons *soxRS* e *oxyR*. O operon *soxRS* responde ao estresse induzido por superóxido , enquanto o operon *oxyR* responde ao estresse por peróxidos (Figura 10) (HALLIWELL & GUTTERIDGE, 2007; LUSHCHAK, 2011).

O regulon *soxRS* é composto por cerca de 17 genes, incluindo genes envolvidos na resposta a estresse oxidativo como MnSOD, glicose-6-fosfato desidrogenase e a nitrorredutase NfsA, bem como genes de reparação de DNA (NUNOSHIBA, 1996). O sensor do regulon é a proteína SoxR, um fator de transcrição dimérico com centros [2Fe-2S] que é oxidado em situações de estresse oxidativo e induzem a expressão do gene *soxS* que, por sua vez, codifica um regulador transcricional responsável pela indução dos genes do regulon *soxRS* (Figura 10A) (LUSHCHAK, 2011). A proteína SoxR é oxidada quando as células de *E. coli* são expostas a um agente gerador de superóxido ou quando a razão entre NADPH e $NADP^+$ diminui (KRAPP *et al.*, 2011; LUSHCHAK, 2011).

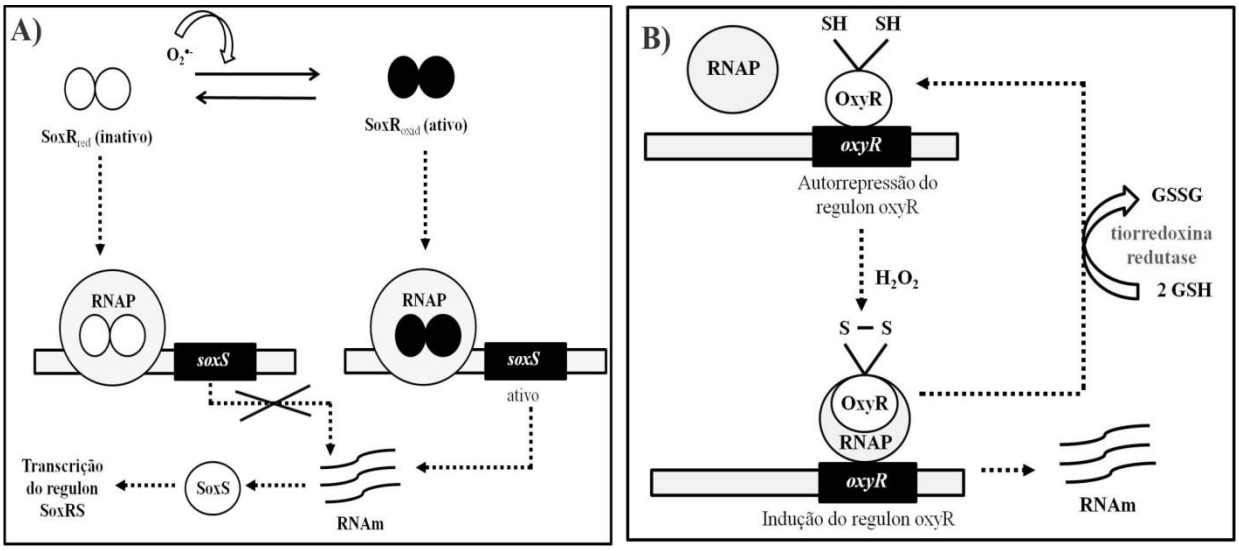


Figura 10: Mecanismos de funcionamento dos regulons soxRS e oxyR em bactéria. A) A forma reduzida de SoxR pode se ligar ao promotor do gene *soxS*, mas somente a forma oxidada ativa a transcrição do regulon. B) A forma reduzida da proteína OxyR reprime a transcrição do regulon *oxyR* e a forma oxidada estimula a sua expressão. Posteriormente, a forma ativa de OxyR é inativada por uma tiorredoxina redutase com utilização de GSH

De modo semelhante à proteína SoxR, OxyR também existe em uma forma oxidada e outra reduzida (CHIANG & SCHELLHORN, 2012). A oxidação direta de OxyR é responsável pela ativação do regulon *oxyR*, que inclui genes como *katG*

(catalase) e *ahpC* (peroxirredoxina) (FARR & KOGOMA, 1991; LUSHCHAK, 2011). Interessantemente, ambas formas podem se ligar ao DNA com diferentes especificidades. Possivelmente, a forma reduzida de OxyR inibe a transcrição do regulon em situações normais e, em situações de estresse oxidativo, OxyR é oxidada e induz a expressão do mesmo (Figura 10B) (HELMANN, 2002; LUSHCHAK, 2011):

8.3. Resposta ao estresse oxidativo em *S. cerevisiae*

A levedura *S. cerevisiae* apresenta uma variedade de mecanismos de defesas antioxidantes (Figura 12). Possui duas enzimas superóxido dismutases: CuZnSOD e a MnSOD, codificada pelo genes *SOD1* e *SOD2*, respectivamente (Figura 11) (MORANO *et al.*, 2012). Fenótipos mutantes de *S. cerevisiae* com disrupção nos genes *SOD1* e/ou *SOD2* fornecem informações com relação à função destas enzimas. Os mutantes *sod1Δ* apresentam uma variedade de fenótipos incluindo crescimento reduzido em condições respiratórias e decréscimo na defesa contra oxidantes externos, como paraquat e menadiona, apresentando ainda deficiência na produção dos aminoácidos lisina e metionina (JENSEN *et al.*, 2004; SEHATI *et al.*, 2011).

Duas catalases foram identificadas, Ctt1p e Cta1p, a primeira citosólica codificada pelo gene *CTT1* e a segunda peroxissomal codificada pelo gene *CTA1*(Figura 11) (MORANO *et al.*, 2012). A proteína Cta1 está envolvida na detoxificação do H_2O_2 gerado pela acil-CoA oxidase durante a β-oxidação de ácidos graxos nos peroxissomos (HILTUNEN *et al.*, 2003). Enquanto acatalase Ctt1p possui um papel não muito claro, já que os mutantes não apresentam defeitos no crescimento nem sensibilidade aumentada a peróxidos, mas o duplo mutante apresenta hipersensibilidade a peróxidos (HALLIWELL & GUTTERIDGE, 2007).

Em levedura existem três GPx, Gpx1, Gpx2 e Gpx3 (Figura 11). Entre os diferentes tipos de GPx a mais importante na resposta a estresse oxidativo parece ser a Gpx3, pois a linhagem mutante apresenta hipersensibilidade a peróxidos, a metais e a ácidos graxos insaturados. Enquanto, as proteínas GPx1 e GPx2 parecem ter uma menor participação na resposta a este tipo de estresse (TEMPLE *et al.*, 2005; MORANO *et al.*, 2012).

O gene *GSH1* codifica a γ-glutamil-cisteinil sintetase, enzima responsável pelo primeiro passo e ponto limitante para a síntese de GSH. Os mutantes são hipersensíveis a peróxidos e a outros oxidantes (BRENDEL *et al.*, 1998; MANFREDINI *et al.*, 2005). A levedura *S. cerevisiae* apresenta ainda duas tiorredoxinas redutases citosólicas (Trx1 e Trx2) e uma mitocondrial (Trx3) (MORANO *et al.*, 2012).

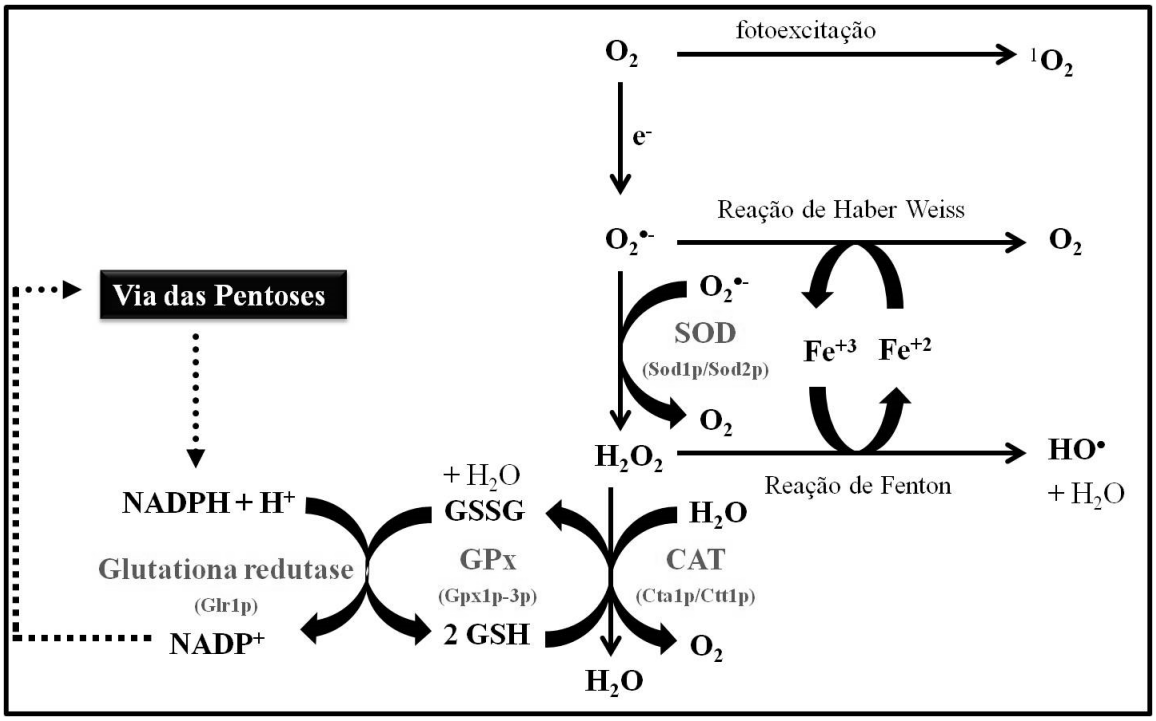


Figura 11: Resumo das principais ERO e defesas antioxidantes em levedura. Adaptado de Temple e colaboradores (2005).

O fator de transcrição Yap1p, homólogo ao fator humano AP1, é apontado como um dos mais importantes mediadores das respostas adaptativas de *S. cerevisiae*, modulando a transcrição de genes envolvidos na defesa contra oxidantes, agindo por mecanismos diretos e indiretos (RODRIGUES-POUSADA *et al.*, 2010; MORANO *et al.*, 2012). Alguns dos alvos da regulação direta que foram identificados são os genes: *TRX1* e *TRX2*, que codificam tiorredoxinas redutases; *GSH1*; *GLR1* que codifica uma GSSG-redutase (JUN *et al.*, 2012; ORUMETS *et al.*, 2012). A via de reparação por excisão de bases (BER) também é regulada por este fator de transcrição em resposta a oxidantes (ROWE *et al.*, 2012). Algumas respostas mediadas por Yap1p exigem uma cooperação de outro fator de transcrição, o fator Skn7p (CHEN *et al.*, 2009; MULFORD & FASSLER, 2011).

8.5. Resposta celular ao estresse oxidativo

Os organismos respondem a ERO principalmente por alterar a expressão de genes que codificam proteínas que destoxificam as espécies reativas ou que reparam os danos causados por estas espécies (HALLIWELL & GUTTERIDGE, 2007). Neste sentido, as células podem responder pelo aumento da transcrição de genes específicos que codificam proteínas envolvidas na defesa antioxidante e inibindo a expressão de outros genes (MORANO *et al.*, 2012). Em adição, é reconhecido que há uma clara alteração no padrão de síntese proteica na célula exposta a oxidantes (CAUSTON *et al.*, 2001; MORANO *et al.*, 2012). Há também um marcante aumento da transcrição de genes envolvidos na biogênese do ribossomo, provavelmente devido ao fato de que, durante um insulto oxidativo, muitas proteínas são danificadas e degradadas, sendo necessário um incremento na síntese protéica (GARDNER, 2010). A dinâmica de degradação de RNAm pode ser altamente regulada em condições de estresse,

modulando a taxa de síntese protéica de modo a redirecionar as prioridades metabólicas e fisiológicas da célula até o retorno ao ponto homeostático pós desbalanço redox (MORANO *et al.*, 2012).

Alterações na expressão gênica também são importantes para reconfiguração metabólica que há durante o estresse oxidativo. Um exemplo disto são as alterações no metabolismo de carboidratos, redirecionando o fluxo do metabolismo de glicose para a via das pentoses com consequente aumento da produção de NADPH (KRUGER *et al.*, 2011; MORANO *et al.*, 2012). O NADPH é importante durante a exposição a oxidantes por doar elétrons para diversas enzimas antioxidantes, como a glutationa redutase (TEMPLE *et al.*, 2005).

Por outro lado, a homeostase metálica deve ser mantida, já que alguns íons, como Fe^{+2} e Cu^{+2}, podem atuar nos ciclos redox e produzir ERO (HALLIWELL & GUTTERIDGE, 2007).

Outro aspecto a ser considerado são as modificações na membrana plasmática em situações de estresse oxidativo, como a redução da razão entre ácidos graxos saturados e insaturados de cadeia longa, bem como nos tipos de fosfolipídeos (DE FREITAS *et al.*, 2012). Há também alterações na permeabilidade da membrana em resposta a alguns oxidantes. Um exemplo disso é a adaptação de levedura a peróxidos, na qual ocorre o aumento da síntese de ergosterol e a redução da permeabilidade da membrana (KELLEY & IDEKER, 2009).

9. A levedura *S. cerevisiae* como modelo de estudo

A levedura *S. cerevisiae* é um fungo unicelular e tem sido amplamente estudada, tornando-se importante nas pesquisas em razão da sua simplicidade e similaridade genética, bioquímica e funcional com mamífero e outros organismos. Assim, esse organismo é um modelo útil para o entendimento de diversas funções biológicas (ZIMMERMANN *et al.*, 1984; MATUO *et al.*, 2012; NATTER & KOHLWEIN, 2013). Além disso, a levedura *S. cerevisiae* é utilizada na indústria química e de alimentos; na indústria farmacêutica, com ênfase na produção de proteínas terapêuticas, incluindo proteínas humanizadas, vacinas e probióticos; e na tecnologia ambiental, com fins de biorremediação, tratamento de resíduos e recuperação do solo (FORSBURG, 2001; IDIRIS *et al.*, 2010; NA *et al.*, 2010; SOARES & SOARES, 2012).

Algumas das propriedades que fazem a levedura particularmente apropriada para estudos biológicos incluem o seu rápido crescimento, segurança, possuir um sistema genético bem definido e mais significativamente, o conhecimento da sequência completa do seu genoma. *S. cerevisiae* foi o primeiro organismo eucarioto a possuir o genoma completamente sequenciado e depositado em bancos de dados especializados (http://www.yeastgenome.org). Também existem ferramentas moleculares apropriadas para manipulação gênica e estudos de expressão, como o transcriptoma, proteoma, metaboloma e interatoma, bem como esclarecimento de diversas rotas metabólicas (OLIVER, 2006; MATUO *et al.*, 2012; FREEBERG *et al.*, 2013; SHIRAI *et al.*, 2013).

Os avanços na análise genômica têm mostrado que muitos genes e proteínas estão envolvidos em resposta ao estresse oxidativo. Outro fator que contribui enormemente para a elucidação das vias de resposta a estresse oxidativo é o desenvolvimento de diversas ferramentas, como a construção de linhagens mutantes de *S. cerevisiae* com deleção para genes não essenciais e extensivos registros de interações

proteína-proteína (MATUO *et al.*, 2012; MORANO *et al.*, 2012; REECE-HOYES & MARIAN WALHOUT, 2012). Desta forma, as leveduras estão sendo essenciais na detecção de novos genes e proteínas importantes na regulação do metabolismo redox (TEMPLE *et al.*, 2005; HERRERO *et al.*, 2008). Assim, não surpreende o uso de células de levedura como um sistema modelo com relevante contribuição para compreender o mecanismo molecular básico da resposta ao estresse oxidativo.

9.1. Ciclo de vida e metabolismo

As células de *S. cerevisiae* se dividem por brotamento logo após a duplicação de seu DNA, com tempo de geração de aproximadamente 90 minutos, quando incubadas a temperaturas de 28-30ºC. Além disso, existe a possibilidade de células diploides poderem ser induzidas à meiose e a uma consequente esporulação. A esporulação origina células haplóides que, por sua vez, se multiplicam por divisão mitótica e brotamento (HERMAN, 2002; LAFAVE & SEKELSKY, 2009; NEIMAN, 2011).

A levedura *S. cerevisiae* pertence ao grupo das leveduras anaeróbias facultativas. Isto significa que fermenta hexoses, como a glicose e a frutose, independente da concentração de oxigênio. A glicose é a principal fonte de carbono da levedura, uma preferência que é mediada por um complexo processo de repressão e ativação de genes e de proteínas, usualmente conhecido como repressão da glicose ou repressão catabólica (USAITE *et al.*, 2008; BLOUNT *et al.*, 2012). Quando a concentração de glicose cai para menos de 0,2% no meio, há a indução das enzimas que participam da biossíntese da mitocôndria e de outros genes necessários para o crescimento respiratório (LIPINSKI *et al.*, 2010; RINALDI *et al.*, 2010). Esse

crescimento apresenta fases distintas do ponto de vista metabólico e cinético (Figura 12).

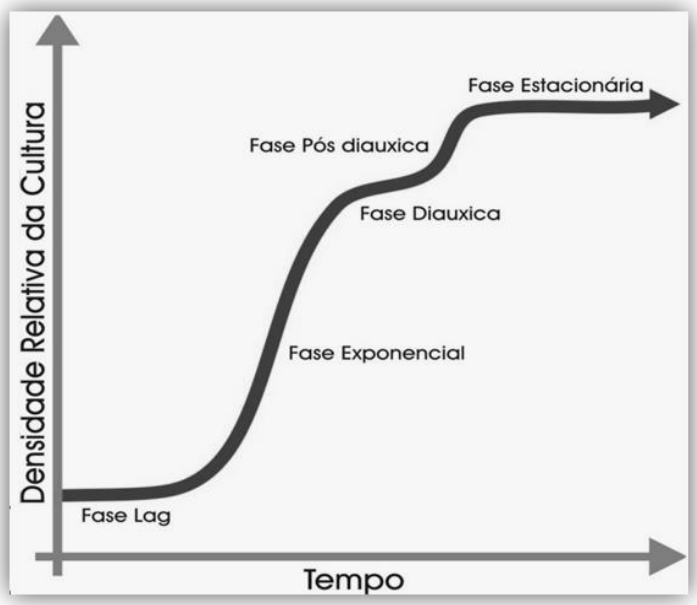


Figura 12: Curva de crescimento de uma linhagem selvagem de *S. cerevisiae*. Adaptado de Herman (2002).

Após um breve período de adaptação em meio rico (YPD - 2% glicose), chamado de fase lag, as células iniciam uma divisão celular a cada hora e meia (fase exponencial), com energia proveniente da fermentação da glicose. Ao diminuir a disponibilidade de glicose no meio, ocorre a desrepressão catabólica (transição diáuxica), na qual há uma parada transiente na divisão celular, enquanto as células são preparadas para o metabolismo respiratório. Após, ela reassume a divisão celular em um ritmo mais lento (uma divisão a cada três ou quatro horas), utilizando o etanol como fonte de carbono produzido durante a fermentação (fase pós-diáuxica). Quando todas as fontes de carbono forem exauridas, as células entram na fase estacionária na qual podem sobreviver por muito tempo na ausência de nutrientes (HERMAN, 2002; BUSTI *et al.*, 2010; GALDIERI *et al.*, 2010; CAI & TU, 2012).

10. Biologia de Sistemas

A Biologia de Sistemas estuda a interação entre os componentes de um sistema biológico e como estas interações determinam ou regulam comportamentos e funções dentro do sistema. Exemplos de componentes que interagem dinamicamente incluem os genes, enzimas e metabólitos derivados de uma determinada via metabólica (BARABASI & OLTVAI, 2004; BONATTO, capítulo em preparação). Assim, a Biologia de Sistemas busca observar a relação entre componentes de um sistema biológico e os processos associados, seguindo a geração de hipóteses e permitindo a construção de modelos que possam ser testados experimentalmente e aperfeiçoados (IDEKER *et al.*, 2001; KITANO, 2002). As técnicas de análise em larga escala de processos biológicos permitiram a obtenção de uma grande quantidade de informações a respeito do comportamento celular em determinadas condições fisiológicas (ALMAAS, 2007). Assim, a Biologia de Sistemas integra as ferramentas computacionais disponíveis para a extração e análises dos dados depositados bancos de dados, que constituem a fonte de informações a respeito das interações funcionais relacionadas às principais macromoléculas, como proteínas, DNA e RNA (ALBERT, 2005).

Para realizar a integração das informações extraídas dos bancos de dados, é necessário o embasamento em modelos matemáticos, isto é, em teorias que representem a relação entre componentes de um determinado conjunto, como a Teoria dos Grafos. Os grafos consistem em uma estrutura de dados e permitem representar o conhecimento obtido a partir de dados "ômicos", como mecanismos regulatórios e redes metabólicas, por exemplo (HUBER *et al.*, 2007).

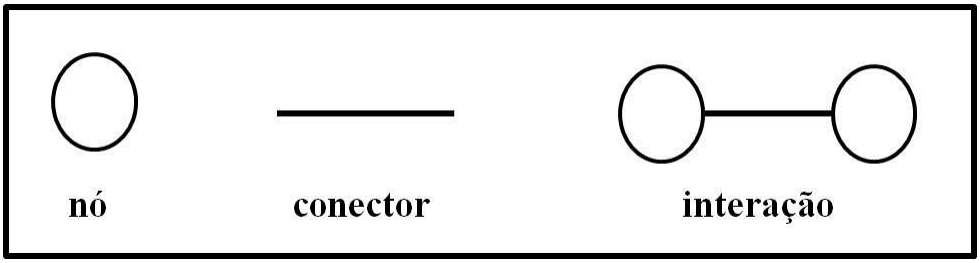


Figura 13: Figura representando um nó (esquerda), conector (centro) e a interação entre dois nós (direita).

Um grafo é especificado por um conjunto de nós e conectores, sendo que cada conector contém um par de nós (Figura 13) (BARABASI & OLTVAI, 2004). Os nós são as entidades de interesse e os conectores representam as relações entre estas entidades. Essas relações podem ser interações físicas diretas, ativações, inibições, co-regulação ou qualquer outra relação entre os nós (BROWN *et al.*, 2004; HUBER *et al.*, 2007). Se os nós forem proteínas, por exemplo, os conectores podem representar a existência de interação física ou funcional entre as proteínas consideradas. Dessa maneira, uma célula pode ser descrita como uma conjunto de nós conectados, formando uma rede de interações. O grafo compõe, portanto, a base da Biologia de Sistemas que é uma rede de interações. Os termos "grafo" e "rede", neste caso, podem ser usados como sinônimos. É importante salientar que o termo grafo se refere ao conceito matemático, enquanto que o termo rede está associado à funcionalidade de um sistema (HUBER *et al.*, 2007).

Os grafos permitem a geração de modelos onde é possível mensurar os dados biológicos. Algumas ferramentas matemáticas são frequentemente usadas para estudar as redes, incluindo a análise de uma estrutura global de rede, a presença de módulos, agrupamentos e centralidade (HUBER *et al.*, 2007). Essas medidas geram informações importantes a respeito do sistema em estudo (VALENTE *et al.*, 2008; PARK & KIM, 2009).

10.1 O uso de centralidades na Biologia de Sistemas

As centralidades constituem um conjunto de ferramentas matemáticas que permitem identificar aqueles nós importantes na arquitetura global de uma rede (ESTRADA & BODIN, 2008). A avaliação de centralidades podem indicar proteínas que são essenciais para um organismo ou para um determinado processo biológico (KONIG & TESSONE, 2011).

Entre as medidas de centralidade, o *degree* (grau de conectividade) indica quanto o nó está conectado a outros nós adjacentes (SCARDONI *et al.*, 2009). Nós com alto *degree* são denominados de *hubs* (Figura 14), representando proteínas importantes para o organismo (JIN *et al.*, 2007; LIN, C. Y. *et al.*, 2008). Outro parâmetro de centralidade é o *closeness* (proximidade), que indica o nível de proximidade de um nó a outros nós. O *stress* é representa quanto um nó é atravessado por rotas ideais ou curtas em uma rede. O *betweenness* (intermedialidade) é similar ao *stress* e revela o quanto um nó específico está entre todos os outros nós na rede (SCARDONI *et al.*, 2009). O *bottleneck* (gargalo) (Figura 14) define todos os nós com altos valores de *betweenness*, configurando-se como pontos centrais que controlam a propagação de informação para outros nós integrantes da rede. Os *bottleneck* também indicam os nós que estão entre agrupamentos altamente interconectados, onde a remoção destes nós pode dividir a rede inteiramente (YU *et al.*, 2007). De forma geral, o *degree*, o *stress* e o *betweenness* demonstram a importância que o nó tem em uma rede.

Tendo os parâmetros de centralidade em vista, mais especificamente o *degree*, e o *betweenness*, as proteínas (nós) em uma rede podem ser classificadas em quatro categorias: (1) não *hub* – não *bottleneck* (NH-NB); (2) *hub* – não *bottleneck* (H-NB); (3) não *hub* – *bottleneck* (NH-B) e (4) *hub* – *bottleneck* (H-B) (Figura 15). Proteínas classificadas como NH-B e H-B têm maior tendência a serem produtos de genes

essenciais, sugerindo que o *betweenness* é o maior determinante da essencialidade de uma proteína em uma rede do que o *degree* (YU *et al.*, 2007).

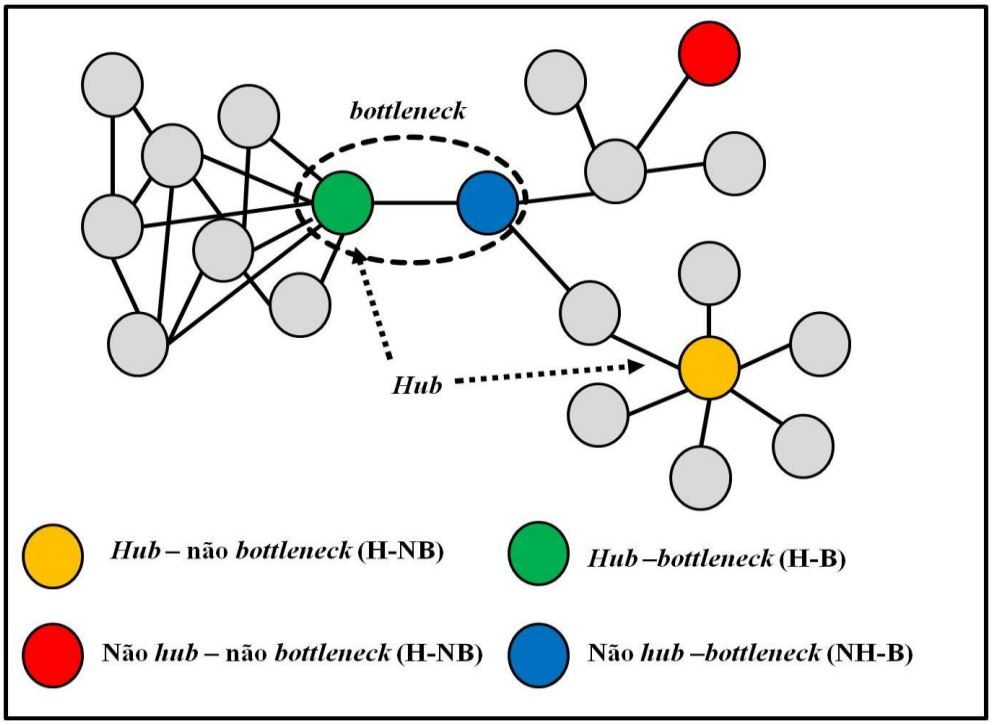


Figura 14: Esquema mostrando um *bottleneck*, *hubs* e quatro categorias de nós em uma rede. Adaptado de Yu e colaboradores (2007).

As redes de interações entre moléculas podem ser formadas por inúmeras sub-redes distintas (SANZ-PAMPLONA *et al.*, 2012). Uma sub-rede pode ser considerada estrutura funcional que é unida a outras semelhantes para a construção de um objeto mais complexo. Por exemplo, grupos de genes, proteínas ou metabólitos são capazes de formar sub-redes, onde a união destas constitui processos biológicos. Os processos biológicos, por sua vez, estão ordenados hierarquicamente em um conjunto de entradas lógicas denominadas de ontologias gênicas (MAERE *et al.*, 2005; RIVALS *et al.*, 2007). Assim, de uma forma bastante simplificada, um sub-rede ou agrupamento aparece em uma rede como um grupo de nós altamente interconectados (SANZ-PAMPLONA *et al.*, 2012).

Objetivos

OBJETIVOS

Objetivo geral

Este estudo tem como objetivo investigar possíveis funções das nitrorredutases no metabolismo de *Escherichia coli* e *Saccharomyces cerevisiae*, com ênfase na possível participação dessas enzimas na resposta ao estresse oxidativo.

Objetivos específicos

- Elaborar novas hipóteses em relação ao papel das nitrorredutases NfsA e NfsB no metabolismo de *E. coli*.

- Construir linhagens simples e duplo mutante de *S. cerevisiae* para os genes *FRM2* e *HBN1* que codificam para as nitrorredutases Frm2p e Hbn1p, respectivamente.

- Avaliar a resposta das linhagens mutantes *frm2*Δ e *hbn1*Δ e da respectiva linhagem isogênica a oxidantes.

- Investigar a indução de mutantes citoplasmáticos "*petites*" nas linhagens mutantes *frm2*Δ e *hbn1*Δ e na linhagem isogênica;

- Construir a rede de interação das nitrorredutases Frm2p e Hbn1p de *S. cerevisiae* identificando os agrupamentos e processos biológicos envolvidos, bem como definir quais nós são considerados *bottleneck.*

- Investigar a sensibilidade a oxidantes de linhagens de *S. cerevisiae* deficientes nas proteínas identificadas como *bottleneck*.

- Construir linhagens duplo e triplo mutante de *S. cerevisiae* para os genes *FRM2*, *HBN1* e os genes que codificam proteínas *bottleneck*, cujas linhagens simples mutante apresentaram as respostas mais evidentes na exposição a oxidantes.

- Investigar a influência das interações das nitrorredutases Frm2p e Hbn1p com outras proteínas na resposta a estresse oxidativo.

Capítulo I

APRESENTAÇÃO DO CAPÍTULO I

Nitrorredutases: enzimas com importância ambiental, biotecnológica e clínica.

Na presente revisão foi abordada e discutida a importância das nitrorredutases na redução de nitrocompostos e como isso pode influenciar na ciência ambiental e na saúde humana, além de suas aplicações biotecnológicas e clínicas. A presença de dessa classe de compostos no ambiente oferece riscos à saúde humana, uma vez que bactérias da microbiota intestinal metabolizam estes compostos, convertendo-os para formas mais tóxicas. Essa capacidade de "ativação" é utilizada em promissoras terapias para o tratamento de certos tumores. Neste caso, células alvo expressando nitrorredutases bacterianas ou ligadas a um anticorpo carregando a enzima são capazes de converter o nitrocomposto CB1954 em agentes citotóxicos. Além disso, as nitrorredutases podem contribuir com infecções hospitalares, pois linhagens da bactéria *Helicobacter pylori* deficientes nestas enzimas não metabolizam o antibiótico metronidazol, tendo resistência a este nitrocomposto. Linhagens de *Salmonella typhimurium* deficientes ou superexpressando nitrorredutases são empregadas no estudo e avaliação do potencial mutagênico de compostos nitroaromáticos e nitroheterocíclicos. As nitrorredutases são utilizadas na biorremediação de nitrocompostos recalcitrantes no ambiente, como TNT, por formar espécies mais facilmente degradadas. Para tanto são utilizadas bactérias ou plantas geneticamente modificadas expressando nitrorredutases bacterianas.

A revisão foi publicada como um capítulo do livro *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*

Nitroreductases: Enzymes with Environmental, Biotechnological and Clinical Importance

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The nitroreductase family comprises a group of flavin mononucleotide (FMN)- or flavin adenine dinucleotide (FAD) - dependent enzymes that are able to metabolize nitroaromatic and nitroheterocyclic derivatives (nitrosubstituted compounds) using the reducing power of nicotinamide adenine dinucleotide (NAD(P)H). These enzymes can be found in bacterial species and, to a lesser extent, in eukaryotes. The nitroreductase proteins play a central role in the activation of nitrocompounds and have received a lot of attention in recent decades based on their (a) environmental and human health importance due to their central role in mediating nitrosubstituted compound toxicity; (b) biotechnological application for bioremediation biocatalysis; and (c) clinical importance in chemotherapeutic tumor treatment, ablation of specific cells and antibiotic resistance. Nitrosubstituted compounds are mainly produced by industrial processes or other human activities and have become an important group of environmental pollutants. Human health concerns have arisen with regard to these compounds because their metabolization leads to the formation of potent genotoxic and mutagenic metabolites and to the generation of reactive nitrogen oxide species, which readily react with biological macromolecules. In addition, many genotoxic tests have been performed using mutant strains of bacteria such as *Salmonella typhimurium* that do not express or overexpress these enzymes, to identify and elucidate the molecular mechanism of mutagenesis caused by several nitrocompounds. Bioremediation treatments for nitroaromatic and nitroheterocyclic compounds, in particular phytoremediation using transgenic plants expressing bacterial nitroreductases or soil bacteria such as *Bacillus* sp., may be effective in decontaminating soil *in situ.* The nitroreductases have clinical application due to their ability to convert non-toxic prodrugs such as CB1954 (5-[aziridin-1-yl]-2,4-dinitrobenzamide) into a potent DNA-crosslinking cytotoxic agent that kills tumor cells. These enzymes have great clinical interest because they are used in techniques such as gene (or virus) directed enzyme prodrug therapy (GDEPT or VDEPT) and antibody-directed enzyme prodrug therapy (ADEPT) for potential use in the treatment of certain tumors. Nitroreductases are also involved in resistance to metronidazole, a drug used mainly in infections caused by *Helicobacter pylori*, which causes gastric ulcers and constitutes a risk factor for adenocarcinoma and gastric lymphoma. In this chapter, the most relevant aspects of nitroreductases enzymes are presented and discussed: the occurrence of these enzymes in organisms, their catalytic reduction mechanism, physiological role and importance in mediating the toxicity of nitrocompounds, and their influence on the environment and human health, as well as their potential biotechnological and medical applications.

Keywords nitroreductases, nitrocompounds; bioremediation; cancer therapy

1. Introduction: Nitrocompounds and Nitroreductases

Nitrocompounds such as nitroaromatic and nitroheterocyclic derivatives (nitrosubstituted compounds) constitute a large group of chemicals that are characterized by the presence of one or more nitro groups [1]. The toxic effects of nitrosubstituted aromatics have been well-established, and many of these compounds have been reported as toxic, mutagenic, or carcinogenic [2]. However, nitroaromatic compounds, including nitrofurans, nitropyrenes, nitrobenzenes and several others, have been used in multiple applications as pharmaceuticals, antimicrobial agents, food additives, pesticides, explosives, dyes and raw materials in several industrial processes. As a result, they are distributed widely in the environment and are categorized as an important group of pollutants [3, 4]. Enzymatic reduction is essential for the nitrocompounds to exercise their therapeutic and/or cytotoxic effects, and most nitroaromatics should undergo enzymatic reduction in organisms [1, 5]. The nitroreductases proteins form a group of enzymes that have a central role in the reduction of nitro groups on nitrocompounds [5].

Nitroreductases comprise a family of proteins with conserved sequences that were originally discovered in eubacteria and have been grouped together based on their sequence similarity [6]. These enzymes are capable of catalyzing the reduction of nitrosubstituted compounds using flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) as prosthetic groups and nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) as reducing agents [5, 6]. These proteins have recently raised enormous interest both in the environmental engineering community (due to their central role in mediating nitroaromatic toxicity and their potential use in bioremediation and biocatalysis) and in the medical community (as agents used to activate prodrugs in directed anticancer therapies). In addition, many studies have associated nitroreductases with susceptibility to antibiotics [7, 8]. Therefore, in this chapter will discuss the importance of nitroreductase in the environment and human health by

mediating the toxicity of nitro, biotechnology in bioremediation, removal of cell populations and clinical resistance to antibiotics and treatment of tumors as well as their occurrence and possible physiological functions.

2. Where nitroreductases are and how they work

In the nitro group, the bond between the oxygen and nitrogen atoms is polar because oxygen is more electronegative than nitrogen, attracting nitrogen's electrons to form partially negative and positive poles. The positive pole tends to attract electrons, and therefore, it has a great tendency to undergo reduction [3]. The reduction of nitro groups can be catalyzed by nitroreductase enzymes that can perform one- or two-electron transfers [5, 6]. Thus, the nitroreductases have been grouped into two categories based on their ability to reduce nitro groups in the presence of oxygen by one-electron or two-electron transfers (Figure 1):

(i) Type I (oxygen-insensitive) nitroreductases catalyze the sequential transfer of two electrons from NAD(P)H to the nitro groups of nitrosubstituted compounds, in the presence or absence of oxygen, resulting in nitroso and hydroxylamine intermediates and finally primary amines. In general, type I nitroreductases perform two-electron transfers using a ping–pong, bi–bi kinetic mechanism, and the FMN group cycles between the oxidized and the reduced states with a flavin two-electron reduction [5, 6]. The formation of the hydroxylamino intermediate is well-established, because it has been detected in numerous studies of nitro group reduction. However, because the nitroso intermediate is so reactive and the second two-electron reaction has a much faster rate than the first two-electron transfer, it is difficult to isolate. However, its role can be inferred from studies of nitrocompounds that are reduced in controlled chemical reactions [3, 6].

(ii) Type II (oxygen-sensitive) nitroreductases catalyze one-electron reductions of the nitro group in the presence of oxygen, producing a nitro anion radical that subsequently reacts with molecular oxygen, forming a superoxide radical and regenerating the original nitroaromatic compound. This "futile redox cycle" can cause oxidative stress by producing large amounts of superoxides. Type II nitroreductases perform single-electron reactions; they stabilize the formation of nitro anion radicals, and the enzyme transfers one electron to oxygen to generate the superoxide anion. Thus, these enzymes can mediate the reduction of nitroaromatics by two-electron transfers only under anaerobic conditions [9].

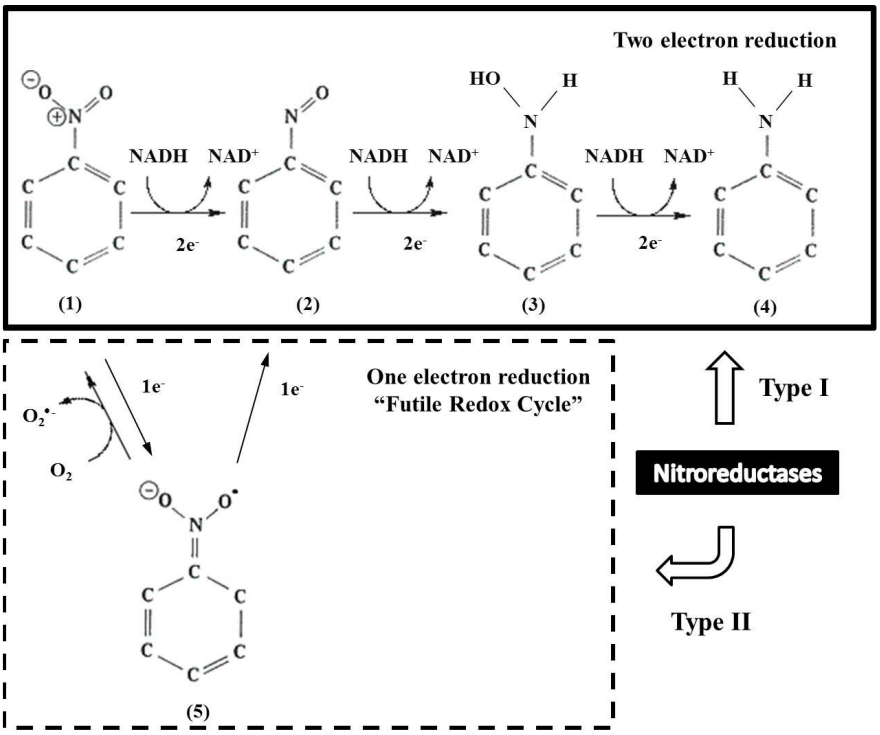

Figure 1. The mechanism of action of type I and type II nitroreductases: An example of a nitroaromatic compound (1). Type I nitroreductases can transfer two electrons from NAD(P)H to form the nitroso (2) and hydroxylamino (3) intermediates and finally the amino group (4). Type II nitroreductases transfer a single electron to the nitro group, forming a nitro anion radical (5), which in the presence of oxygen generates the superoxide anion in a futile redox cycle, regenerating the nitro group.

The nitroreductases proteins are widespread in eubacteria, but nitroreductase-*like* proteins are also found in *Archaea* and in eukaryotes [6]. Type I nitroreductases participate in the reduction of a variety of nitrocompounds, including nitrofurans, nitrobenzene, nitrophenols, nitrobenzoate, nitrotoluenes (TNT), and nitroimidazoles [1, 10]. Because of

their importance, many cloning, gene isolation, structural analysis and functional characterization studies have been carried out on nitroreductases isolated from various organisms [1].

Type I or oxygen-insensitive nitroreductases, can be classified into two main groups or families, according to their similarity with *Escherichia coli* nitroreductases NfsA (group A) and NfsB (group B) [6]. These are known as the major and minor oxygen-insensitive nitroreductases, respectively. NfsA is the major oxygen-insensitive nitroreductase and uses NADPH as an electron source, whereas NfsB is a reductase that can use either NADH or NADPH as a source of reducing equivalents. Almost all nitroreductases share similar biochemical properties. They are usually homodimeric proteins of approximately 30 kDa and have broad substrate specificity, contain FMN as a cofactor and catalyze the reduction of various nitrocompounds using a two-electron transfer mechanism [1, 6, 11]. However, newly discovered nitroreductase-*like* proteins that belong to new families uncharacterized [12-14]. The most relevant nitroreductases are presented in Table 1.

Table 1. The major nitroreductases

Organism	Nitroreductases			Reference
	Type I Group A	**Type I Group B**	**New Groups**	
Bacillus amyloliquefaciens	YwrO			[15, 16]
Bacillus licheniforms		Yfk0		[17]
Bacillus subtilis	NfrA1 (YwcG)			[18]
Clostridium acetobutylicum	NitA and NitB			[19]
Enterobacter cloacae		NR		[20]
Escherichia coli	NfsA	NfsB		[6, 11, 21, 22]
Helicobacter pylori	RdxA			[23]
Klebsiella sp.		NTR I		[24, 25]
Pseudomonas pseudoalcaligenes				[26, 27]
Pseudomonas putida	PnrA	PnrB		[28]
Rhodobacter capsulatos		NprA and NprB		[29]
Salmonella typhimurium	SrnA	Cnr		[30-32]
Staphylococcus aureus	NfrA		NtrA	[12, 33]
Synechocystis sp	DrgA			[34]
Vibrio fischeri		FRase I		[35]
Vibrio harveyi	Frp			[36]
Homo sapiens	Iodotirosina deiodinase			[37]
Saccharomyces cerevisiae			Frm2 and Hbn1	[13, 14]

[a] - bacterial nitroreductases are shown in light grey and eukaryotic nitroreductases in dark grey

There are few records of nitroreductases in eukaryotic cells. In mammals, some enzymes exist that are functionally related to type I nitroreductases such as NAD(P)H-quinone oxidoreductase (DT-diaphorase) and xanthine dehydrogenase, but these enzymes are not phylogenetically related and do not exhibit the domain characteristic of this family. Similarly, nitroreductase enzymes that are functionally related to type II enzymes are also found in various organisms, especially in eukaryotes. These include aldehyde oxidase, cytochrome c oxidase, and NADPH cytochrome P-450 reductase and others [9]. In humans, iodotyrosine deiodinase catalyzes the deiodination of mono- and diiodotyrosine and contains an NfsA nitroreductase domain [37]. Recently in the yeast *Saccharomyces cerevisiae,* two putative nitroreductase-*like* proteins – Frm2 and Hbn1 – have been identified and characterized *in silico*, and these proteins belong to a new family of nitroreductases and have homologues present in several bacteria and fungi [13].

Interestingly, the genes encoding enzymes that catabolize aromatic compounds are frequently associated with transposable elements on conjugative plasmids and genomic islands, facilitating their dispersal via horizontal transfer. Transferable degradative plasmids play an important role in the adaptation of microbial communities to the presence of xenobiotics in their environments, as do other mobile genetic elements, including conjugative transposons, integrons, genomic islands and phages [38]. Based on these findings, it has been hypothesized that nitroreductase-*like* sequences present in the genomes of several protozoan species have been acquired by lateral gene transfer, for example, the oxygen-insensitive nitroreductase of *Giardia lamblia*, the sequence of which demonstrates great similarity to that of *Clostridium acetobutylicum* [39].

3. The physiological function of nitroreductases: a mystery to be unraveled

The biological function of the nitroreductase family of proteins is largely unknown. It has been postulated that the *E. coli* nitroreductase, NfsB, may be able to reduce 3-nitrotyrosine (3-NT) residues in proteins. However, a study conducted by Lightfoot et al. (2000) indicated that neither NfsA nor NfsB reduced 3-NT [40]. Some studies suggest the

possible involvement of nitroreductases in the oxidative stress response [40-42]. For instance, the *E. coli nfsA* gene, which encodes the nitroreductase NfsA, is part of the *SoxRS* regulon and is strongly induced by paraquat, a well-known superoxide generator [41, 42]. The genes *snrA* in *Salmonella typhimurium* and *nprA* in *Rhodobacter capsulatos* are also induced by paraquat [35, 39], and some NfsA-*like* proteins of *Bacillus subtilis* and *Staphylococcus aureus* help to maintain the cell thiol-disulfide balance [33]. Very recently, de Oliveira et al. (2010) showed that yeast *S. cerevisiae* strains deficient in Frm2p and Hbn1p nitroreductase-*like* proteins have reduced basal activity of superoxide dismutase (SOD), ROS production, lipid peroxidation, and petite induction (respiratory deficient yeast), higher sensitivities to 4-nitroquinoline-oxide (4-NQO), N-nitrosodiethylamine (NDEA), and superoxide generating agents, higher basal activities of catalase (CAT) and glutathione peroxidase (GPx), and reduced glutathione (GSH) content in the single and double mutant strains *frm2Δ*, *hbn1Δ* and *frm2Δ hbn1Δ*. These strains exhibited less ROS-accumulation and lipid peroxidation when exposed to peroxides, H_2O_2, and *t*-BOOH. Therefore, the Frm2p and Hbn1p enzymes influence the response to oxidative stress in *S. cerevisiae* by modulating GSH contents and antioxidant enzymatic activities, such as SOD, CAT and GPx activity [14]. In addition, the *S. aureus* NtrA nitroreductase-*like* enzyme, a member of a novel family of nitroreductases, exhibits *S*-nitrosoglutathione (GSNO) reductase and nitroreductase activity, which protect the cell against transnitrosylation and promote nitrofuran activation [12].

Some nitroreductases are associated with metabolic pathways such as the nitroreductase-*like* BluB, a member of the NADH/flavin mononucleotide (FMN)-dependent nitroreductase family found in *Selenomonas ruminantium*, *Sinorhizobium meliloti* and *R. capsulatus* that has been implicated in the biosynthesis of cobalamin (B_{12}). B_{12} is a cofactor for several enzymatic reactions in animals, protists, and some prokaryotes, such as the biosynthesis of fatty acids, methionine and deoxynucleotides) [43]. The DrgA protein of cyanobacterium *Synechocystis* sp. and NfsB in *E. coli* show ferric reductase activities that potentially play a role in iron metabolism and can catalyze the Fenton reaction (the reaction by which iron and hydrogen peroxide react, generating the hydroxyl radical) [34]. McHale et al. (1996) indicated that the nitroreductase-*like* Frm2p of *S. cerevisiae* might be involved in the lipid signaling pathway [44]. In *Vibrio fischeri,* the nitroreductase FRaseI may be involved in bioluminescence, as the enzyme can provide the reduced form of flavin required for the luciferase reaction by catalyzing the reduction of FMN by NAD(P)H [21, 22, 35].

A question can be asked: Do nitroreductases have adaptive and evolutionary importance? In this regard, Roldán et al. (2008) called attention in their review to the adaptive advantage of nitroreductase. They suggested that the primitive physiological function of these enzymes could have been lost or modified to allow the reduction of different nitroaromatic and nitroheterocyclic compounds. Bacteria that are able to deal with these chemicals have a selective advantage and may survive in polluted environments [45].

The diversity and versatility of nitroreductases may be involved in certain metabolic pathways and mainly metabolize several nitrosubstituted compounds to make enzymes that are important in relation to human exposure to environments contaminated with nitrocompounds and that are very attractive in biotechnological and clinical applications.

4.Environmental contamination and human health: the role of nitroreductase

Several nitrocompounds that are released into the environment are generated only as a result of anthropogenic activities. Some compounds are produced by the incomplete combustion of fossil fuels; others are used as synthetic intermediates in industrial processes and as dyes. Recently, mutagenic aromatic nitrocompounds have been found in photocopies, the urban atmosphere, automobile exhaust, wastewater from gasoline stations and cigarettes [2, 3, 45]. Therefore, we are continuously exposed to environment nitrocompounds through inhalation, ingestion, and skin contact [2, 3]. These nitrosubstituted compounds have generated considerable health concerns because their metabolization by microorganisms leads to the formation of nitroso and hydroxylamino derivatives, which are very reactive and, in many instances, more toxic than the parent molecules and can be potent genotoxic and / or mutagenic metabolites [2-4]. In addition, many nitrosubstituted compounds are able to generate reactive nitrogen oxide species (RNOxS) that react with biological macromolecules, inducing changes in their functions [2, 45].

The metabolism of nitrocompounds in the human body can be accomplished by the intestinal microflora that comprise a complex and relatively stable community of obligate anaerobes, especially *Clostridium* species [46]. Therefore, the microflora exhibit many kinds of physiological enzyme activities that play an important role in the metabolism of environmental chemical compounds and are consequently of importance in human health and disease [46, 47]. In this sense, the nitroreductases present in the intestinal microbial communities play a key role in the metabolism of the exogenous nitroaromatic chemicals to which the host is exposed [48]. The products of these biotransformations can be toxic to the host [46-48]. When the intestinal microflora is exposed to antibiotics, their metabolic activity is altered. Consistently, there are differences between animals that have been treated with antibiotics and untreated animals with respect to their reabsorption and excretion of mutagenic environmental pollutants [49]. One example is 1-nitropyrene, an important environmental pollutant that can be issued directly from diesel engines and that has been shown to be mutagenic and tumorigenic. There is good evidence that 1-nitropyrene can be reduced by anaerobic human, monkey, and rat intestinal microflora to aromatic amines with greater toxicity [48]. Other examples include the *N*-nitroso compounds (i.e., the NDEA) that are suspected to be involved in gastrointestinal tumors in

humans, as these compounds can form secondary amines by reactions with nitroso agents. Nitrosamines are widespread and commonly ingested as food constituents, food additives, agricultural chemical residues and pharmaceutical drugs. The nitroreductases of the intestinal microflora play a role in stimulating the toxicity of these compounds [50, 51].

Interestingly, diet can greatly influence the development of tumors, and a contributing factor to this phenomenon is the metabolism of compounds by bacterial microflora, which has been demonstrated as an important factor in tumor initiation [46-51]. Cancer of the colon can be reduced by changes in the microfloral profile; i.e., changes in the numbers and types of microbes caused by changes in diet and the consumption of live bacteria (probiotics) or other substances such as oligosaccharides (prebiotics) and polyphenols (synbiotics) [52]. Some human studies suggest that the consumption of foods such as fiber may protect against the growth of colon adenomas [47, 51]. The precise mechanism by which fiber exerts its effects remains elusive. However, studies in rats have shown that a diet based on fiber reduces the activity of nitroreductase in the gut microflora, whereas a meat-based diet increases this activity. These data may suggest that part of the protective effect of fiber occurs via modulation of the activities of these enzymes during the metabolism of nitrocompounds in the gut, possibly by altering the profile of bacterial microflora [51].

a. Use of nitroreductases in elucidating the toxicity of nitrocompounds: the Ames test

The mutagenicity of nitrocompounds is associated with the products formed during reduction of the nitro groups. Hydroxylamino derivatives can interact with biomolecules, including DNA, causing toxic and mutagenic effects [2, 3]. Thus, the genotoxicity and carcinogenicity of nitroaromatic compounds has been studied intensively over the last 50 years beginning with 4-NQO, which was found to be a potent carcinogen [2]. Subsequently, several other compounds, including nitroarenes, nitrofluorenes, and nitropyrenes, were also analyzed and have been demonstrated potential mutagenic [2-4, 45].

The *Salmonella* / microsome assay, developed by Dr. Bruce Ames and co-workers in the 1970s, is a short-term bacterial test used to identify individual compounds and complex mixtures that cause genetic damage and mutation. Identifying the mutagenic potential of several agents has become increasingly important in the minimization of human and environmental exposure to these compounds. This assay is based on the induction of reverse mutations in strains of *Salmonella typhimurium*. These strains are unable to synthesize the amino acid histidine and are therefore unable to grow and form colonies in medium lacking this amino acid. For cells that can grow in the absence of histidine and form colonies, it is necessary for a mutation to occur in the histidine *locus* to reverse this auxotrophic phenotype to a prototrophic phenotype that is able to synthesize this amino acid [53]. In addition to carrying a mutation in a histidine gene, most of the Ames strains also contain additional mutations or genetic factors designed to enhance the sensitivity of the bacterial strains to mutagens. The first of these mutations were the *ΔuvrB* alleles, which eliminated the nucleotide-excision-repair system and conferred enhanced sensitivity to many mutagens. Later, cell wall mutations called “deep rough” (*rfa*) were included to permit the passage of large molecular weight compounds into the cell. A third critical addition was the inclusion of the pKM101 plasmid, which provided inducible SOS repair that enhanced the mutagenicity of certain compounds. Different strains can therefore be used in the Ames test. Each strain of *S. typhimurium* carries a different mutation in the histidine operon, which confers greater specificity for the detection of a particular type of mutation. Strains TA97, TA98, TA100, and TA1535 are used most often in this assay. Strain TA97 has a mutation in the histidine operon that detects mutagens that cause an error in the reading frame of DNA. The mutation detected by this strain results from the addition of one cytosine to the histidine operon, forming a sequence of six cytosines (CCCCCC). This alters the reading frame and, consequently, leads to reversion to a prototrophic character. The mutation carried by strain TA98 is a -1frameshift mutation which affects the reading frame of a nearby repetitive –C–G–C–G–C–G–C–G– sequence. Therefore, strain TA98 detects mutagens that cause errors in the DNA reading frame. Presented as a preferred point of injury are eight residues in the repetitive GC operon gene, which encodes the enzyme histidinol dehydrogenase. Strains TA100 and TA1535 detect mutagenic compounds that cause base-pair substitution in genes that encode the first enzyme in the biosynthetic pathway of histidine. These strains reverse G-C pairs, leading to the substitution of proline by leucine. In contrast to its isogenic strain TA1535, TA100 does not possess the plasmid pKM101 [53, 54]. Additional mutations were engineered into these strains to make them more sensitive to specific classes of substances. Therefore, strains of *S. typhimurium* were developed that either lacked nitroreductase completely (e.g., TA98 NR, TA1535 NR and TA100 NR) or that overproduced it (e.g., YG1021, YG1026) [50, 55]. Either the mutagenicity of many nitroaromatic compounds is substantially reduced in the nitroreductase deficient strain compared to the normal strain or the mutagenicity is often greater in the enriched strain than in the normal strain. More recently, tester strains have been constructed by introducing plasmids that encode the major and minor nitroreductase genes, *nfsA* and *nfsB*, of *E. coli* [56].

Tests employing combinations of these strains can facilitate the elucidation of the importance of these enzymes and the role of nitro group reduction in the mutagenicity of certain nitroaromatic compounds [60, 68]. Watanabe et al. (1989) conducted an early study that demonstrated the utility of this area of investigation. Initially, they constructed the *S. typhimurium* tester strains YG1021 and YG1026, which overproduce *S. typhimurium* Cnr nitroreductase, by introducing a plasmid carrying the gene in strains TA98 and TA100, respectively. These strains were more sensitive to the mutagenic activities of 2-nitrofluorene, 1-nitropyrene and 2-nitronaphthalene compared to the corresponding strain

YG1020, which does not express the plasmid-encoded nitroreductase [50, 55, 56]. Thereafter, many other studies using these strains or strains lacking nitroreductases have identified the role of reduction in the mutagenicity of nitrocompounds such as nitrobenzothiophenamines, nitrophenanthrene derivatives and *N*-nitroso compounds, drugs such as the antiepileptic AMP397, environmental samples such as atmospheric particles collected from various locations, contaminated soil or complex samples such as cigarettes and dyes [2-4, 45, 57].

The nitroreductase-modified *Salmonella* assay appears to be useful for studying the mutagenicity of nitroaromatic compounds when one wants to predict their possible effects on human health and elucidate their mechanisms of action [50, 55, 56].

5. Nitroreductase in biotechnology: a good tool

Currently, nitroreductases have received much attention, mainly because they can be used as biosensors and for the bioremediation of nitroaromatic compounds [7, 58]. Approaches for monitoring environmental contamination by the anthropogenic release of nitrocompounds are increasingly used, and there is a need to develop more efficient methods for the detection and bioremediation of these compounds in the environment [3, 7]. This is true especially for environmental contamination by explosives, which can readily enter groundwater supplies from contaminated soil and hence pose environmental concerns. Several laboratory-based methods are available to measure contaminants in solution, including such conventional methods as high performance liquid chromatography (HPLC), gas chromatography–mass spectroscopy (GC/MS), fluorimetric methods, and capillary electrophoresis (CE). However, these instruments are very expensive, and many of the sensors cannot be used to screen individuals. There is a need for *in situ* and highly selective continuous analysis in real time, but the techniques needed for this kind of detection are slow and expensive. Consequently, biosensors have been developed for rapid, sensitive and specific detection [7, 58]. In this respect, Gwenin et al. (2007) reported the preliminary stages of the development of an *in situ* electrochemical biosensor for the detection of trace levels of nitroaromatic compounds. The sensor is a gold electrode onto which an enzyme, the nitroreductase NfsB of *E. coli*, is directly immobilized *via* thiol linkages. The function of the enzyme is to provide selectivity by virtue of its biological affinity for the dinitroethylbenzene used in the study as a test explosive material [58].

Soil and groundwater have been polluted because of the production, deployment, and disposal of industrially important nitroaromatic compounds [45, 59]. Additionally, public health has been threatened as a consequence of this contamination. Explosives including nitro-substituted compounds such as TNT, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and glycerol trinitrate (GTN) are the main toxic pollutants, contaminating numerous military sites [7]. Few microorganisms can metabolize these compounds to form non-toxic, and therefore, they are persistent environmental contaminants [59, 60]. TNT is a nitroaromatic that has been used since 1902 as an explosive and as a chemical intermediate in the manufacture of dyes and photographic chemicals. It has been released into ecosystems principally as the result of military activities [3, 7]. TNT is one of the most recalcitrant and toxic of all the military explosives used, and its metabolites are toxic, mutagenic, and carcinogenic to many organisms, including humans [2, 3, 7]. TNT has accumulated in areas of manufacturing, storage, and decommissioning over recent decades [7]. Various government agencies, such as the U.S. Environmental Protection Agency, have listed TNT as a priority pollutant and have recommended that it be removed from contaminated sites to prevent environmental and health problems [59].

Traditional methods for the remediation of contaminated sites are often invasive, costly to the environment and, in many cases, ineffective for the level of contamination concerned. For example, the excavation of soil before treatment by incineration only moves, but does not remove the contaminants and can damage the environment [59]. In this context, bioremediation consists of the application of organisms that can metabolize contaminants, thereby removing pollutants from the environment [61]. Therefore, bioremediation using microorganisms or plants represents an attractive alternative for the treatment of contaminated sites [59]. Nitroreductases are able to degrade several compounds, including TNT [7, 59]. The biodegradation of this compound by these enzymes has been studied in several microorganisms including *Klebsiella* sp., *Pseudomonas pseudoalcaligenes* JS52 and *Pseudomonas putida* JLR1. In these studies, the nitroreductases have been cloned and characterized [29, 32, 34].

The use of plants for the bioremediation of pollution (phytoremediation) is a potentially useful technology for the treatment of soil contaminated by toxic chemicals, including nitrocompounds [59]. Phytoremediation has advantages including easy installation, low maintenance costs and a low environmental impact [7, 59]. However, unlike bacteria and mammals, plants are autotrophic organisms that lack the enzymatic machinery necessary for the efficient metabolism of organic compounds, which often results in slow and incomplete remediation performance, leading to the genetic modification of plants via the introduction of bacterial or mammalian genes involved in the breakdown of toxic chemicals [7, 61]. In this way, transgenic plants have also been used for the phytoremediation of sites contaminated with high levels of explosives. Examples of this include the use of the tobacco plant (*Nicotiana tabacum*) constitutively expressing the *nsfI* nitroreductase gene from *Enterobacter cloacae*, *Arabidopsis thaliana* expressing a nitroreductase gene of *E. coli* and Aspen expressing the nitroreductase gene *pnrA* of *P. putida* JLR1. These trangenic plants are able to degrade nitroaromatic compounds [7, 60, 61]. Although transgenic plants have not yet been used in field applications,

this strategy is expected to be efficient in decontaminating procedures to facilitate the effective future cleanup of contaminated sites [59].

Other compounds, such as the severe environmental pollutant hexavalent chromium [Cr(VI); chromate], are amenable to bacterial bioremediation. Interestingly, NfsA of *E. coli* and nitroreductase of *Vibrio harveyi* are able to reduce chromate to the less soluble and less toxic trivalent chromium [Cr(III)] [62, 63].

6. Clinical significance of nitroreductases: heroes or villains?

Nitroreductases are also of great clinical interest for use in cancer treatments and play a role in antibiotic resistance [1, 8, 64].

a. Nitroreductases in cancer treatments

Chemotherapy is the use of chemical agents in the treatment of disease and is the most widely used modality to treat cancer. The first antineoplastic chemotherapeutic agent was developed from mustard gas, which was used in both World Wars as a chemical weapon. After exposure of soldiers to this agent, it was found that they developed marrow hypoplasia and lymphoid tissues, leading to its use in the treatment of malignant lymphomas [64]. However, chemotherapy has several limitations, including limited amount of drug that can be administered to the patient, drug-resistant tumor cells, and a lack of selectivity for tumor cells over normal cells (the agents used can affect both normal and neoplastic cells, leading to systemic toxicity) [64, 65]. Enzyme prodrug therapy is a very promising strategy to address these problems. Prodrugs are chemicals that are pharmacodynamically and toxicologically inert but can be converted to highly active species [64]. These prodrugs can be activated by specific enzymes when they are expressed at a higher level in tumor cells than in normal cells. This allows for differential effects between these cells, which enhances the specificity of chemotherapy [8, 64, 65]. However, tumors that express adequate levels of enzymes capable of activating these prodrugs are rare and are not associated with any particular type of tumor [64]. Therefore, new therapies have been proposed to overcome this limitation of prodrug therapy. Gene- (or virus-) directed enzyme prodrug therapy (GDEPT or VDEPT) and antibody-directed enzyme prodrug therapy (ADEPT) represent attempts to overcome this problem [64, 65]. GDEPT is the use of DNA complexes, Clostridia, or viruses as vectors for the efficient delivery of the gene encoding an enzyme to tumor cells. This enzyme is capable of converting an inherently nontoxic prodrug into a cytotoxic metabolite that kills the tumor cells [64]. In ADEPT, the enzyme is conjugated to tumor cell-specific antibodies to direct the enzyme to the tumor. After the antibody binds to specific proteins on the surfaces of tumor cells, the prodrug is administered and is converted by the enzyme linked to the antibody on the tumor surface into a toxic molecule that can diffuse within the tumor tissues, resulting in cytotoxic effects [64, 65].

CB 1954 (5- aziridinyl 2,4-dinitrobenzamide) is the prototype of the dinitrobenzamide family of prodrugs, which are of increasing interest as potential cancer therapeutics, mainly as the result of the observation that this agent is activated by DT-diaphorase and causes complete regression of the Walker-256 carcinoma tumor in rats with minimal toxic side effects [65]. CB 1954 entered into clinical trials in the 1970s, but little antitumor activity was observed because human DT-diaphorase is much less active in the reduction of CB 1954 than is the rat enzyme. The difference in catalytic activity between the rat and human enzymes arises from differences in their amino acid sequences [65]. The nitroreductase NfsB of *E. coli* has been demonstrated to activate the prodrug CB 1954 to its toxic form more rapidly than does rat DT-diaphorase, raising the possibility of using CB 1954 with this nitroreductase in ADEPT and GDEPT therapies [64, 65]. The nitroreduction of CB 1954 by *E. coli* nitroreductase results in the formation of the cytotoxic 4-hydroxylamine, which then undergoes further reaction with thioesters, such as acetyl CoA, to form a potent DNA alkylating agent. This agent generates highly cytotoxic interstrand crosslinks in DNA and reduction of the 2-nitro group to either a 2-hydroxylamine or a 2-amine group, which are also potent cytotoxins [65]. In contrast, DT-diaphorase reduces only the 4-nitro moiety [64, 65]. These crosslinks are poorly repaired and lead to cell death in both dividing and non-dividing cells via p53-independent apoptosis [66]. The ability of activated CB1954 to kill cells independently of the cell cycle is an advantage when the target cells are not proliferating [64-66]. Another advantage is that whereas many prodrugs exhibit a bystander effect (they cause the death of adjacent cells that do not express the activating enzyme), this system does not display such an effect. Therefore, the anticancer potential of this therapy has been evaluated in preclinical studies and in phase I/II clinical trials in human patients with prostate cancer, and the results suggest that this direct cytotoxic strategy can also stimulate tumor-specific immunity, inducing the expression of a range of stress proteins including heat shock protein HSP70 and, in patients with liver cancer, a dose-limiting hepatotoxicity [64, 67].

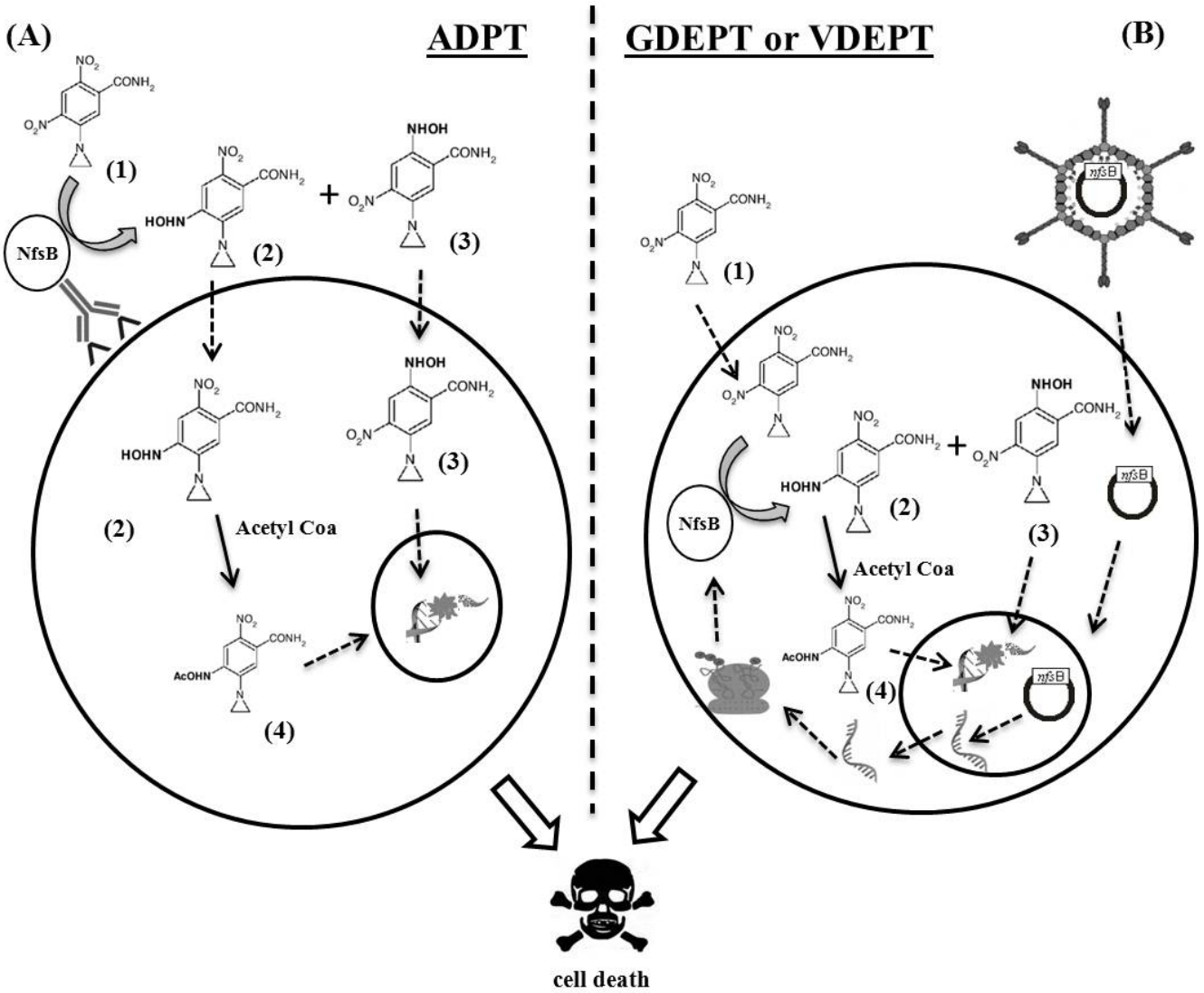

Figure 2. General outline of all approaches for enzyme/prodrug cancer therapy and bioactivation of CB 1954. **Panel (A)** antibody-directed enzyme prodrug therapy (ADEPT), the enzyme is conjugated to cell antibodies to direct the enzyme to tumor cell. After the antibody to bind to specific proteins on the surface of tumor cell, the prodrug CB 1954 **(1)** is then administrated in its inactive form non-toxic and is converted by the Nfs*B* nitroreductase of *E. coli* linked to antibody on the cell surface into 4-hydroxylamine **(2)** or 2-hydroxylamine **(3)** that can diffuse to within the cell resulting in DNA damage in cell, 4-hydroxylamine reacts with Acetyl coa to form 4-N-acetoxy **(4)** a potent DNA cross-linking specie, ,the 2-hydroxylamine is less toxic but can form DNA mono-adducts **Panel (B)** Gene (or virus) directed enzyme prodrug therapy (GDEPT or VDEPT), use of a viral vector for the efficient delivery of the gene *nfsB* of *E. coli* to into the tumor cell, where it will be over-expressed, thus inside the cell, where it is transcribed into mRNA in the nucleus, later translated in the cytoplasm forming the nitroreductase NfsB that will convert the prodrug CB 1954 **(1)** administered in 4-hydroxylamine **(2)** and after 4-*N*-acetoxy **(4)** or 2-hydroxylamine **(3)** that will cause damage to DNA. The lesions in DNA caused by metabolites of are poorly repaired, and lead to cell death.

However, the use of therapeutic NfsB / CB 1954 has disadvantages, because nitroreductase has a low affinity for the prodrug substrate and, consequently, a low reaction rate [11]. Thus, the use of other substrates, nitroreductases from other organisms, and alterations in the structure of the enzyme are being investigated as alternatives [11, 65]. Other nitroreductases have been shown to activate CB1954, including YwrO isolated from *B. amyloliquefaciens* and NbzA isolated from *P. pseudoalcaligenes.* Both of these enzymes share moderate sequence homology with the major nitroreductase of *E. coli,* NfsA, which has been demonstrated to activate the prodrug but with a lower *K*m than that determined for NfsB [18, 33]. Interestingly, it has been recently verified that the nitroreductase NfsA of *E. coli* has a higher catalytic efficiency than NfsB in trials with purified enzymes; however, the enzymes exhibit similar efficiencies when expressed in HCT-116 human colon carcinoma cells, making these cells sensitive to the prodrug [65]. The *E. coli* nitroreductases exhibit maximum activity at 30^{o}C, but human physiological temperatures is 37^{o}C, although *E. coli* nitroreductases possess sufficient catalytic activity at 37^{o}C for use in prodrug therapy. However, it would be interesting discover an enzyme with a higher efficiency of catalysis at body temperature. Bearing this in mind, the nitroreductase of

the Gram-positive thermophile bacterium *B. licheniformis* would be a potential candidate for prodrug therapies due to its bacterial origin, the high activity observed with CB1954 and its great stability [15]. The NfsB enzyme was optimized to increase the efficacy of the system by altering specific amino acids in protein and these modified enzymes are more efficient in activating CB1954 than is the parental enzyme [84]. Additionally, other prodrug substrates for *E. coli* NfsB nitroreductase have been tested and developed, including various dinitrobenzamide mustards, oxazino-acridines and nitrobenzyl and nitroheterocyclic carbamates [45].

Most studies that have described new or modified prodrug-activating nitroreductases have focused on enzyme kinetics at the purified protein level and/or growth inhibition in transfected tumor cell lines. Apart from cancer gene therapy, nitroreductases and CB1954 are also being used increasingly for other applications that require conditional cell killing, including the killing of cells responsible for the loosening of orthopedic implants in patients, and targeted, controllable tissue ablation in transgenic animals [45, 68].

b. Unraveling the role of cells and tissues: Nitroreductases in the ablation of specific cells

Conditional targeted ablation is the ability to temporally and spatially control specific tissue damage and remove a specific cell population. It has wide applications as a powerful tool in studying the role of specific cell lineages, cell-cell interactions, develop and regeneration of tissue, screening abnormalities organism, cellular degeneration or physiological processes *in vivo* [68, 69]. It has been used to develop several toxic protein systems or "suicide genes" to destroy selective cell populations while leaving the remaining tissues unharmed [69]. Prodrug-dependent cell ablation is a method that is based mainly on the ability of the enzyme nitroreductase NfsB of *E. coli* to convert the nontoxic prodrug CB1954 or metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] into cytotoxic metabolites [45, 69]. The prodrug is converted into its toxic form only in nitroreductase-expressing cells, leading to specific, inducible and spatially restricted cell destruction without affecting neighboring cells [69]. Interestingly, metronidazole is a better nitroreductase substrate than others that have been described previously, such as CB1954, which exhibits a slight "bystander effect" that can damage neighboring cells [68, 69].

To monitor the course of ablation, it can be used in association with fluorescence techniques. Thus, one can insert a vector expressing a fluorescent protein – nitroreductase fusion protein (*i.e.*, NfsB - GFP) in target tissues. Once the prodrug is administered, the cells expressing the fluorescent protein – nitroreductase fusion protein are destroyed and the ablation process can be monitored [69]. This technique offers many advantages, including spatial specificity and temporal control. The effects of the absence of a cell population or a tissue can provide a better understanding of their role in morphogenesis, patterning or cell survival, tissue recovery, and molecular and cellular mechanisms during regeneration and development [68, 69]. For this purpose, transgenic animals such as mice, rats and zebrafish can be constructed with specific cells that express a fluorescent protein – nitroreductase fusion protein [87]. This procedure has been used successfully to ablate cells such as cardiomyocytes, hepatocytes and pancreatic β-cells in zebrafish embryos and larvae. Interestingly, these studies have also shown that this technique is reversible, because the tissue can recover after ablation; this makes the technique very useful in regeneration studies [68-70].

Several studies have employed this technique. The NfsB/CB1954 system has been used to specifically ablate progenitor stem cells in the CNS to elucidate more thoroughly the complex and multiple functional roles of these cells in both early postnatal and adult brains and the role of astrocytes in the adult brain has been investigated using this system [71]. The feasibility of ablating differentiated adipocytes in mice with a suitable prodrug activating system has been described [70]. Transgenic zebrafish that express the gene encoding *E. coli* nitroreductase have been used to ablate pancreatic β cells and the male germ line of zebrafish to create a model of inducible male sterility [72, 73]. Recently, a model of retinal ablation was used in zebrafish: rod cells expressing NfsB *E. coli* nitroreductase were treated with metronidazole to study the involvement of these cells in the regeneration of retinas [68].

c. Nitroreductases and antibiotic resistance: when they are or are not presents...

Nitroreductase also plays a role in antibiotic resistance, mainly against metronidazole [74]. Metronidazole was originally developed to treat *Trichomonas* infections but has also been used in the treatment of infections caused by *Entamoeba*, *Giardia*, and the mainly anaerobic bacteria *Helicobacter pylori*, a common bacterial pathogen in humans found in association with gastric inflammation, peptic ulcer disease, gastric carcinoma, mucosa-associated lymphoid tissue and lymphoma of the stomach [10, 74]. However, resistance to metronidazole is increasingly a problem in the treatment of *H. pylori* infection [74]. Therefore, understanding the molecular basis of antibiotic resistance is becoming of paramount importance in the development of new strategies, such as the design of more effective antibiotic compounds, and in the development of more rapid and accurate methods of diagnosing the susceptibility of infections to antimicrobials. Proper targeting and shorter time lags in determining the resistance status of an infection can be critical factors in its elimination [10, 74, 75]. Resistance to metronidazole in *H. pylori* is associated with mutations in *rdxA*, which encodes an oxygen-insensitive NADPH nitroreductase, and with mutations in *frxA*, which encodes a NAD(P)H-flavin oxidoreductase [10]. RdxA nitroreductase converts metronidazole from a prodrug to a mutagenic hydroxylamine that damages DNA, resulting in strand breakage, helix destabilization, unwinding, and cell death. FrxA may act

indirectly by affecting the cellular reductive potential. The reductive activation of metronidazole depends on the redox system of the target cell. Therefore, factors that lead to the loss of or a decrease in the activities of the two enzymes may contribute to metronidazole resistance [10, 74, 75]. Clinically, therefore, nitroreductases are considered attractive targets for nitroimidazole-based intervention therapies for the treatment of *H. pylori* infection. *H. pylori* strains can become resistant in three ways: (1) by inactivation of *rdxA* (type I), (2) by inactivation of both *rdxA* and *frxA* (type II) and (3) rarely, if ever, by inactivation of *frxA* alone. In summary, the disruption of *rdxA* alone can produce high-metronidazole resistance at all levels, and mutations in *frxA* alone do not render *H. pylori* metronidazole-resistant but can enhance the level of type I resistance. High-level metronidazole resistance can be caused by *rdxA*/*frxA* double mutations [74]. Single nucleotide transitions that introduce frameshift mutations or stop codons, along with larger DNA insertions or deletions, both inactivate the *rdxA* gene and have been found to be associated with the metronidazole-resistant phenotype [10]. Despite this association, the strict correlation of metronidazole resistance with mutations in *rdxA* or *frxA* remains controversial. Because the inactivation of the *rdxA* gene alone is frequently, but not always, associated with resistance to metronidazole, the described resistance of *H. pylori* strains with an intact *rdxA* gene suggests that other pathways participate in metronidazole resistance. Therefore, inactivation of *rdxA* alone is insufficient to explain the heterogeneity of metronidazole resistance among clinical *H. pylori* isolates [13, 93, 94].

Other antibiotics such as the nitrofuran derivatives nitrofurazone and nitrofurantoin are used to treat infections caused by a broad spectrum of bacteria, mainly genito-urinary infections. These compounds also need to be activated by nitroreductases such as NfsB of *E. coli* [1].

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Capítulo II

APRESENTAÇÃO DO CAPÍTULO II

Estrutura, distribuição e o papel fisiológico de nitrorredutases em procariotos e eucariotos: uma mini revisão.

Este capítulo apresenta uma revisão que começa com uma breve introdução salientando a importância e aplicações das nitrorredutases (abordadas no capítulo anterior). Em seguida é mostrada uma visão geral dos mecanismos pelos quais essas enzimas podem reduzir os nitrocompostos. Posteriormente é discutida a distribuição das nitrorredutases nos organismos, mostrando a presença em bactérias, fungos e mamíferos. Neste sentido, foi realizada uma análise filogenética com as principais nitrorredutases evidenciando a presença de quatro grupos filogeneticamente distintos. Outro aspecto apresentado na revisão é a estrutura geral dessas enzimas. Neste ponto é mostrado que são proteínas com grande similaridade estrutural entre si, sendo conservada tanto em procariotos quanto em eucariotos. É apresentada a relação da estrutura com o mecanismo de redução e a especificidade ao substrato. Por fim, são revisadas e discutidas as possíveis funções fisiológicas dessas enzimas nos diferentes grupos filogenéticos identificados. Neste sentido, as hipóteses do papel biológico dessas enzimas como bioluminescência, homeostase metálica, biossíntese de cobalamina e resposta a estresse oxidativo foram abordadas. É importante salientar que essa é a primeira revisão abrangendo as nitrorredutases presentes em eucariotos.

O manuscrito será submetido ao periódico *FEBS Letters*.

Structure, distribution and physiological role of nitroreductases in prokaryotes and eukaryotes: A minireview

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Abstract

Nitroreductases enzymes comprise a group of flavoproteins able to metabolize nitrocompounds using the reducing power of nicotinamide adenine dinucleotide (NAD(P)H). Nitroreductase genes are widely spread within bacterial genomes and, to a lesser extent, in eukaryotes. In this sense, our phylogenetic analysis indicated the presence of four major groups of nitroreductases in bacteria, fungi and mammals. The nitroreductases play a central role in the nitrocompounds activation and have received a lot of attention in recent decades based on their role in mediating nitrosubstituted compound toxicity, biotechnological application, and clinical importance. Due to its relevance, bacterial nitroreductases have been purified, and their biochemical, kinetic parameters and structure have been determined. Despite their phylogenetic differences, these enzymes display a conserved 3D structure, as shown in this minireview. However, little is known about their physiological functions. In this sense, hypotheses have been proposed to solve the real physiological role of nitroreductases, such as metal homeostasis, cobalamin metabolism, bioluminescence and oxidative stress response. Therefore, in this minireview the mechanisms of nitroreduction, classifications, distribution, structure and possible physiological functions of these enzymes in prokaryotes and eukaryotes are presented and discussed.

Keywords: Nitroreductases; flavoproteins, physiological role; nitrocompounds.

1. Introduction

The nitroreductases proteins form a group of flavoproteins that have a central role in the reduction of nitro groups [1]. They comprise a family of proteins with conserved sequences that were originally discovered in eubacteria [2]. Few data exist on the distribution of nitroreductase sequences in eukaryotic cells. These enzymes are capable of catalyzing the reduction of nitrocompounds using flavin mononucleotide (FMN) as prosthetic groups and nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH) as reducing agents [3].

The knowledge about these proteins is of significant interest due to: (a) environmental and human health importance due to their role in mediating nitrosubstituted compound toxicity; (b) biotechnological application for bioremediation and biocatalysis; and (c) clinical importance in tumor treatment, ablation of specific cells and bacterial antibiotic resistance [4]. These enzymes are used in techniques such as gene (or virus) directed enzyme prodrug therapy (GDEPT or VDEPT) and antibody-directed enzyme prodrug therapy (ADEPT) for potential use in the treatment of certain tumors. In this sense, the non-toxic prodrug CB1954 (5-[aziridin-1-yl]-2,4-dinitrobenzamide) is converted into a DNA-crosslinking cytotoxic agent by tumor cells expressing bacterial nitroreductases [5]. Nitroreductases are also involved in resistance to antibiotic metronidazole, a nitrocompound used mainly in infections caused by *Helicobacter pylori*, which causes gastric ulcers and constitutes a risk factor for adenocarcinoma and gastric lymphoma [6]. In addition, these enzymes are involved in the bioremediation of nitroaromatic and nitroheterocyclic explosives in soil, such as 2,4,6-trinitrotoluene (TNT). In this case, the phytoremediation using transgenic plants expressing bacterial nitroreductases may be effective in decontaminating soil *in situ* [7]. Genotoxic tests have been performed using mutant strains of bacteria *Salmonella*

typhimurium that do not express or overexpress these enzymes to identify and elucidate the molecular mechanism of mutagenesis caused by nitrocompounds [8]. Because of their importance, cloning, purification, structural analysis and functional characterization studies have been carried out on nitroreductases isolated from various organisms [2,9]. However, the knowledge about the physiological role of nitroreductases is very limited and an open question to be answered. Therefore, different physiological functions have been suggested for these enzymes. In this minireview are presented and discussed important aspects of the nitroreductases: reduction mechanisms, structure and the most relevant studies about the physiological role of these interesting enzymes.

2. General mechanism of nitroreductases reduction

Two types of bacterial nitroreductases have been characterized and grouped into two categories based on mechanism to reduce nitro groups: type I (oxygen-insensitive) and type II (oxygen-sensitive) (Fig. 1) [1]. Type I nitroreductase catalyzes a series of two electron reductions of the nitro group, which produces the corresponding nitroso-, hydroxylamino, and amino derivatives and are known to participate in the reduction of a variety of nitrocompounds such as nitrofurazone and nitrofurantoin [10]. Type II nitroreductase catalyzes one electron reduction of the nitro group, which produces a nitro anion radical, and this radical subsequently reacts with oxygen to yield superoxide and regenerate the parent nitrocompound [11]. This "futile redox cycle" can cause oxidative stress by producing large amounts of superoxide (Fig. 1). Thus, these enzymes can mediate the reduction of nitroaromatics by two-electron transfers only under anaerobic conditions [12].

3. Classification of nitroreductases enzymes

Some nitroreductases are listed in Table 1, and among them, the bacterial enzymes are especially well studied. Our phylogenetic analysis of main nitroreductases sequences suggests that there are four major groups of phylogenetically distinct enzymes: NfsA/Frp, NfsB/FRaseI, Frm2p/Hbn1p and IYD/BluB families (Fig. 2). Therefore, bacterial nitroreductases, represented by type I nitroreductases, can be classified into two families or groups: NfsA/Frp (group A) and NfsB/FRaseI (group B), that share very low identity with each other at the level of primary structure [11]. The group A nitroreductases includes also proteins such as SnrA of *S. typhimurium* [13], PnrA of *Pseudomonas putida* JLR11 [14] and Frp of *Vibrio harveyi* [15]. The group B includes the NR of *Enterobacter cloacae* [16], Cnr of *S. typhimurium* [13] and FRaseI of *Vibrio fischeri* [17].

In contrast to bacteria, there are few records of nitroreductases in eukaryotic cells. In fungi, two nitroreductase– Frm2p and Hbn1p – were identified by *in silico* analysis in the yeast *Saccharomyces cerevisiae* [18]. Recently, Frm2p was purified and characterized as nitroreductase according to their ability to reduce 4-nitroquinoline-oxide (4-NQO) [19]. However, Frm2p and Hbn1p display low primary sequence similarity with *E. coli* nitroreductases (Fig. 2), and thus constituting a new family of nitroreductases with homologues present in several bacteria, such as CinD nitroreductase of *Lactococcus lactis* (Fig. 2), and also some species of protozoan [18]. The IYD/BluB group includes the mammalian enzyme iodotyrosine deiodinase (IYD), which contains the highly conserved C-terminal domain and resembles NfsA of *E. coli* [20]. Interestingly, our phylogenetic analysis indicated a greater similarity of IYD with

the bacterial nitroreductase BluB of *Rhodobacter capsulatus* (Fig. 2). Curiously, IYD is the only example of nitroreductase identified in mammals until the moment. In this aspect, it has also been hypothesized that the nitroreductase-like sequences present in the genomes of eukaryotes have been acquired by lateral gene transfer [18]. Supporting this idea, it was reported that the anaerobic protozoan *Giardia lamblia* also has an oxygen-insensitive nitroreductase-like sequence with high similarity to *Clostridium acetobutylicum* nitroreductase sequence [21]. It is important to highlight, that there are enzymes in eukaryotes functionally related to type I nitroreductases such as NAD(P)H-quinone oxidoreductase (DT-diaphorase) and xanthine dehydrogenase, but these enzymes are not phylogenetically related and do not exhibit the domain characteristic of this family [22].

4. Structure of nitroreductases

The crystal structures of many nitroreductases have been determined [3,15,20]. Although the variation in amino acid sequence, the 3D structure of the nitroreductases are very similar (Fig. 3A). Nitroreductases do not have metals in the structure of enzyme or sulfhydryl groups at the active site [23]. Moreover, nitroreductases share a homodimeric globular structure, $\alpha + \beta$ fold of the subunits, with monomeric molecular weight of 20–30 kDa. The nitroreductases have a central hydrophobic core consisting of stranded β-sheets surround by α-helices with where are located conserved domains for FMN binding (Fig. 3A) and the active site of the enzyme for interactions with the NAD(P)H and substrates (Fig. 3B) [23].

The FMN prosthetic groups are bound in deep pockets at the dimer interface and interact with residues of both monomers, forming hydrogen bounds with one

subunit and hydrophobic contacts to both (Fig. 3B and 3C) [23]. This set of interactions with FMN is well conserved in all nitroreductases and involves identical or similar residues (Fig 3E) [3].

The nitroreductases of NfsA/Frp group use NADPH as an electron source, whereas NfsB/FRaseI group can use either NADH or NADPH [24-25]. The basis for NADPH specificity is believed to be associated with two requirements: the 2'-phosphate group of NADPH interacts with Arg203 and the nicotinamide ring of NADPH is located near the isoaloxazine ring of FMN for hydride ion transfer between the two rings, as deduced from the position in the NfsB protein of the analogous ligand nicotinic acid (Fig. 3C) [26]. In this sense, it has been previously reported that positively charged residues, such as arginine, lysine and histidine, are involved in the recognition of the 2'-phosphate group of NADPH through a hydrogen bond [15,25]. In NfsB, the nicotinamide ring of NAD(P)H is placed between the flavin isoaloxazine ring and the Phe124 residue [23]. In NfnB from *M. smegmatis* the 2'-phosphate make only a salt bridge with Arg105 residue (Phe124 in NfsB), which may explain why NfnB does not show significant specificity for either NADPH or NADH). A similar mechanism should occur in other enzymes of group B, explaining the plasticity for these two cofactors [27].

In addition, the oxidation state of FMN influences the spatial orientation of the substrate in the active site [25]. Therefore, the charge distribution on the FMN rings, which alters upon reduction, could be a key determinant of substrate binding and reactivity in flavoproteins with broad substrate specificity [23]. In NfsB, the broad substrate specificity is explained by the inherent plasticity of the helix 8 which contains residue Phe124 (or similar in other nitroreductases), showing an elevated variability in position for accommodating substrates of different sizes (Fig. 3D) [23,25]. In addition,

mutation of Arg203 in NfsA equivalent to Phe124 in NfsB affects substrate specificity [24-25]. These and others residues of active site vary among nitroreductases suggesting that they may play a role in determining substrate preferences [23,25].

The inspection of nitroreductase structures suggest a common substrate binding mode: nitrocompounds are all positioned with their nitrobenzene moiety stacking parallel to the FMN isoalloxazine ring and the nitro group pointing at ~ 3.5 Å to FMN. Due to the limited space between of active site, the initial substrate (NADPH/NADH) and the second substrate (nitrocompound) cannot simultaneously bind to the active site [23]. Such a mechanism implies that NAD(P)H and the second substrate must bind to the active site of nitroreductase such that the reduced nicotinamide ring of NAD(P)H approaches the isoalloxazine ring of FMN sufficiently closely to allow efficient hydride transfer, then reducing the FMN. Subsequently, $NAD(P)^+$ is released and the second substrate binds in the active site pocket allowing for reverse electron transfer from the flavin moiety to reduction effect. Therefore, is consistent with substrate reduction process via a ping-pong mechanism [15,23-24].

The nitroreductases of fungi have their structures not yet determined, but the hypothetical structure of the nitroreductase of *S. cerevisiae* was constructed by de Oliveira et al. [18] using the template sequence of *Streptococcus mutans* nitroreductase. The *in silico* structure analysis suggests that fungal nitroreductases can be monomeric or homodimeric FMN-containing proteins with a subunit size of approximately 25 kDa, composed of β strands and α chains and that use NAD(P)H as a reductant. Consistently, the CinD of *L. lactis* resembles a typical nitroreductase. There is one profound difference between CinD and the majority of the reported nitroreductases. The prominent α-helice that usually shields the active site is substantially shortened in CinD

[28]. These helices are believed to have an influence on substrate specificity and recognition.

The active site of IYD protein is structurally similar to NfsB active site, where the substrate monoiodotyrosine (MIT) binds in a similar form to nicotinic acid in NfsB active site (Fig. 3D) [20]. The chemistry promoted by this enzyme is likely quite different from the oxygen-insensitive nitroreductases because its biological function appears to involve degradation of FMN-bound by molecular oxygen [20]. A similar mechanism occurs in the reduction carried out by the enzyme BluB [30].

The structure analyses of nitroreductases reveals an inherent plasticity of the active site for the substrate binding and variability in the amino acid residues participating in these interactions, thus explaining the different nitroreductase activities for nitroaromatics and other compounds and also suggest that these enzymes may participate in different metabolic functions in the organisms.

5. The physiological role of nitroreductases

The physiological role of nitroreductases is a mystery to be revealed. Considering that nitroreduction increases the solubility of organic compounds, facilitating microbial biodegradation. It may be assumed that these enzymes play a role in general detoxification of nitrocompounds [31].Thus, it might be supposed that the microorganisms may be exposed to nitrocompounds naturally formed in the environment, such as the reaction of nitrite/nitrate with phenolic compounds present in the soil humic matter forming nitrosophenols, and their toxicity can be avoided by the action of the nitroreductases [32]. In contrast, bacterial mutants lacking nitroreductases were more resistant to some nitrocompounds, due to the greater toxicity of the products

of reduction [10]. However, the nitroaromatic compounds used in the previous studies are not the ones that elicited the evolution of nitroreductases [33]. In this context, Roldan et al. [11] proposes that it is not likely that nitroaromatic compounds could be the physiological substrates of nitroreductases, since most nitroaromatic compounds have been very recently released into the environment by human activities. The primitive physiological function of these enzymes could have been lost or modified to allow the reduction of different nitrocompounds. In this sense, it is possible that nitroreductases have distinct roles in cellular metabolism unrelated to nitrocompounds reduction. The Table 1 shows possible physiological functions of some nitroreductases.

NfsA/Frp and NfsB/FRaseI families

Some bacterial nitroreductases seem to be specialized in the transformation of a specific nitroaromatic compound, such as nitrobenzene and nitrophenol, playing a role in specific degradation pathways [31]. In this sense, it was postulated primarily that *E. coli* nitroreductases could be able to reduce 3-nitrotyrosine (3-NT). However, a study conducted by Lightfoot et al. [34] indicated that neither NfsA nor NfsB can reduce 3-NT. Therefore, it is possible that the nitroreductases are only capable of reducing small substrates and not free 3-NT.

Interestingly, NfsA and NfsB of *E. coli* and *V. harveyi* are able to reduce ions, such as chromate to less soluble and less toxic Cr^{3+} [35-36]. In this context, Takeda et al. [37] showed that DrgA nitroreductases of *Synechocystis* and NfsB of *E. coli* function as ferric reductases. DrgA utilizes both a synthetic iron chelator, such as EDTA, and natural chelators such as citric acid. In addition, small molecular-weight chemical chelators, such as iron transporter protein, transferrin, and an iron storage protein,

ferritin, turned out to be substrates of the DrgA protein, suggesting that it might play a role in iron metabolism in *Synechocystis,* under physiological conditions [37].

In *V. fischeri* and *V. harveyi,* the nitroreductases FRaseI and Frp, respectively, may be involved in bioluminescence by catalyzing the FMN reduction dependent of NAD(P)H and provide the flavin required for the luciferase reaction [38]. FRaseI and Frp can reduce FMN, but also shows nitroreductase activity [39-40]. In contrast, little FMN reductase activity is associated with NfsA and NfsB. However, a single amino acid change at the active site of the *E. coli* NfsA or NfsB protein converts these nitroreductases to FMN reductase with similar properties to the Frp and FRaseI, respectively. The amino acid substitution (Glu99 to Gly in NfsA and Phe124 to Ser in NfsB) may destroy some hydrogen bonds in the active center, so that large molecules such as FMN can be properly accommodated in the active center and recognized as efficient substrates [39-40]. These facts suggest that the genes encoding nitroreductases and flavin reductases shared a common progenitor. It is possible that progenitors of the NfsA-Frp and NfsB-FraseI pairs lost FMN reductase activity during evolution in *E. coli* cells or acquired FMN reductase activity during evolution in luminescent bacteria.

The nitroreductase Pyr-1 of *Mycobacterium* sp. seems to be a lipoamide dehydrogenase, as deduced from its N-terminal sequence [41]. Lipoamide dehydrogenase belongs to a family of pyridine-nucleotide:disulfide oxidoreductases and catalyzes flavin-dependent regeneration of the lipoamide cofactor involved in production of reducing equivalents, such as NADH [42]. However, there is no biochemical evidence of nitroreductase activity for this enzyme. Also, sequences of the N-terminal ends of the nitroreductase NfsB and a dihydropteridine reductase from *E. coli* are almost identical, suggesting that both proteins are homologous, but no functional studies have been performed to demonstrate the enzymatic activity of these

proteins [43]. However, *R. capsulatus* NprA nitroreductase showed dihydropteridine reductase activity [44]. *R. capsulatus* is able to use phenylalanine or tyrosine as the sole nitrogen source and metabolism of these aromatic amino acids involves hydroxylation of Phe to Tyr [45]. Aromatic amino acid hydroxylases use tetrahydrobiopterin as cofactor and needs to be regenerate by NADH-dependent reaction catalyzed by the dihydropteridine reductase [46].

The largest number of evidences regarding the physiological role of nitroreductases is about their involvement in the oxidative stress response [11]. For instance, the *E. coli nfsA* gene is part of the SoxRS regulon and is strongly induced by paraquat [47], a well-known superoxide generator [48]. The *snrA* gene in *S. typhimurium* and *nprA* in *R. capsulatos* are also induced by paraquat [44,49] and some NfsA-like proteins of *Staphylococcus aureus* help to maintain the cell thiol–disulphide balance [50]. The *S. aureus* NtrA nitroreductase exhibits S-nitrosoglutathione (GSNO) reductase activity, which protects the cell against transnitrosylation [51].

NfrA1 *B. subtilis* nitroreductase has probably a dual role in the regulation of hydrogen peroxide (H_2O_2),showing NADH oxidase activity. NfrA1 produce high concentrations of H_2O_2 that can serve as a defense agent against invading strains. In contrast, and due to abundant and accessible methionine residues NfrA1 can also scavenge H_2O_2, probably by converting these residues to the corresponding sulfoxides [52].

Frm2p/Hbn1p family

The nitroreductase Frm2p in *S. cerevisiae* was initially identified as a repressor of a regulon controlled by exogenous fatty acids. These observations led to the

conclusion that Frm2p functions in the fatty acid signaling pathway and is regulated by fatty acids [53]. However, this family of nitroreductases should be involved with oxidative stress response. In this sense, de Oliveira et al [54] showed that yeast *S. cerevisiae* strains deficient in Frm2p and Hbn1p nitroreductase-like proteins have reduced basal activity of superoxide dismutase (SOD), higher ROS production, lipid peroxidation, petite induction (respiratory deficient yeast) and sensitivity to 4-NQO and N-nitrosodiethylamine (NDEA) (4-NQO and NDEA are superoxide-generating compounds by redox cycle). The single and double mutant strains *frm2*Δ, *hbn1*Δ and *frm2*Δ*hbn1*Δ have higher basal activities of catalase (CAT) and glutathione peroxidase (GPx), and reduced glutathione (GSH) content, consequently less ROS-accumulation and lipid peroxidation when exposed to peroxides, H_2O_2, and *t*-BOOH. Therefore, Frm2p and Hbn1p enzymes influence the response to oxidative stress in *S. cerevisiae* by modulating GSH contents and antioxidant enzymatic activities. In addition, it was reported that the *FRM2* gene promoter contains aYap2p (or Cad1p) binding site [55]. *YAP2* was identified in a genetic screen for genes conferring resistance to metal and chemical stress when overexpressed, mainly associated with ion Cd^{2+} resistance [56]. Indeed, Northern blot analysis shows that *FRM2* gene is as a Yap2 target gene regulated by Cd^{2+} in a strictly Yap2-dependent way. The metabolism of polyunsaturated fatty acids and Cd^{2+} generate large amounts of ROS [58]. Consistently, Horan et al. [59] demonstrated that the yeast *S. cerevisiae* under nitrosative stress has *FRM2* and *YAP2* genes overexpressed, suggesting that this pathway is important in the response to RNOxS.

The CinD is component of a regulon in the copper-responsive system of *L. lactis* and reduces 2,6-dichloro-phenolindophenol and 4-NQO using NADH as a reductant. *cinD* gene expression is induced by copper, cadmium, and silver. CinD is a

nitroreductase that can protect *L. lactis* against oxidative stress exerted by nitroaromatic compounds and metals [28]. Consistently, the strains deficient in CinD is more sensitive to oxidative stress exerted by 4-NQO and copper. Interestingly, CinD also exhibited significant catalase activity *in vitro* [28]. These evidences emphasize the hypothesis of nitroreductases participation in the response to oxidative stress and metal metabolism.

IYD/BluB group

This group contains representatives of nitroreductases in bacteria and mammals with enzymes apparently involved metabolism pathways. Thus, the bacterial nitroreductase BluB has been implicated in the biosynthesis of cobalamin (vitamin B_{12}). It was proposed that BluB is involved in unusual fragmentation of the FMN to 5,6-dimethylbenzimidazole (DMB), a lower ligand of cobalamin [30]. B_{12} is a cofactor for several enzymatic reactions in animals, protists, and some prokaryotes, such as the biosynthesis of fatty acids, methionine and deoxynucleotides [60].

The other member of this group is the nitroreductase IYD involved in thyroid hormone (TH) synthesis. The TH plays critical roles in adult organs and during development in vertebrates [61]. Iodine is an indispensable component of TH and is mainly taken up from the diet. The thyroid gland has two systems to ensure sufficient iodine for TH synthesis. One of them is iodine uptake at the basolateral membrane by the sodium-iodine symporter [61]. Another one is the regeneration of iodide through the deiodination of diiodotyrosine (DIT), the main iodinated product of TH synthesis. IYD facilitates iodide salvage by catalyzing deiodination of MIT and DIT in the thyroid [62]. Failure of IYD function causes hypothyroidism with elevated levels of iodotyrosine in serum and urine [63]. Interestingly, the phenotypes of all four mutations of human IYD

described are consistent with mutations in the human gene encoding proteins with structural changes, particularly in residues that bind to FMN, causing reduction or loss of enzyme activity [20].

Final considerations

Increasingly, new nitroreductases have been identified in different organisms, purified, their biochemical and kinetic parameters have been characterized, and the crystal structures have been solved. Nitroreductases are enzymes normally involved in degradation pathways of specific nitrocompounds. However, the versatility of these enzymes since the phenomena of gene duplications, lateral gene transfer and accumulation of different mutations at specific sites have created a set of nitroreductase proteins that should perform a wide variety of metabolic functions in the different organisms, such as metals homeostasis, bioluminescence, B_{12} biosynthesis and oxidative/nitrosative stress response. Having in mind that nitroreductase play a central role in the activation of nitrocompounds, their possible physiological functions, as well as potential industrial, biotechnological and clinical applications, the increased knowledge about these enzymes is very important.

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Table 1. The most relevant nitroreductases and proposed physiological functions

Nitroreductase	Organism	Possible Physiological role	References
NfsA/Frp group			
Frp	*Vibrio harveyi*	Chromate reduction	[36]
		Bioluminescence	[15]
NfrA	*Staphylococcus aureus*	Oxidative stress response	[50-51]
NfrA1	*Bacillus subtilis*	Oxidative stress response	[52]
NfsA	*Escherichia coli*	Oxidative stress response	[47,64]
		Chromate reduction	[35]
NitA and NitB	*Clostridium acetobutylicum*	ND	[65]
PnrA	*Pseudomonas putida*	ND	[14]
SnrA	*Salmonella typhimurium*	Oxidative stress response	[49]
NfsB/FRaseI group			
Cnr	*Salmonella typhimurium*	ND	[13]
DrgA	*Synechocystis sp*	Ferric reductase	[37]
FRaseI	*Vibrio fischeri*	Bioluminescence	[17]
Flv	*Paenibacillus polymyxa*	ND	[66]
NbzA	*Pseudomonas pseudoalcaligene*	ND	[67]
NfnB	*Mycobacterium smegmatis*	ND	[27]
NfsB	*Escherichia coli*	Ferric reductase activity	[68]
		Dihydropteridine reductase	[43]
NR	*Enterobacter cloacae*	ND	[16]
PnbA	*Lactobacillus plantarum*	ND	[69]
PnrB	*Pseudomonas putida*	ND	[14]
RdxA	*Helicobacter pylori*	ND	[70]
YfkO	*Bacillus licheniforms*	ND	[71]
Frm2p/Hbn1p group			
CinD(YtjD)	*Lactococcus lactis*	Oxidative stress response	[28]
		Defense against copper toxicity	
Frm2p	*Saccharomyces cerevisiae*	Oxidative stress response	[54]
		Fatty acid metabolism	[53]
		Defense against cadmium toxicity	[55]
Hbn1p	*Saccharomyces cerevisiae*	Oxidative stress response	[54]
IYD/BluB group			
BluB	*Rhodobacter capsulatus* *Sinorhizobium meliloti* *Selenomonas ruminantium*	Biosynthesis of vitamin B_{12} (cobalamin)	[72]
IYD (DEHLA1)	*Homo sapiens* *Mus musculus*	Enzymatic deiodination in the formation of the thyroid hormones	[20]
Nitroreductases-like			
NprA	*Rhodobacter capsulatus*	Dihydropteridine reductase	[73]
NtrI	*Klebsiella sp.*	ND	[74]
Pyr-1	*Mycobacterium sp.*	Lipoamide dehydrogenase	[41]
YcnD	*Bacillus licheniforms*	ND	[71]
YdjA	*Escherichia coli*	ND	[29]
YwrO	*Bacillus amyloliquefaciens* *Bacillus subtilis*	ND	[75]

ND =not determined

Figure legends

Fig. 1. The mechanism of action of type I (oxygen-insensitive) and type II (oxygen-sensitive) nitroreductases: A nitrocompound exemplified in (1). Type I catalyze the reduction of the nitro group of nitroaromatics by transference of pair of electrons from NAD(P)H to form the nitroso (2) and hydroxylamino (3) intermediates and finally the amino group (4). Type II nitroreductases transfer a single electron to the nitro group, forming a nitro anion radical (5), which can be reoxidized aerobically to the original structure with the concomitant generation of the superoxide in a futile redox cycle or can form nitroso (2) intermediate by transfer of another electron.

Fig. 2. Topological view of an unrooted phylogenetic tree obtained from global alignment of some proteins listed in Table 1. There are three main groups or families of nitroreductases: (1) bacterial nitroreductases; (2) Frm2p/Hbn1p and (3) IYD/BluB families.

Fig. 3. Structure of the nitroreductases. (A) Global fold of the homodimer of NfsB; the FMN prosthetic group is shown as 'Van der Waals' spheres. The α-helices (red) are labeled from 1 to 12 and β-strands (yellow) are represented from 1 to 5. (B) The active site is located at the interface of the dimer, and residues from both monomers (chain A in red and chain B in blue) contribute to FMN-binding. (C) The structure of NfsB complexed with nicotinic acid. The protein surface, shown on the background, is colored according to its electrostatic potential. Blue indicates positive charge, red indicates negative charge and White neutral charge. (D) Figure emphasizing the α-helix 8 (with Phe124 residue in red) in contact with the active site of the NfsB nitroreductase. (E) Ionic interactions and hydrogen bonding stabilize the FMN in active site. The represented amino acid residues are well conserved in all nitroreductases (or similar residues).

Fig. 1.

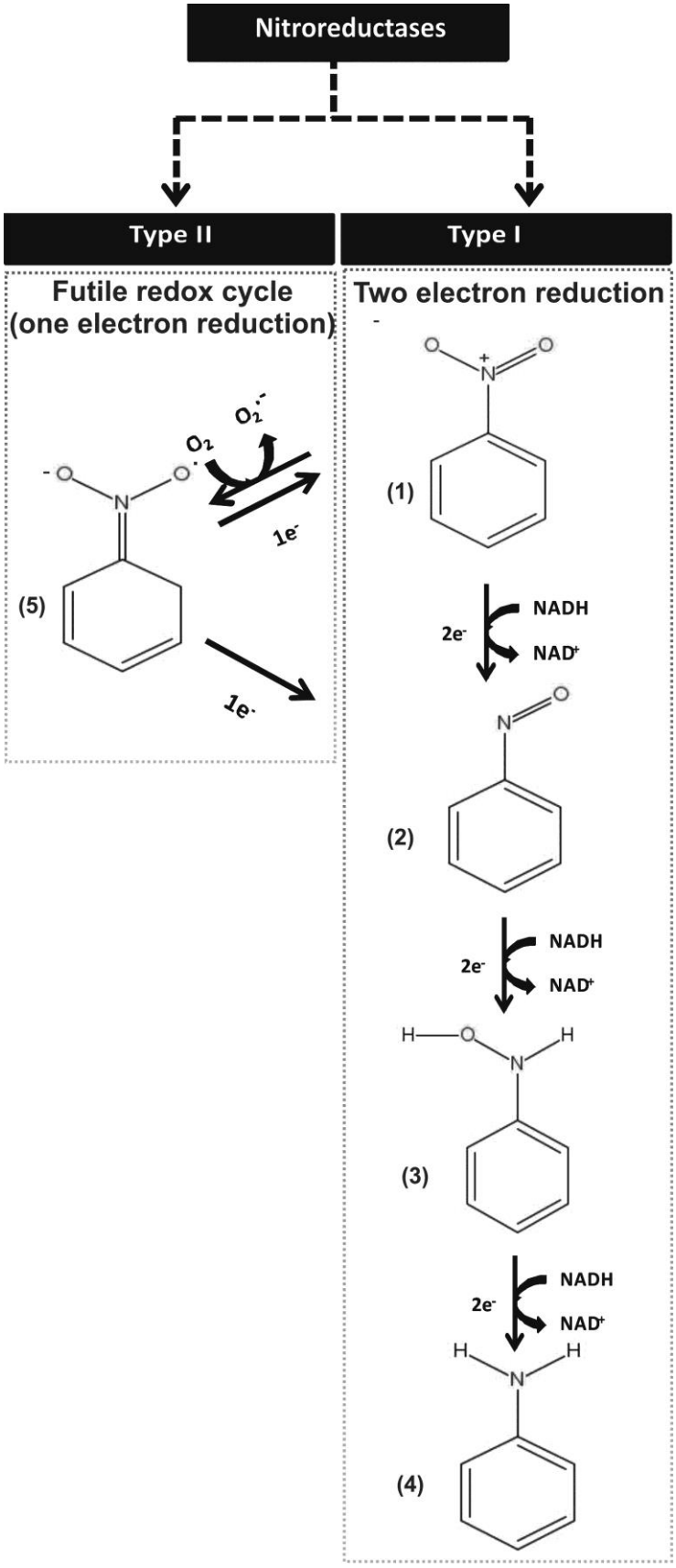

Nitroreductases
Type II
Type I
Futile redox cycle
(one electron reduction)
Two electron reduction
(1)
(2)
(3)
(4)
(5)
NADH
NAD+
2e-
1e-

Fig. 2.

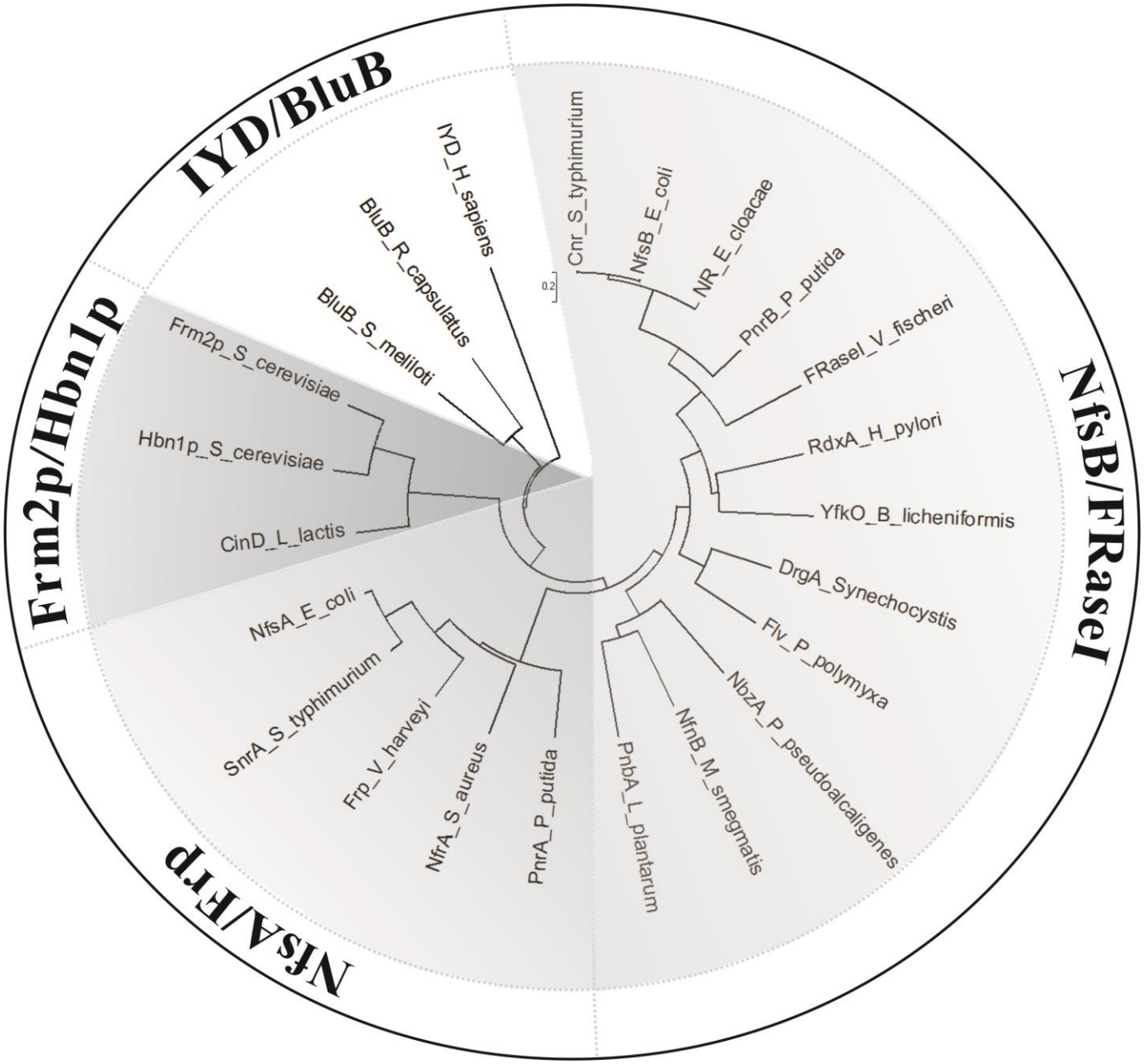
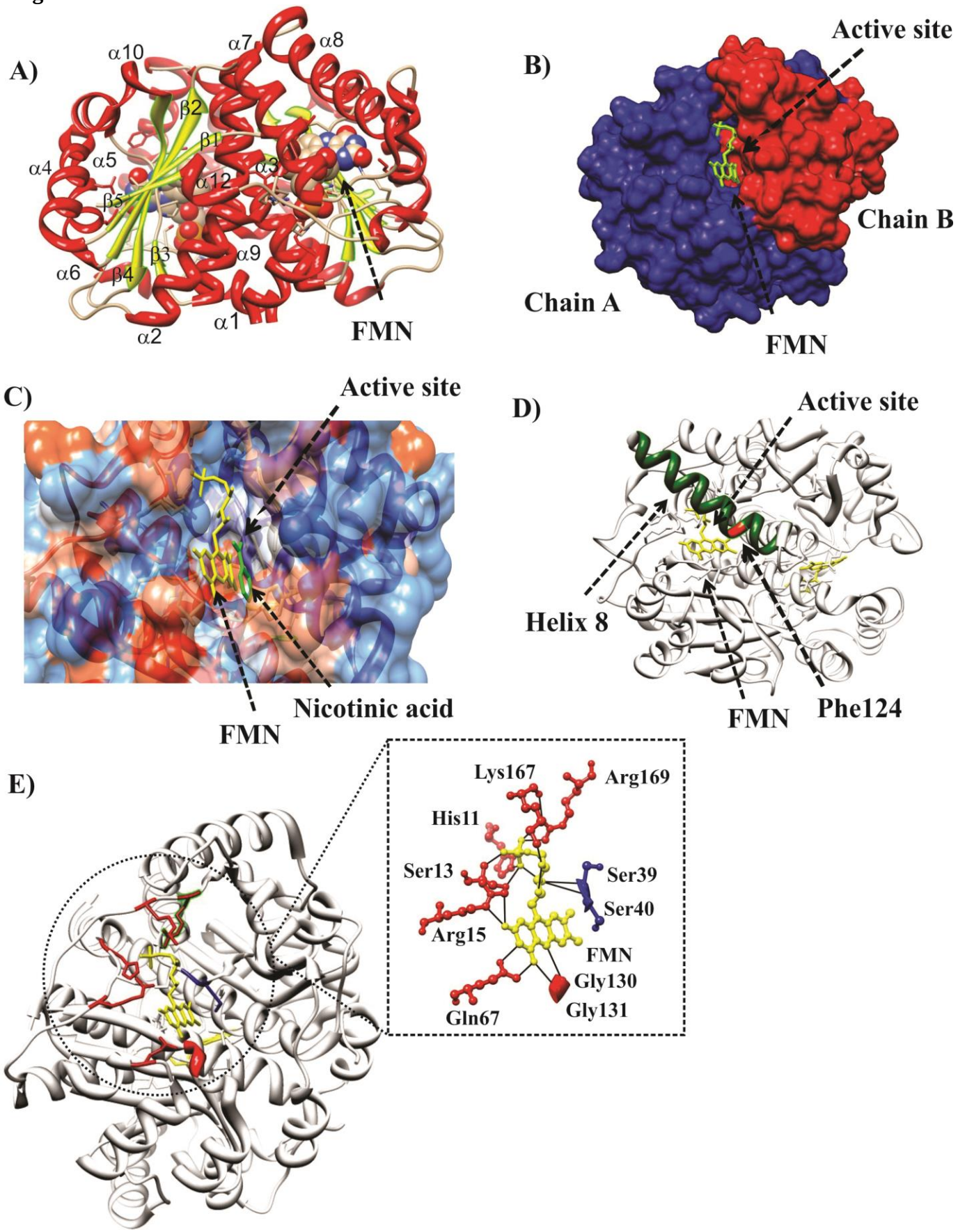

IYD/BluB
NfsB/FRaseI
NfsA/FRP
Frm2p/Hbn1p
IYD_H_sapiens
BluB_R_capsulatus
BluB_S_melliloti
Frm2p_S_cerevisiae
Hbn1p_S_cerevisiae
CinD_L_lactis
Cnr_S_typhimurium
NfsB_E_coli
NR_E_cloacae
PnrB_P_putida
FRaseI_V_fischeri
RdxA_H_pylori
YfkO_B_licheniformis
DrgA_Synechocystis
Flv_P_polymyxa
NbzA_P_pseudoalcaligenes
NfnB_M_smegmatis
PnbA_L_plantarum
PnrA_P_putida
NfrA_S_aureus
Frp_V_harveyi
SnrA_S_typhimurium
NfsA_E_coli
0.2

Fig. 3.


A)
α10
α7
α8
β2
β1
α4
α5
α3
α12
β5
β3
α9
α6
β4
α1
α2
FMN
B)
Active site
Chain B
Chain A
FMN
C)
Active site
FMN
Nicotinic acid
D)
Active site
Helix 8
FMN
Phe124
E)
Lys167
Arg169
His11
Ser13
Ser39
Ser40
Arg15
FMN
Gly130
Gln67
Gly131

Capítulo III

APRESENTAÇÃO DO CAPÍTULO III

O papel fisiológico das nitrorredutases de *Escherichia coli*: novas ideias pela análise da rede de interações de proteínas.

Neste trabalho foi analisada a rede de interações das nitrorredutases NfsA e NfsB de *E. coli*; utilizando ferramentas de biologia de sistemas, com o objetivo de gerar hipóteses a respeito das suas possíveis funções no metabolismo da bactéria. Os resultados obtidos permitiram a elaboração de modelos, sugerindo que as nitrorredutases de *E. coli* podem participar do metabolismo de ferro, manutenção do conteúdo de NADPH, metabolismo de compostos aromáticos e síntese de glicogênio. Estas vias podem contribuir nas respostas ao estresse oxidativo e a limitação de nutrientes.

Manuscrito a ser submetido.

Physiological role of *Escherichia coli* nitroreductases: New insights by protein interaction network analyses

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Short title: Physiological role of bacterial nitroreductases by protein interaction network analyses

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Abstract

Nitroreductases are flavoproteins that catalyze nicotinamide adenine dinucleotide (NAD(P)H) dependent reduction of nitroaromatic compounds. These enzymes are of special interest due to their significant human health, environmental implications, potential use in bioremediation and their activation of prodrugs in directed anticancer therapy. Due to its relevance, different bacterial nitroreductases have been purified, and their biochemical, kinetic parameters and structure have been determined. In this sense, two proteins, NfsA and NfsB, with oxygen-insensitive nitroreductase activity in *Escherichia coli* were identified. However, little is known about their physiological functions. Therefore, hypotheses have been proposed to solve the physiological role of nitroreductases, such as oxidative stress response, although the mechanism is still unknown. To better understand the possible biological roles of NfsA and NfsB nitroreductases in *E. coli* metabolism, a systems biology study was performed employing the construction of protein–protein interactions (PPI) and five subnetworks representing different biological processes were observed. The results obtained in this work allow us to draw some models suggesting that *E. coli* nitroreductases can participate in iron metabolism, NADPH pool maintenance, aromatic compound metabolism, methionine and glycogen synthesis. Through these pathways, possibly contribute to adaptation to stress oxidative response and also nutrients limitation.

Keywords: Nitroreductases; systems biology, physiological role; *Escherichia coli*.

Introduction

Nitroreductase are enzymes involved in the reduction of nitro-containing compounds utilizing flavin mononucleotide (FMN) as prosthetic groups and NAD(P)H as reducing agents [1]. They comprise a family of proteins with conserved sequences and structures that were discovered in eubacteria. Few data exist on the distribution of nitroreductase sequences in eukaryotic cells, but recently have been also described in protozoan, fungi and mammalian systems [2-3]. In this sense, two nitroreductases in *Escherichia coli*, NfsA and NfsB, were purified and enzymatic activities have been characterized [4-5]. In *E. coli*, nitroreductases are known to catalyze the reduction of organic nitroaromatic and nitroheterocyclic compounds such as nitrophenols, nitrobenzenes, and nitrobenzoates [6]. Therefore, these proteins have raised enormous interest because of their central role in mediating nitroaromatic toxicity, because their metabolization leads to the formation of potent genotoxic and/or mutagenic metabolites or generate reactive nitrogen oxide species (RNOxS), which readily react with biological macromolecules [4-6]. The nitroreductases are also biotechnologically attractive, showing potential applications for bioremediation, biocatalysis and chemotherapeutic tumor treatment [7-10]. In contrast, the physiological role of these enzymes remains unclear, although different tentative functions have been suggested, such as oxidative stress response, bioluminescence, cobalamin synthesis and metal homeostasis [11-13]. Therefore, the physiological context in which these enzymes may be playing is uncertain and unknown. Considering these facts, in the present work a construction and analysis of protein–protein interactions (PPI) network was performed with the aim of better understanding the possible biological roles of NfsA and NfsB nitroreductases in *E. coli* metabolism.

Material and methods

Protein–protein network design

To design PPI networks related to "classical" nitroreductases NfsA and NfsB of *E. coli*, the metasearch engine STRING 9.0 [http://string.embl.de/] was initially applied by using NfsA and NfsB proteins. The STRING 9.0 software allowed us to visualize the interaction among proteins related to *E. coli* nitroreductases. In this sense, the following parameters were used within STRING 9.0: active prediction methods all enabled except text mining; no more than 50 interactions; medium confidence score (0.400); and network depth equal to 2. In addition to STRING 9.0, iHop [http://www.ihop-net.org/UniPub/iHOP/] search engines were also employed using the default parameters. The results gathered from these search engines were subsequently analyzed using Cytoscape 2.8.3 [14]. To select the most relevant protein subnetworks from the interactome, the initial PPI network was analyzed using Molecular Complex Detection (MCODE) [15], which is a Cytoscape plugin freely available at http://baderlab.org/Software/MCODE. The parameters used in MCODE to generate the subnetworks were: loops included; degree cutoff 2; deletion of single connected nodes from cluster (haircut option enabled); expansion of cluster by one neighbor shell allowed (fluff option enabled); node density cutoff 0.1; node score cutoff 0.2; kcore 2; and maximum depth of network 100. A MCODE score was calculated for each protein/small compound present in the interactome networks.

Gene Ontology Analysis

Gene ontology (GO) clustering analysis was performed using Biological Network Gene Ontology (BiNGO) software, a Cytoscape plugin available at [http://chianti.ucsd.edu/cyto_web/plugins/index.php] [16]. The degree of functional enrichment for a given cluster and category was quantitatively assessed (*p* value) by hypergeometric distribution [17] and a multiple test correction was applied using the false discovery rate (FDR) [18] algorithm, fully implemented in BiNGO software. Overrepresented biological process categories were generated after FDR correction, with a significance level of 0.05.

Results and Discussion

To gain insights and new perspectives into possible metabolic pathways in which bacterial nitroreductases may be acting, we performed unbiased searches for proteins interacting with the "classical" *E. coli* nitroreductases NfsA and NfsB by means of systems biology analyzing PPI network, an increasingly emerging method to allow hypothesis formulation [19]. For this purpose, a binary PPI network containing 213 bacterial proteins (nodes) and 1,904 interactions (edges) was employed. These nodes are distributed among five subnetworks as identified by the MCODE program (Figure. 1; Table 1).The subnetworks represent some discrete biological processes, as identified by gene ontology (GO) analysis (Figure. 1; Table 1): (i) enterobactin metabolic process/ribonucleoside-diphosphate reductase activity/iron-sulfur cluster assembly; (ii) methionine biosynthetic process; (iii) glycogen metabolic process/aromatic compound catabolic process; (iv) NAD(P)H transhydrogenase activity and (v) riboflavin biosynthetic process.

Considering these results, the subnetworks were subjected to analysis to gather information about the *E. coli* nitroreductases and their biologically associated processes.

E. coli nitroreductases in enterobactin metabolic process, ribonucleoside reductase activity and iron-sulfur cluster assembly

Some interesting aspects of the PPI network were generated in this work. The enterobactin metabolic process/ribonucleoside-diphosphate reductase activity/iron-sulfur cluster assembly subnetwork was gathered from the major PPI networks (Figure. 2A). An efficient mechanism of iron acquisition from the environment by bacteria under iron-limiting conditions involves the synthesis and secretion of the siderophore such as enterobactin (a cyclic trimer of 2,3-dihydroxybenzoyl serine) [20]. This low-molecular-weight and high-affinity chelators sequesters ferric iron of extracellular medium and facilitates its transport to the cytoplasm of the cell [21]. The subnetwork 1 shows proteins involved in enterobactin siderophore system (Figure 2A), which comprises specific steps required for ferric iron uptake in *E. coli*: (1) enterobactin synthesis (EntC, EntA, EntD, EntE, EntF, and EntB proteins); (2) export of enterobactin (EntS and TolC); (3) reception, internalization of the Fe^{+3}-enterobactin (FepA and TonB-ExbDB complex) and (4) iron release in the cytoplasm (FeS and YqjH proteins) [20, 22-23]. The mechanisms to iron release in the cell comprises Fe^{+3}-enterobactin hydrolysis, leading to loss of complex stability and hence facilitating the removal of the iron as Fe^{+2} as performed by FeS esterase [24]. However, the subsequent steps of hydrolysis of Fe^{+3}-enterobactin are not fully understood. In this sense, YqjH protein is capable of catalyzing reductive iron release in a step that directly follows the hydrolysis of Fe^{+3}-enterobactin [25]. YqjH is similar to members of the NAD(P)H:flavin oxidoreductase

family and could reduce ferric complexes in the presence of NADPH as electron donor [25]. Interestingly, some members of this family have similarity with nitroreductases [5, 25]. In view of these facts, the model of Figure 2B considers that *E. coli* nitroreductases play a role similar to YqjH in reduction of Fe^{+3}-enterobactin hydrolyzed and further release of Fe^{+2}. Consistent with our hypothesis, Takeda et al. [26] showed that DrgA *Synechocystis* and *E. coli* NfsB nitroreductases functioning also as ferric reductase in the presence of synthetic iron chelators, such as EDTA and natural chelators such as citric acid. These observations indicate that DrgA and NfsB proteins might function in iron metabolism [12, 26].

In subnetwork 1 can also check for the presence of ribonucleotide reductases (RNRs) (NrdH, NrdF, NrdE, NrdI and NrdR proteins) (Figure 2A). RNRs providing the *de novo* pathway for the biosynthesis of deoxyribonucleotides, the immediate precursors of DNA synthesis and repair [27]. *E. coli* contain genes encoding two class I RNRs (Ia and Ib) and a class III RNR, which is active only under anaerobic conditions [28]. The class Ia RNR, composed of NrdA and NrdB, is expressed under normal aerobic growth conditions. The class Ib RNR, composed of NrdE and NrdF, is expressed under oxidative stress and iron-limited growth conditions [29]. Interestingly, *E. coli* nitroreductases have direct interaction with NrdI and NrdR proteins (Figure 2A). NrdI is a flavodoxin, functioning as a two-electron reductant to the metal bonded in NrdF, using FMN as electron donor [30]. Thus, NrdI can play a role in NrdEF cluster assembly and in the maintenance pathway in *E. coli* class Ib RNRs to reactivate these enzymes when the metal bounded becomes reduced, whether by cellular regulatory mechanisms (e.g., cell cycle) or external environmental stresses (e.g., a host's immune response) [30-31]. Interestingly, was suggested that NrdI need of reductases to help in the reduction of FMN to FMNH [30]. In this sense, flavin reductase activity in bacterial nitroreductases

was mainly characterized to FRaseI and Frp nitroreductases of *Vibrio. fischeri* and *Vibrio harveyi*, respectively [32-33]. NfsA and NfsB have lower flavin reductase activity that FRaseI and Frp enzymes [32, 34], but it is possible that *E. coli* nitroreductases assist NdrI in the reduction of FMN (Figure 2B). NrdR is a repressor of *nrdHIEF* operon [35]. In this case, we hypothesized that *E. coli* nitroreductases may be acting as a kind of sensor to NrdR about the intracellular iron content and/or redox status. Thus, NrdR is inactivated in reduced intracellular concentration of iron, inducing *nrdHIEF* transcription (Figure 2B). Interestingly, Torrents et al. [35] suggests that NrdR may respond to a nucleotide or metal ion, likely iron. It also draws attention to the analysis of this subnetwork 1 the presence of proteins of SUF system (SufA, SufB, SufC, SufD, SufS, and SufE) (Figure 2A and 2B), which make the assembly of iron-sulfur (Fe-S) clusters and maturation of Fe-S proteins in iron starvation and oxidative stress [36-37].

E. coli nitroreductases in methionine biosynthetic process

Another interesting aspect of the subnetwork 2 is the presence of several enzymes involved in methionine biosynthesis (MetA, MetB, MetC/MalY, LuxS, MetK, MetH and MetE) in subnetwork 2 (Figure 3A). Methionine is an essential precursor for the synthesis of GSH and is required for the metabolic pathways such as gene expression and protein synthesis [38]. The methionine biosynthesis pathway is shown in Figure 3B. The final step of methionine biosynthesis is catalyzed by either cobalamin (B_{12}) - independent methionine synthase (MetE) or (B_{12})-dependent methionine synthase (MetH). In this sense, the BluB, a NADH/ FMN-dependent enzyme, is a member of nitroreductase family found in *Selenomonas ruminantium*, *Sinorhizobium*

meliloti and *Rhodobacter capsulatus* [11]. It has been demonstrated that BluB triggers an unusual fragmentation of the FMN cofactor to form the 5,6-dimethylbenzimidazole (DMB) ligand of B_{12} [40]. However, until now there is no evidence that nitroreductases NfsA and NfsB can fragment FMN and consequently participates in the synthesis of cobalamin. However, a direct association of *E. coli* nitroreductases with LuxS was revealed in this PPI network (Figure 3A). LuxS protein is an autoinducer 2 (AI-2) synthase and many bacteria, LuxS functions as a quorum-sensing molecule [41]. However, it also has a second, more central metabolic function in the activated methyl cycle in bacterial cells (Figure 3B) [41]. Methyl groups donated by this cycle are critical for the activity of methyltransferases that are associated with bacterial DNA methylation, chemotaxis, motility, and a variety of other metabolic and biosynthetic reactions [41-42]. The role of LuxS in the methyl cycle is to catalyze cleavage of *S*-ribosylhomocysteine to 4,5-dihydroxy-2,3-pentanedione (DPD, a precursor of the AI-2) and homocysteine, a precursor of methionine biosynthesis (Figure 3B) [42-43]. In this sense, was shown that deletion of *luxS* gene affects genes associated with methionine biosynthesis [43], such as *metE* that has much lower expression in the Δ*luxS* mutant than in the wild-type strain, possibly due fact that homocysteine is required for the full induction of *metE* expression [44]. On the other hand, homocysteine was shown to play an inhibitory role in the expression of *metA*, which catalyzes the conversion of o-acetylhomoserine to homoserine [45]. In agreement with this, *metA* expression is higher in the Δ*luxS* mutant and the expression level of *metB* gene was also increased by *luxS* deletion [46]. Thus, in our proposed model, the interaction of *E. coli* nitroreductases with LuxS could contribute in the conversion of s-ribosylhomocysteine to homocysteine (Figure 3B). In addition, LuxS participates in the luminescence system (lux system) in *V. fischery* [47]. Interestingly, FRaseI nitroreductase of *V. fischery* have been involved

in the bioluminescent process, catalyzing the reduction of FMN by NAD(P)H to provide the reduced form of the flavin required for the luciferase reaction [47]. Consistently, NfrA1 nitroreductase in *Bacillus subtilis* have direct interaction with proteins of lux system [48].

E. coli glycogen metabolic process and aromatic compound catabolic process

The PPI network shows direct interaction of *E. coli* nitroreductases with GlgB, connecting to subnetwork 3, which include proteins involved in glycogen metabolism (Glk, PgmA, GlgC, MalY, GlgA, GlgX, GlgP and GlgB) (Figure 4A). The biosynthesis of glycogen or starch is one of the main strategies developed by living organisms for the intracellular storage of carbon and energy [49] and the pathway of synthesis is showed in Figure 4B. The precise role that glycogen may play in bacteria is still not clear; however, the accumulation of glycogen is increased by nutritional stress, such as iron, magnesium and amino acid limitation, factors determining intercellular communication, aggregative and social behavior modes (which in turn determine the nutritional status of the cell) [50-51]. In addition, the study conducted by Yamamotoya et al. [52] indicated the importance of glycogen participation in the lag phase for the growth of *E. coli*. The model developed in this study considers that *E. coli* nitroreductases possibly participate in the glycogen formation by the interaction with the enzyme GlgB as shown in Figure 4B.

The subnetwork 3 also show direct interaction of *E. coli* nitroreductases with MhpD protein, connecting to a set of enzymes encoded by the *mhp* cluster (MhpA, MhpB and MhpCDFE complex) (Figure 4A) [53]. These enzymes participate in *meta*-fission pathway that degrades 3-hydroxyphenylpropionic acid (3HPP) [54]. It is likely that aromatic compounds can also be a frequent carbon source for *E. coli* different

environmental, such as soil, water or animal gut [53]. In this sense, phenylpropanoic and phenylpropenoic acids and their hydroxylated derivatives are widely distributed in the environment, arising from digestion of aromatic amino acids or as breakdown products of lignin and other plant-derived phenylpropanoids and flavonoids [55]. The bacterial catabolism of these aromatic compounds plays a key role in recycling of such carbon sources in the ecosystem [53]. The 3HPP catabolism in *E. coli* is show in Figure 4C, that considers the possible involvement of *E. coli* nitroreductases in this degradation pathway.

E. coli nitroreductases in NAD(P)$^+$ transhydrogenase and riboflavin biosynthetic process.

E. coli nitroreductases exhibit direct interaction with PtnA and PtnB enzymes, in subnetwork 4, which participate in the production of NAD(P)H by reduction of NAD(P)$^+$ and proteins involved in the biosynthesis of FMN, such as Rib complex in cluster 5 (Figure 5A). *E. coli* and a variety of other bacteria, possess a membrane-bound nicotinamide nucleotide transhydrogenase composed of two subunits, α and β, encoded by the *pntA* and *pntB* genes, respectively [56]. The PtnAB enzyme use the electrochemical proton gradient as driving force for the reduction of NADP$^+$ to NADPH by oxidation of NADH to NAD$^+$. The physiological function of these transhydrogenases is to provide NADPH for reactions in the cell [57]. The *ribADCH* operon encodes the Rib complex that is enzymatic complex involved in FMN biosynthesis [58]. These interactions are expected once nitroreductases have FMN as prosthetic group and NAD(P)H as cofactor [1]. Therefore, PtnAB may possibly provide NAD(P)H cofactor for *E. coli* nitroreductases and FMN by Rib proteins (Figure 5B).

E. coli nitroreductases in oxidative stress response and environmental adaptation

The largest number of evidences regarding the physiological role of nitroreductases is about their possible involvement in the oxidative stress response [6, 10, 13, 59]. In this sense, SnrA in *S. typhimurium* and NprA in *R. capsulatos* are induced by paraquat (a superoxide generator) [60-61], and NfrA nitroreductase of *Staphylococcus aureus* help to maintain the cell thiol–disulphide balance and exhibits S-nitrosoglutathione (GSNO) reductase, which protect the cell against transnitrosylation [62-63]. The *B. subtilis nfrA* gene has their expression increased with exposure to paraquat and H_2O_2. In addition, NfrA1 nitroreductase also can scavenge H_2O_2 [64]. CinD is a nitroreductase that can protect *L. lactis* against oxidative stress exerted by nitroaromatic compounds and copper [65-66]. Interestingly, CinD also exhibited significant catalase activity *in vitro* [66]. In addition, the Frm1p and Hbn1p nitroreductases of *Saccharomyces cerevisiae* influence the response to oxidative stress by modulating the GSH contents and antioxidant enzymatic activities, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [59]. However, the pathways by which nitroreductases act in oxidative stress response are still unknown. In this sense, based on the analysis of PPI network, we propose possible ways in which *E. coli* nitroreductases may be operating in response to oxidative stress.

E. coli nfsA gene is part of the SoxRS regulon and is strongly induced by paraquat [13]. Therefore, our model (Figure 6) considers that during oxidative stress, the *soxRS* operon is induced. The SoxR protein is a dimeric sensor with two [2Fe–2S] centers that detects oxidative stress and induces expression of *soxS* gene, which, in turn, induces the members of this regulon [67]. The *soxRS* operon is also influenced by the

levels of NADPH [68]. In this sense, NADPH is the reductant or essential cofactor for several enzymes involved in protective and/or antioxidant activities, including glutathione reductase, thioredoxin reductase, NADPH-dependent alkylhydroperoxidase and nitroreductases [69]. Consistently, exposure of *E. coli* to H_2O_2 induced the activity of enzymes that synthesize NADPH, such as NADPH/NAD^+ transhydrogenases [56]. However, the increased levels of NADPH may have unwanted consequences. Accumulation of the reduced nucleotide is expected to downregulate the soxRS system, thus slowing down or even switching off the entire response [68]. In addition, NADPH may favor the propagation of deadly hydroxyl radicals through the Fenton reaction by redox-cycling the free iron [70]. This process is important for the regulation of NADPH pool. In this sense, some proteins such as Fpr have been suggested to oxidize NADPH to $NADP^+$, thus decreasing NADPH pool, amplifying the *soxRS* induction and also reducing the reaction of NADPH with metals [68]. It was considered in the model, that *E. coli* nitroreductases may play a function similar to Fpr protein, in regulation of NADPH pool, especially in regulating levels of NADPH produced by PtnAB transhydrogenases (Figure 6).

Interestingly, *soxRS* induces inhibition of enterobactin [71], possibly because during oxidative stress is important to reduce intracellular iron levels to prevent the Fenton reaction and the hydroxyl radical generation [72]. This reduction in intracellular iron level by *E. coli* nitroreductases may induce inactivation of NrdR leading to expression of *nrdIEF* operon, producing RNRs Mn – dependent and decreasing the iron use [73]. Thus, the iron can be directed to assembly Fe-S clusters of proteins important in metabolism, oxidative stress response and DNA repair, possibly realized with the contribution of the SUF system, which has increased expression in these conditions [37].

Another aspect to be considered is the interaction with LuxS, which was shown to have involvement in cellular oxidative stress response [74-75]. In this sense, mutation in *luxS* downregulates *ahpC* (encoding alkyl hydroperoxide reductase) and *tpx* (encoding thiol peroxidase) genes in the presence of H_2O_2 in *Campylobacter jejuni*. Other genes responsible for synthesis of some stress response proteins such as the heat shock chaperon DnaK, PerR (peroxide stress regulator), and KatA (catalase) had reduced expression in Δ*luxS* [46].

There are also an association between stress conditions and methionine limitation in *E. coli*. Studies found that strains lacking the genes coding for the SOD had requirement for methionine [76]. Sob oxidative stress, MetE is inactivated by oxidation, resulting in cellular methionine limitation, in these circumstances the expression of *metE* is increased, possibly with the contribution of LuxS by the production of homocysteine [44, 46]. Interestingly, homocysteine is nitrosated by reactive nitrogen species (RNS), thus depleting the pathway leading to methionine biosynthesis, then is expected compensatory increase in homocysteine production by MetA and MetB and active methyl cycle with LuxS participation [77]. In view of these facts, it is proposed that under oxidative stress, *E. coli* nitroreductases can contribute in maintaining the levels of homocysteine, methionine and also induced the set of genes of response to oxidative stress controlled by LuxS (Figure 6).

The model also considers that *E. coli* nitroreductases can contribute by interacting with GlgB in the accumulation of glycogen. Grundel et al. [78] suggest a role for glycogen in the tolerance to oxidative stress in *Synechococcus elongatus* cyanobacterium. The strains with deficiency of glycogen synthesis and accumulation were incapable of adapting to oxidative stress and consequently showed growth inhibition [78]. However, during oxidative stress glycogen synthesis is reduced, because

it increases the glucose consumption by the energy metabolism [79]. It is possible that *E. coli* nitroreductases and GlgB interaction controls the glycogen synthesis depending of redox balance of the cell. Interestingly, GlgB of *Mycobacterium tuberculosis* has interaction with WhiB1 protein, a thioredoxin like protein [80].

In addition, another possible participation of *E. coli* nitroreductases is adapting to environments with limited nutrients such as iron, contributing in this metal uptake. Under limitation of nutrients such as amino acids and metals, but glucose availability, an adaptive response is an increase in glycogen synthesis [50]. It can also be considered a possible involvement of *E. coli* nitroreductases in the use of alternative sources of carbon, such as 3HPP by interaction which MhpD protein. Interestingly, it is likely that aromatic compounds can also be a frequent carbon source for *E. coli* in the soil, water and animal gut [53].

Final considerations

Nitroreductases are enzymes involved in degradation of nitrocompounds. However, little is known about their physiological functions. In this sense, different tentative physiological roles have been suggested for these enzymes, such as B_{12} biosynthesis and bioluminescence. The most studies indicate the involvement of nitroreductases in the oxidative stress response, being that the mechanisms are not clear yet. In this view, systems biology was used in this work to generate new hypotheses and insights about possible metabolic pathways which these enzymes may participate. This work suggests that *E. coli* nitroreductases can participate in iron metabolism, methionine and glycogen synthesis, utilization of alternative carbon sources and control of the NADPH pool. These pathways are possibly important also in adverse conditions

such as changes in the redox status of the cell, suggesting mechanisms by which these enzymes may act in oxidative stress response and in nutrient limitation adaptation. Experimental data are currently being obtained to confirm the systems biology data of this article.

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Table 1. Specific gene ontology (GO) classes derived from *E. coli* nitroreductases protein-protein interactions

GO biological process category	GO number	*p*-Value[a]	Corrected *p*-Value[b]	*k*[c]	*f*[d]
enterobactin metabolic process	9238	1.63×10^{-12}	2.62×10^{-10}	14	19
ribonucleoside-diphosphate reductase activity	4748	1.96×10^{-4}	2.43×10^{-3}	5	9
iron-sulfur cluster assembly	16226	7.67×10^{-5}	1.40×10^{-3}	6	18
methionine biosynthetic process	9086	2.61×10^{-12}	2.45×10^{-10}	12	20
aromatic compound catabolic process	19439	7.51×10^{-9}	2.71×10^{-6}	6	24
glycogen metabolic process	5977	2.72×10^{-8}	3.28×10^{-6}	8	10
NAD(P)H transhydrogenase activity	8746	1.76×10^{-7}	3.65×10^{-5}	2	2
riboflavin biosynthetic process	9231	1.18×10^{-4}	2.71×10^{-3}	5	8

a: *p*-Values calculated by hypergeometric distribution of a given gene ontology cluster observed in the network.
b: Values calculated from *p*-value after FDR application.
c: Total number of proteins found in the network that belong to a specific gene ontology.
d: Total number of proteins belonging to a specific gene ontology.

Figure legends

Fig. 1. A protein–protein interaction network of NfsA/NfsB nitroreductases.

Fig. 2. Panel (A): An amplified view of proteins that compose subnetwork 1 from the PPI network, associated with enterobactin metabolic process, ribonucleoside-diphosphate reductase activity and iron-sulfur cluster assembly. *E. coli* nitroreductases is represented by a dark square. NrdR and NrdI proteins directly connected to NfsA/NfsB. Panel (B): System contributing to siderophore-mediated iron uptake in *E. coli.* The model considers that *E. coli* nitroreductases or YqjH can act to reduce Fe^{+3}-enterobactin hydrolyzed and removal as Fe^{+2}. The iron concentration can regulate the NrdR inhibitory activity in *nrdHIEF* operon. NfaA/NfsB nitroreductases also may be acting in RNRs maintenance pathway, possibly by help reduce FMN of NrdI, which is utilized to metal group of NrdEF, recovering in this way the RNRs enzymes.

Fig. 3. Panel (A): An amplified view of proteins that compose subnetwork 2 from the PPI network, associated with methionine metabolic process. *E. coli* nitroreductases is represented by a dark square. NfsA/NfsB directly connected with LuxS protein. Panel (B): Pathway of methionine biosynthesis, emphasizing the interaction of *E. coli* nitroreductases with LuxS, possibly contributing in the formation of homocysteine and consequently in methionine synthesis. *Abbreviations*: DPD, 4,5-dihydroxy-2,3-pentanedione.

Fig. 4. Panel (A): An amplified view of proteins that compose subnetwork 3 from the PPI network, associated with glycogen metabolic process and aromatic compound catabolic process. *E. coli* nitroreductases is represented by a dark square. NfsA/NfsB directly connected with GlgB and MhpD proteins. Panel (B): Pathway of glycogen biosynthesis, emphasizing the interaction of *E. coli* nitroreductases with GlgB, possibly

contributing in the formation of glycogen. Panel (C): Pathway for the catabolism of 3HPP in *E. coli*, emphasizing the interaction of *E. coli* nitroreductases with MhpD, possibly contributing in the use of 3HPP as alternative carbon source. *Abbreviations*: DHPP, 2,3-dihydroxyphenylpropionate; HKNDA, 2-hydroxy-6-keto-nona-2,4-diene 1,9 dioic acid; HPDA, 2-hydroxy-penta-2,4-dienoic acid; HKP, 4-hydroxy-2-ketopentanoic acid.

Fig. 5. Panel (A): An amplified view of proteins that compose subnetworks 4 and 5 from the PPI network, associated with $NAD(P)^+$ transhydrogenase activity and riboflavin biosynthetic process. *E. coli* nitroreductases is represented by a dark square. NfsA/NfsB directly connected with PtnA and PtnB proteins. Panel (B): The model considers that PtnAB proteins and Rib complex can provide NADPH and FMN, respectively to *E. coli* nitroreductases.

Fig. 6. Schematic model representing possible pathways of metabolism that *E. coli* nitroreductases may be participating and contributing in oxidative stress response. The *soxRS* operon is induced by ROS and increased expression of some genes, including *E. coli* nitroreductases, that can interact with PtnAB proteins and to oxidize NADPH to $NADP^+$, do not allowing increasing of NADPH pool and maintaining the *soxRS* expression. *E. coli* nitroreductases possibly contribute in iron uptake by iron reduction of bonded Fe^{+3} in ferric enterobactin hydrolyzed to Fe^{+2} form, but during oxidative stress, iron uptake is decrease due to enterobactin synthesis inhibition by *soxRS*. It is possible that *E. coli* nitroreductases signalize to NrdR the decreased iron levels, allowing NrdR inhibition and RNRs Mn – dependent expression. The possible interaction of *E. coli* nitroreductases with NrdI may contribute to maintenance pathway of RNRs due to reduction of FMN used by NrdI in metal group reduction of RNRs. The interaction of NfsA/NfsB with LuxS can influence the increased production of

homocysteine and methionine, consequently. It is also possible that this interaction contributes in expression of a set of stress response proteins. Finally the interaction with GlgB protein can inhibit glycogen synthesis under oxidative stress.

Fig. 1

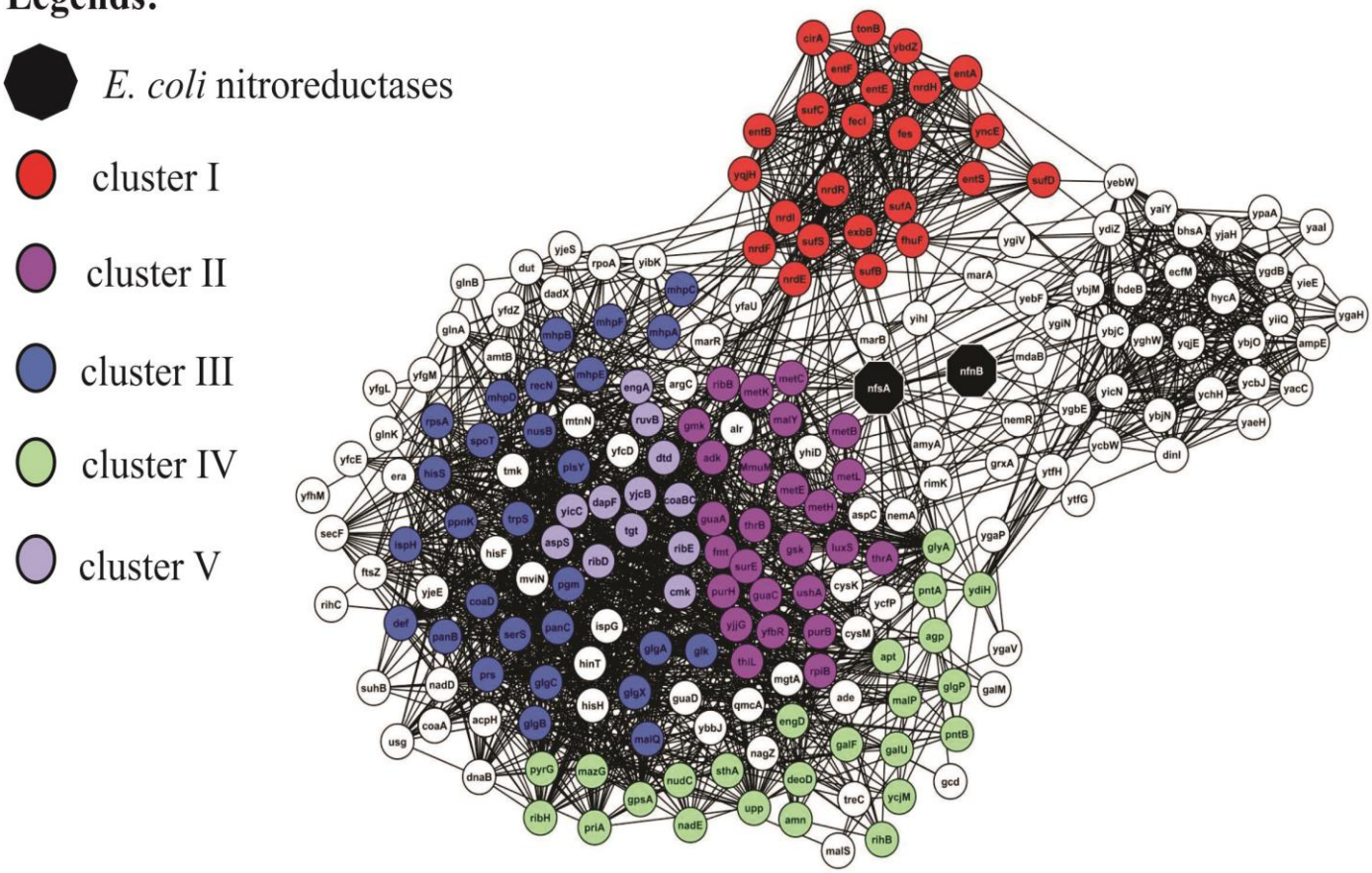

Legends:
E. coli nitroreductases
cluster I
cluster II
cluster III
cluster IV
cluster V
nfsA
nfnB

Fig. 2.

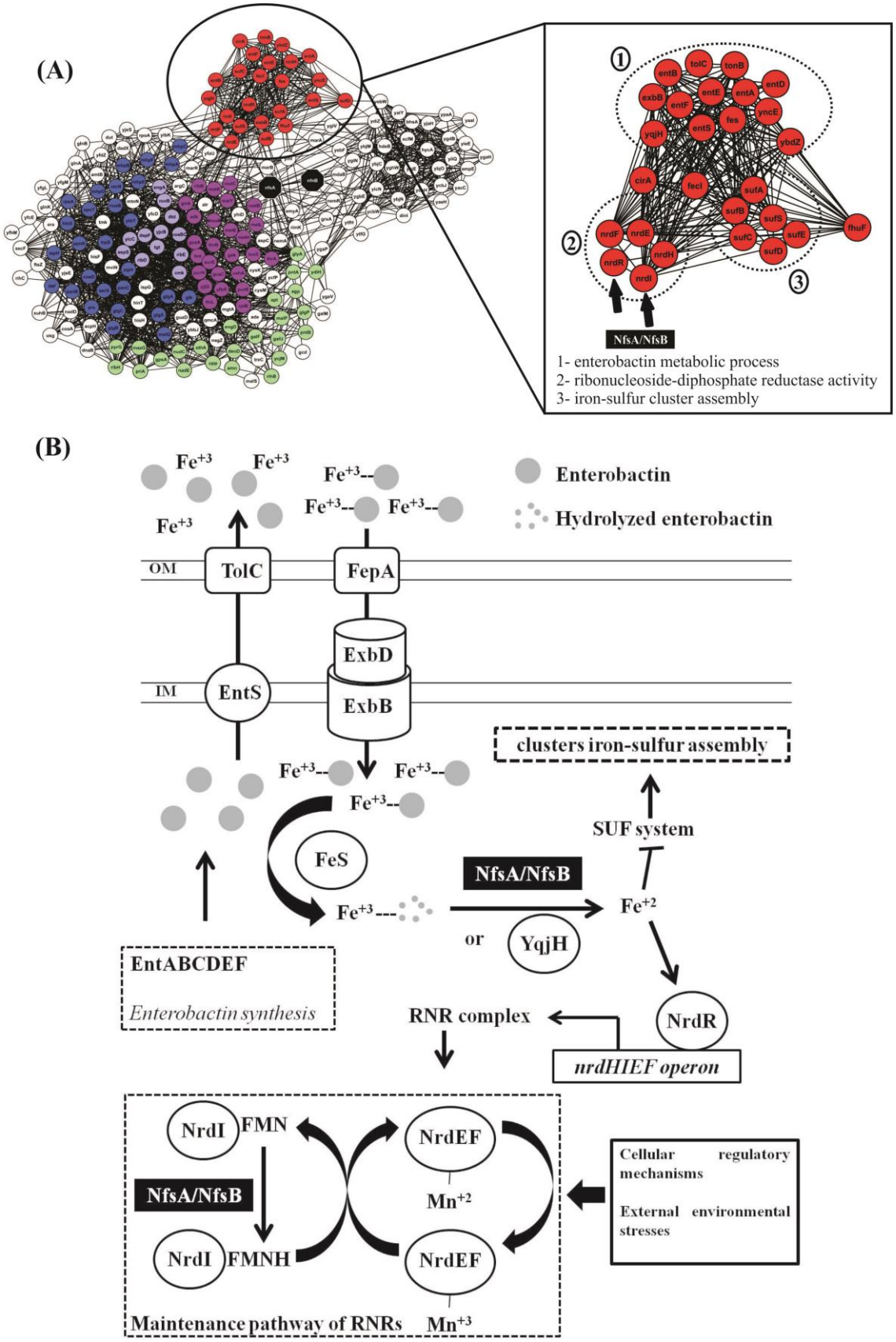

(A)
1- enterobactin metabolic process
2- ribonucleoside-diphosphate reductase activity
3- iron-sulfur cluster assembly
NfsA/NfsB
(B)
Enterobactin
Hydrolyzed enterobactin
OM
IM
TolC
FepA
ExbD
ExbB
EntS
clusters iron-sulfur assembly
SUF system
FeS
NfsA/NfsB
or
YqjH
EntABCDEF
Enterobactin synthesis
RNR complex
NrdR
nrdHIEF operon
NrdI
FMN
FMNH
NrdEF
Mn+2
Mn+3
Cellular regulatory mechanisms
External environmental stresses
Maintenance pathway of RNRs

Fig. 3.

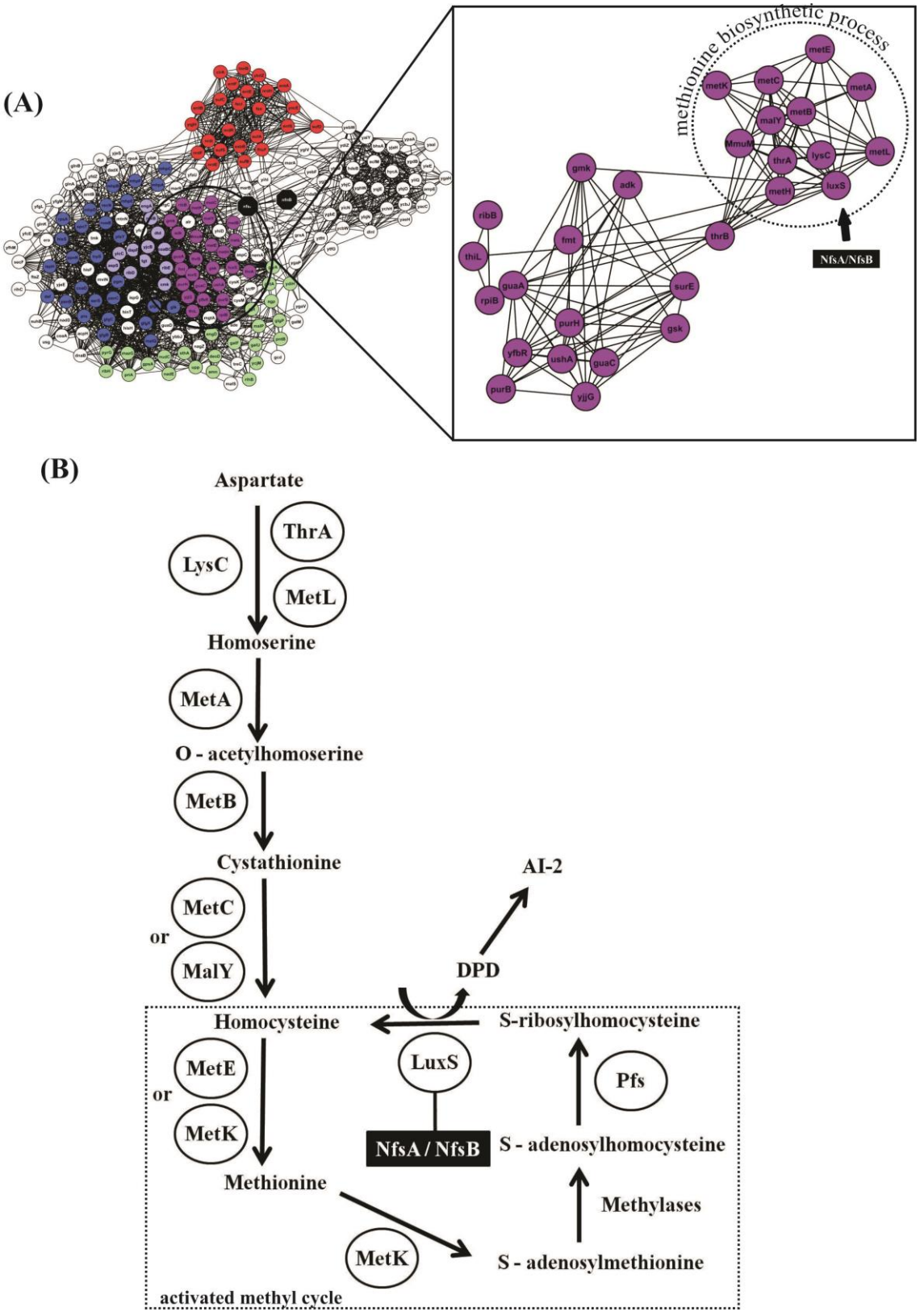

(A)
methionine biosynthetic process
metE
metK
metC
metA
malY
metB
MmuM
thrA
lysC
metL
metH
luxS
NfsA/NfsB
gmk
adk
ribB
fmt
thrB
thiL
guaA
surE
rpiB
purH
gsk
yfbR
ushA
guaC
purB
yjjG
(B)
Aspartate
ThrA
LysC
MetL
Homoserine
MetA
O - acetylhomoserine
MetB
Cystathionine
MetC
or
MalY
AI-2
DPD
Homocysteine
S-ribosylhomocysteine
MetE
or
MetK
LuxS
Pfs
NfsA / NfsB
S - adenosylhomocysteine
Methionine
Methylases
MetK
S - adenosylmethionine
activated methyl cycle

Fig. 4.

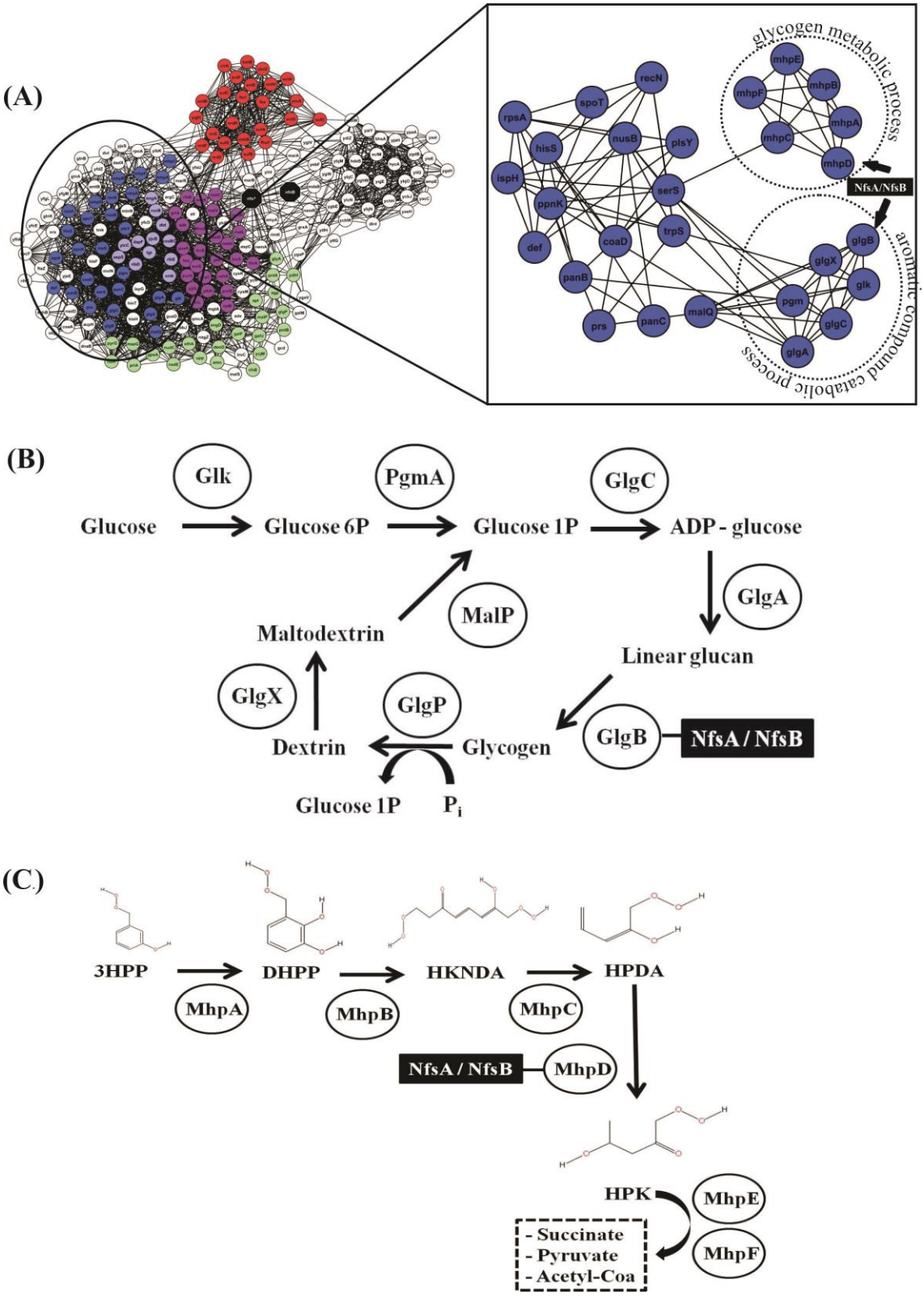
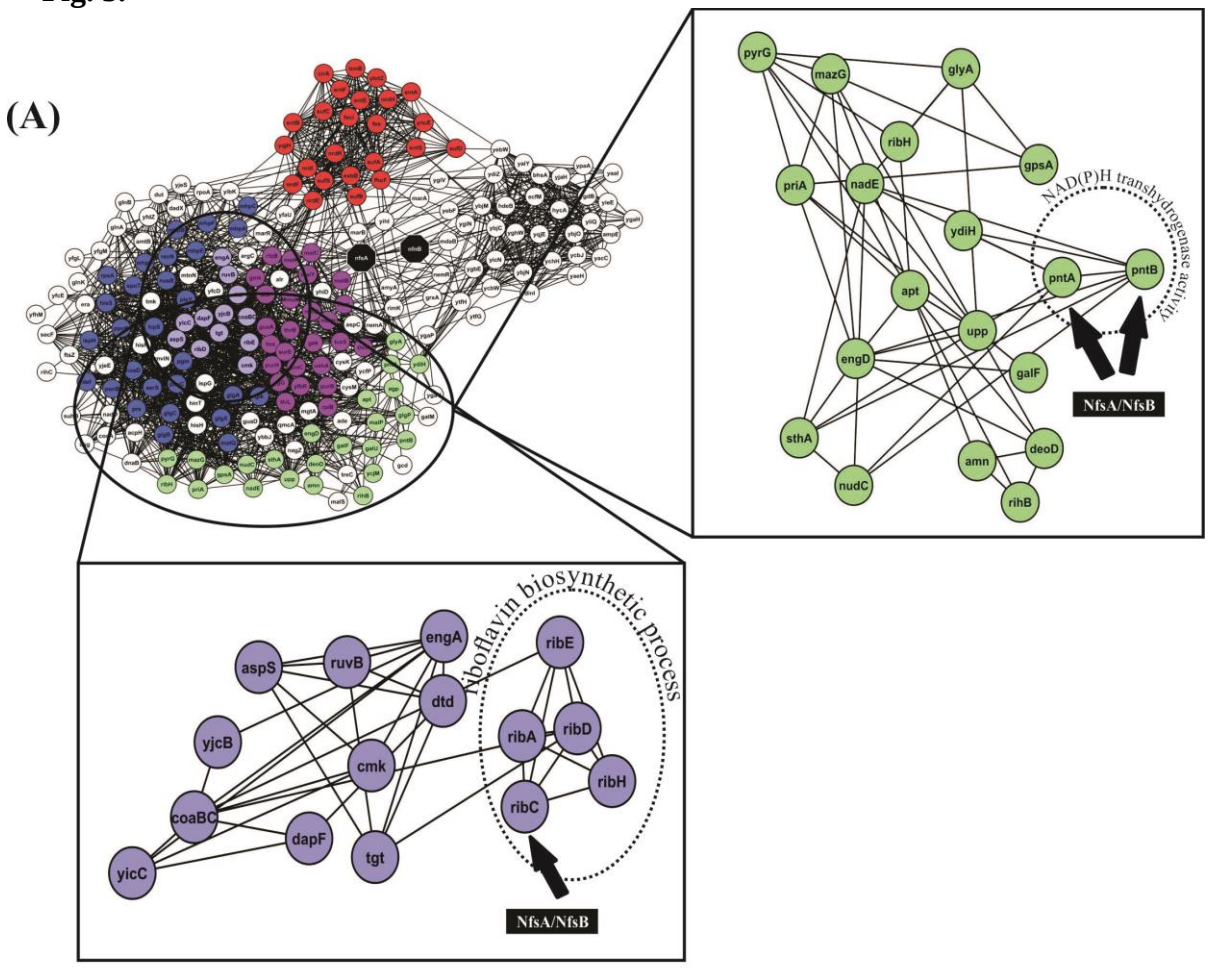

(A)
glycogen metabolic process
aromatic compound catabolic process
NfsA/NfsB
recN
spoT
rpsA
nusB
plsY
hisS
ispH
serS
ppnK
trpS
coaD
def
panB
panC
malQ
prs
mhpE
mhpB
mhpF
mhpA
mhpC
mhpD
glgB
glgX
glk
pgm
glgC
glgA
(B)
Glk
PgmA
GlgC
Glucose
Glucose 6P
Glucose 1P
ADP - glucose
GlgA
MalP
Maltodextrin
Linear glucan
GlgX
GlgP
GlgB
NfsA / NfsB
Dextrin
Glycogen
Glucose 1P
Pi
(C)
3HPP
DHPP
HKNDA
HPDA
MhpA
MhpB
MhpC
NfsA / NfsB
MhpD
HPK
MhpE
MhpF
- Succinate
- Pyruvate
- Acetyl-Coa

Fig. 5.


(A)
pyrG
mazG
glyA
ribH
gpsA
priA
nadE
ydiH
NAD(P)H transhydrogenase activity
pntA
pntB
apt
upp
engD
galF
NfsA/NfsB
sthA
amn
deoD
nudC
rihB
riboflavin biosynthetic process
engA
ribE
aspS
ruvB
dtd
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ribD
yjcB
cmk
ribH
ribC
coaBC
dapF
tgt
yicC
NfsA/NfsB

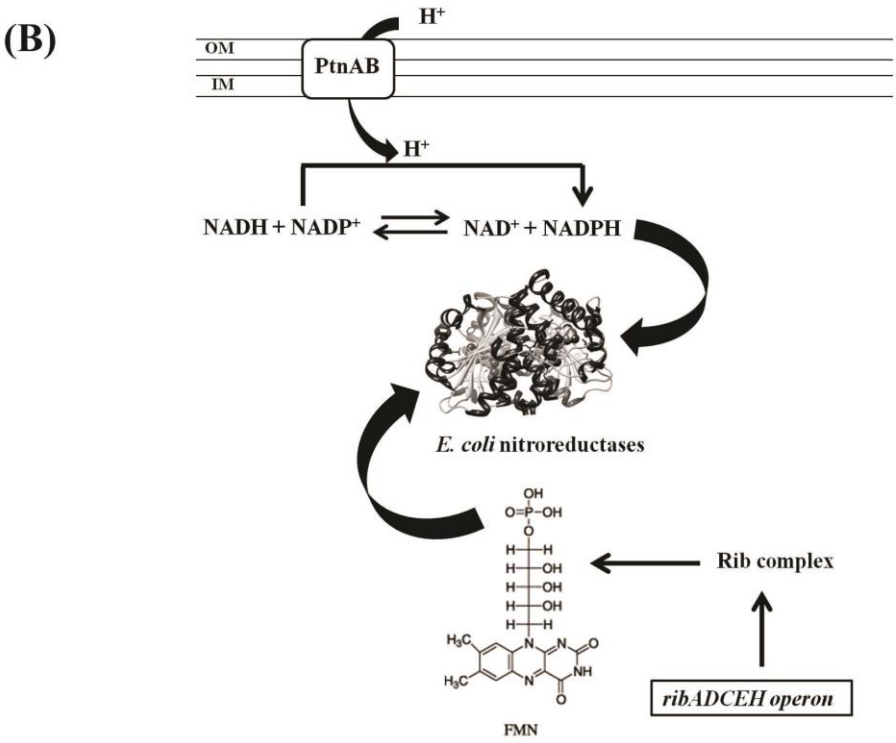
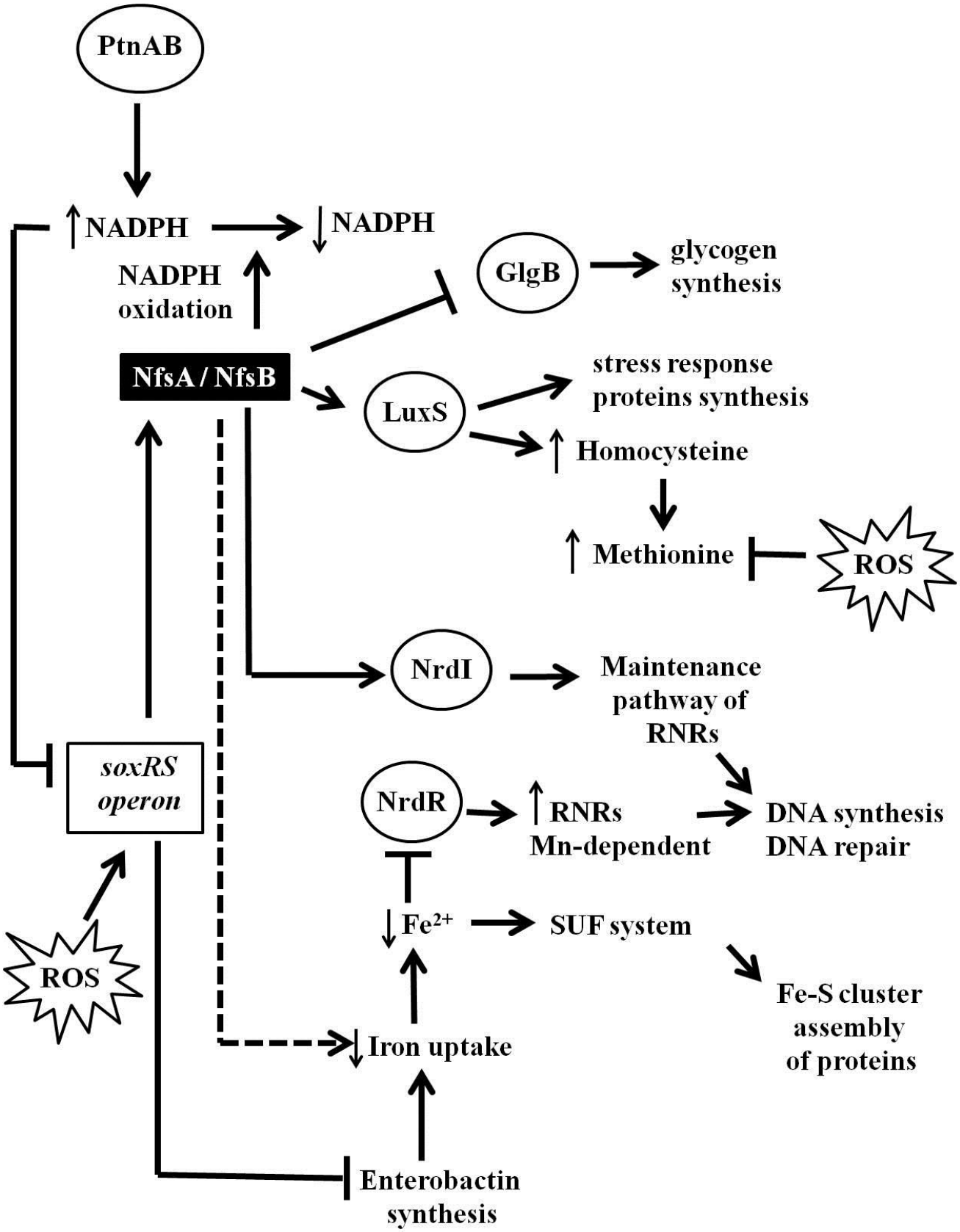

(B)
H+
OM
IM
PtnAB
H+
NADH + NADP+
NAD+ + NADPH
E. coli nitroreductases
Rib complex
ribADCEH operon
FMN

Fig. 6.


PtnAB
↑NADPH
↓NADPH
NADPH oxidation
GlgB
glycogen synthesis
NfsA / NfsB
LuxS
stress response proteins synthesis
↑ Homocysteine
↑ Methionine
ROS
NrdI
Maintenance pathway of RNRs
soxRS operon
NrdR
↑ RNRs Mn-dependent
DNA synthesis DNA repair
↓ Fe2+
SUF system
Fe-S cluster assembly of proteins
ROS
Iron uptake
Enterobactin synthesis

Capítulo IV

APRESENTAÇÃO DO CAPÍTULO IV

O papel de duas prováveis nitrorredutases, Frm2p e Hbn1p, na resposta ao estresse oxidativo em *S. cerevisiae*.

A maior parte das evidências a respeito das funções fisiológicas das nitrorredutases bacterianas é relacionada à resposta ao estresse oxidativo. Após a identificação por análises filogenéticas da presença de duas nitrorredutases, Frm2p e Hbn1p, na levedura *S. cerevisiae*, no presente trabalho foi verificada a influência dessas proteínas no balanço redox de *S. cerevisiae*. Para tanto, foram utilizadas linhagens proficientes e deficientes nas nitrorredutases Frm2p e Hbn1p expostas a agentes oxidantes, seguindo-se a análise da sobrevivência, competência respiratória, acúmulo de espécies reativas (ERO) e peroxidação lipídica, bem como a atividade basal de enzimas antioxidantes. Os resultados mostraram que as nitrorredutases Frm2p e Hbn1p influenciam na resposta ao estresse oxidativo em *S. cerevisiae* por modular conteúdo de glutationa (GSH) e a atividade das enzimas antioxidantes superoxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx).

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Research Article

The role of two putative nitroreductases, Frm2p and Hbn1p, in the oxidative stress response in *Saccharomyces cerevisiae*

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Abstract

The nitroreductase family is comprised of a group of FMN- or FAD-dependent enzymes that are able to metabolize nitrosubstituted compounds using the reducing power of NAD(P)H. These nitroreductases can be found in bacterial species and, to a lesser extent, in eukaryotes. There is little information on the biochemical functions of nitroreductases. Some studies suggest their possible involvement in the oxidative stress response. In the yeast *Saccharomyces cerevisiae,* two nitroreductase proteins, Frm2p and Hbn1p, have been described. While Frm2p appears to act in the lipid signalling pathway, the function of Hbn1p is completely unknown. In order to elucidate the functions of Frm2p and Hbn1p, we evaluated the sensitivity of yeast strains, proficient and deficient in both oxidative stress proteins, for respiratory competence, antioxidant-enzyme activities, intracellular reactive oxygen species (ROS) production and lipid peroxidation. We found reduced basal activity of superoxide dismutase (SOD), ROS production, lipid peroxidation and *petite* induction and higher sensitivity to 4-nitroquinoline-oxide (4-NQO) and *N*-nitrosodiethylamine (NDEA), as well as higher basal activity of catalase (CAT) and glutathione peroxidase (GPx) and reduced glutathione (GSH) content in the single and double mutant strains *frm2* Δ and *frm2* Δ *hbn1* Δ. These strains exhibited less ROS accumulation and lipid peroxidation when exposed to peroxides, H_2O_2 and *t*-BOOH. In summary, the Frm1p and Hbn1p nitroreductases influence the response to oxidative stress in *S. cerevisae* yeast by modulating the GSH contents and antioxidant enzymatic activities, such as SOD, CAT and GPx. Copyright © 2009 John Wiley & Sons, Ltd.

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Keywords: ***Saccharomyces cerevisiae*; Frm2p; Hbn1p; nitroreductases; reactive oxygen species**

Introduction

Nitroaromatic and nitroheterocyclic derivatives (nitrosubstituted compounds) constitute an enormous range of chemicals characterized by the presence of one or more nitro groups in a heterocyclic or aromatic nucleus. For many years, nitro-substituted compounds have been used in several industrial processes, and they have become an important group of environmental pollutants

(Tazima *et al*., 1975; McCalla *et al*., 1978; Spain, 1995). In addition, nitropolyaromatic compounds have been identified as by-products of a variety of combustion processes (Möller *et al*., 1993; De Marini *et al*., 1996). There are human health concerns with regard to all these compounds because their metabolization leads to the formation of potent genotoxic and/or mutagenic metabolites (Padda *et al*., 2003). In addition, many nitrosubstituted compounds are able to generate reactive nitrogen oxide species (RNOxS), which readily react with biological macromolecules (Sies and de Groot, 1993; Homma-Takeda *et al*., 2002). It has been described that nitrosubstituted compounds such as 4-nitroquinoline-oxide (4-NQO) and *N*-nitrosodiethylamine (NDEA) have their toxic action initiated by the enzymatic reduction of their nitro groups, and thus generating metabolites that are implicated in the formation of DNA adducts or can generate high levels of ROS by producing superoxide during futile redox cycle in the presence of molecular oxygen (Sugimura *et al*., 1968; Aiub *et al*., 2006). The metabolic pathways that result in the activation of nitrosubstituted compounds are complex, and nitroreductase proteins play a central role in the activation of nitroheterocyclic and nitroaromatic compounds. Research on these proteins is of significant interest due to: (a) their central role in mediating nitrosubstituted compound toxicity; and (b) its potential industrial applications (Kitts *et al*., 2000; Hannink *et al*., 2001; Kadiyala *et al*., 2003; Vass *et al*., 2009).

Nitroreductases comprise a family of proteins with conserved sequences originally discovered in Eubacteria and grouped together according to their sequence similarity. These enzymes are capable of catalysing the reduction of nitrosubstituted compounds, using FMN or FAD as prosthetic groups and NADH or NADPH as reducing agents (Bryant and DeLuca, 1991). To date, two types of bacterial nitroreductases have been biochemically characterized: (a) type I (oxygen-insensitive), which catalyse the reduction of nitro groups by sequential two-electron reductions to nitroso, hydroxylamine intermediates and finally primary amines (Haack *et al*., 2001; Kobori *et al*., 2001; Sarlauskas *et al*., 2004); and (b) type II (oxygen-sensitive), which catalyse one electron reduction of the nitro group, producing a nitro anion radical that subsequently reacts with oxygen, forming a superoxide radical and regenerating the original nitroaromatic compound. This 'futile cycle' can cause oxidative stress by producing large amounts of superoxides (Peterson *et al*., 1979; Angermaier and Simon, 1983). Type I nitroreductases are known to catalyse the reduction of organic nitroaromatic and nitroheterocyclic compounds, such as nitrobenzene, TNT, nitrofurazone, metronidazole and nitrofurantoin (McCalla *et al*., 1978; Kadiyala *et al*., 2003; Caballero *et al*., 2005; Han *et al*., 2007). Type I genes in *Escherichia coli* (Zenno *et al*., 1996a, 1996b), *Salmonella typhimurium* (Watanabe *et al*., 1990; Nokhbeh *et al*., 2002), *Enterobacter cloacae* (Bryant and DeLuca, 1991; Haynes *et al*., 2002) and *Helicobacter pylori* (Olekhnovich *et al*., 2009) have been cloned and their activities characterized. Interestingly, the genes that encode bacterial type II nitroreductases have not been cloned until now (Whiteway *et al*., 1998).

Few data exist on the distribution of nitroreductase-like sequences in eukaryotic cells. In the yeast *Saccharomyces cerevisiae*, two genes, *FRM2* (YCL026c-A) and *HBN1* (YCL026c-B), encode putative nitroreductase-like proteins, as we recently identified and characterized by *in silico* analysis (de Oliveira *et al*., 2007).

Knowledge of the biological functions of nitroreductase family proteins is very limited. Some studies have suggested their possible involvement in the response to oxidative stress (Liochev *et al*., 1999; Paterson *et al*., 2002; Streker *et al*., 2005). Although the functions of the Frm2 protein are not fully known, experimental data from McHale *et al*. (1996) indicated that Frm2p may be involved in the lipid signalling pathway and cellular homeostasis. The function of Hbn1p is as yet completely unknown. In light of the paucity of knowledge about the biochemical functions of nitroreductases and their possible participation in oxidative stress responses, the aim of the present study was to investigate the effect of Frm2 and Hbn1 proteins in redox homeostasis in *Saccharomyces cerevisiae*. To achieve this goal, we evaluated the sensitivity of single and double mutant yeast strains to oxidative agents and investigated respiratory competence, antioxidant enzyme activities, intracellular reactive oxygen species (ROS) accumulation and lipid peroxidation.

DOI: 10.1002/yea

Materials and methods

Chemicals

The chemicals 4-nitroquinoline-oxide (4-NQO), *N*-nitrosodiethylamine (NDEA), hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (*t*-BOOH), reduced glutathione (GSH), oxidized glutathione (GSSG), NADPH, glutathione reductase, thiobarbituric acid (TBA), trichloroacetic acid (TCA), phenylmethylsulphonyl fluoride (PMSF), 5,5-dithionitrobenzoic acid (DTNB), 2,7-dichlorofluorescein diacetate (DCHFDA), nitroblue tetrazolium (NBT), dioxin, 2,3,5-triphenyl tetrazolium chloride (TTC), amino acids (L-histidine, L-threonine, L-methionine, L-tryptophan, L-leucine and L-lysine) and bases (adenine and uracil) were purchased from Sigma (St. Louis, MO, USA). Yeast extract, bacto-peptone and bacto-agar were obtained from Difco Laboratories (Detroit, MI, USA).

Yeast strains and culture media

The *S. cerevisiae* strains used in this study are all isogenic derivatives of the wild-type (WT) strain BY4741 (Table 1). Complete YPD medium containing 0.5% w/v yeast extract, 2% w/v bacto-peptone and 2% w/v glucose was used for the routine growth of yeast cells (yeast-fermenting cells). For plating, the medium was solidified with 2% w/v bacto-agar. Minimal medium (MM) containing 0.67% w/v yeast nitrogen base without amino acids, 2% w/v glucose and 2% w/v bacto-agar was supplemented with the appropriate amino acids. Synthetic complete medium (SynCo) was supplemented with 2 mg adenine, 2 mg arginine, 5 mg lysine, 1 mg histidine, 2 mg leucine, 2 mg methionine, 2 mg uracil, 2 mg tryptophan and 24 mg threonine per 100 ml MM. SynCo medium without uracil (SynCo − ura) was used for the selection of yeast-transformant cells. The mutant strains were checked periodically in Synco − ura and SynCo medium with the addition of 200 μg/ml geneticin (Synco + gen).

Yeast growth conditions

Stationary phase cultures were obtained by the inoculation of *S. cerevisiae* strains in liquid YPD. After 48 h at 30 °C, cells were harvested by centrifugation (1500 × *g*, 1 min), washed twice in phosphate buffer (PBS 0.067 M, pH 7.0) and the cell density was determined microscopically using a Neubauer counting chamber. The cells were then resuspended in the same buffer at a final density of 2×10^7 cells/ml and further tests were carried out.

Table 1. *Saccharomyces cerevisiae* strains used in this study

Strains	Genotypes	Sources
BY4741 (WT)	Wild-type, WT; *MAT***a**; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	EUROSCARF*
frm2Δ	*MAT***a**; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; frm2::kanMX4*	EUROSCARF*
hbn1Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0; hbn1::URA3*	This study
frm2Δ hbn1Δ	*MAT***a**; *his3Δ1; leu2Δ0; met15Δ0; frm2::kanMX4; hbn1::URA3*	This study

* European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF; Johan Wolfgang Goethe-University, Frankfurt, Germany).

Disruption of the *HBN1* gene

The strain *hbn1* Δ and the double mutant *frm2* Δ *hbn1* Δ were generated by one-step gene replacement, as described by Rhotstein (1983). Briefly, the gene *URA3* was amplified by PCR from YCpLac33 vector (Gietz and Sugino, 1988), using the following sense (S) and antisense (A) oligonucleotides:

HBN1-S (5′-atgtctgctgttgcaacttatttgaaaactttaactgct ACCGAGGAACTCTTGGTATT-3′)
HBN1-A (5′-ttaattgaagatttcaacatcgtttttgatgtaggtctt CGCACATTTCCCCGAAAAGT-3′).

Lower case letters indicate the flanking region of the *HBN1* gene, while upper case letters represent the first 20 nucleotide residues complementary to the *URA3* sequence. Haploid BY4741 and *frm2* Δ strains were transformed with PCR product using the lithium acetate/ssDNA protocol, as described by Gietz and Woods (2002). Transformants were selected in Synco − ura medium.

Yeast survival curves

Suspension of 2×10^7 cells/ml cells were treated with 4-NQO at exposure concentrations of

DOI: 10.1002/yea

0–0.3 μg/ml v/v, with NDEA at concentrations in the range 0–500 μM, and with varying amounts of H_2O_2 and *t*-BOOH at exposure concentrations of 0–5 mM in phosphate buffer (PBS 0.067 M, pH 7.0) for 2 h in a rotary shaker at 30 °C.

After the treatments, the suspensions were serially diluted to 2×10^3 cells/ml, plated on solid medium, and incubated at 30 °C for 3 days. The number of colonies was counted to measure the survival rate.

Determination of respiratory deficient yeast colonies (*petites*)

Yeast *petite* colonies were examined for their ability to reduce 2,3,5-triphenyl tetrazolium chloride (TTC). For this assay, suspensions were serially diluted to 2×10^3 cells/ml, plated on solid YPD medium and incubated at 30 °C for 3 days. Then 0.5% w/v TTC was dissolved in premelted agar (1.2% w/v) at 48 °C and overlaid on the yeast colonies. After 1–2 h incubation at 30 °C, red and white colonies were scored. TTC reduction (colourless) requires the activity of the respiratory chain and leads to production of an insoluble red pigment, while *petite* mutants remain white (Ogur *et al*., 1957).

Preparation of yeast cell-free extracts

For preparation of yeast cell-free extracts, the cells were grown as described previously. Yeast extracts were prepared by glass bead lysis as follows: cells were suspended in lysis buffer (50 mM Tris, 150 mM NaCl, 50 mM EDTA, pH 7.2) with an equal volume of acid-washed 425–600 μm glass beads and PMSF, vortexed for 10–15 cycles (30 s each), followed by 30 s of cooling. The mixture was then microcentrifuged for 5 min at $6000 \times g$ to remove the cellular debris and the glass beads (Longo *et al*., 1996). The supernatant was kept on ice for immediate use. Protein concentrations were determined using the Bradford assay (Bradford, 1976).

Antioxidant enzyme activity estimations

Antioxidant enzyme activity of WT and mutant strains not exposed to oxidizing agents was determined by using the yeast cell-free extract, obtained as described previously. In this sense, total superoxide dismutase (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation by spectrophotometry at 480 nm, as previously described by Misra and Fridovich (1972). The results obtained for SOD activity are expressed as U SOD/mg protein. Catalase (CAT) activity was assayed by measuring the decreasing rate of H_2O_2 absorbance at 240 nm (Aebi, 1984). The CAT activity results are expressed as U CAT/mg protein. Glutathione peroxidase (GPx) activity was determined by measuring the rate of NAD(P)H oxidation at 340 nm, as described by Flohé and Günzler (1984). The GPx results are expressed as mM NADPH/min/mg protein. Total glutathione (GSH plus GSSG) was measured by photometric determination of 5-thio-2-nitrobenzoate (TNB) produced from DTNB in a kinetic assay, according to Akerboom and Sies (1981). The results are expressed as percentage GSH in relation the WT.

Determination of yeast intracellular ROS accumulation

Intracellular ROS accumulation was determined by the 2′,7 dichlorodihydrofluorescein diacetate, DCFH-DA-based real-time assay using intact living cells, as described by Wang and Joseph (1999). DCFH-DA enters the cells and predominantly reacts with highly oxidizing ROS to produce the fluorophore dichlorofluorescein (DCF) (LeBel *et al*., 1992; Gomes *et al*., 2005). The procedure for detection of intracellular superoxide accumulation was carried out by NBT reduction, as described by Nabi and Islam (2001). Briefly, suspensions of 6×10^4 cells/ml were incubated for 30 min with 100 μM DCFH-DA dissolved in PBS to allow cellular incorporation, or incubated with nitroblue tetrazolium (NBT) for 1 h at 30 °C. The cells were then centrifuged and the pellet was resuspended in PBS containing the drugs to be tested. DCFH-DA oxidation or NBT reduction were monitored at 5 min intervals at 30 °C, based on fluorescence emission intensity in a 96-well plate fluorescence reader with emission wavelengths set at 535 nm and 560 nm, respectively. Fluorescence values are expressed as arbitrary units.

Thiobarbituric acid reactive species (TBARS)

Formation of the thiobarbituric acid malondialdehyde (TBA–MDA) complex during an

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acid-heating reaction was used as a ROS accumulation index. This technique is widely used as a sensitive method to measure lipid peroxidation, as previously described (Draper and Hadley, 1990).

Briefly, a suspension of 2×10^7 cells/ml were grown as described before and were treated with 4-NQO at exposure concentrations of 0–0.3 μg/ml v/v, with NDEA at concentrations in the range 0–500 μμ and with varying amounts of H_2O_2 and *t*-BOOH at exposure concentrations in the range 0–5 mM in phosphate buffer (PBS 0.067 M, pH 7.0) for 2 h in a rotary shaker at 30 °C. Crude extracts were then prepared as described and the supernatants precipitated with 10% w/v TCA and centrifuged for 5 min at $1000 \times g$. The 300 μl supernatants were mixed with 100 μl 10% TCA and 600 μl 0.67% TBA, and then heated in a boiling water bath for 15 min. Thiobarbituric acid reactive species (TBARS) were determined by absorbance in a spectrophotometer at 532 nm. The results were expressed as nM TBARS/mg protein.

Statistical analysis

All assays were performed at three independent times, each carried out in triplicate. Statistical analyses of the data were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. $p < 0.05$ was considered significant. Data were expressed as mean ± standard deviation (SD).

Results

Frm2p and Hbn1p nitroreductases are involved in defence against oxidative stress

In order to analyse the role of Frm2p and Hbn1p in oxidative stress, we exposed stationary cultures of WT, *frm2* Δ, *hbn1* Δ and *frm2* Δ *hbn1* Δ yeast strains to increasing concentrations of 4-NQO, NDEA, H_2O_2 and *t*-BOOH. The survival data showed that the single mutant *frm2* Δ was sensitive to the nitrocompounds 4-NQO (Figure 1A) and

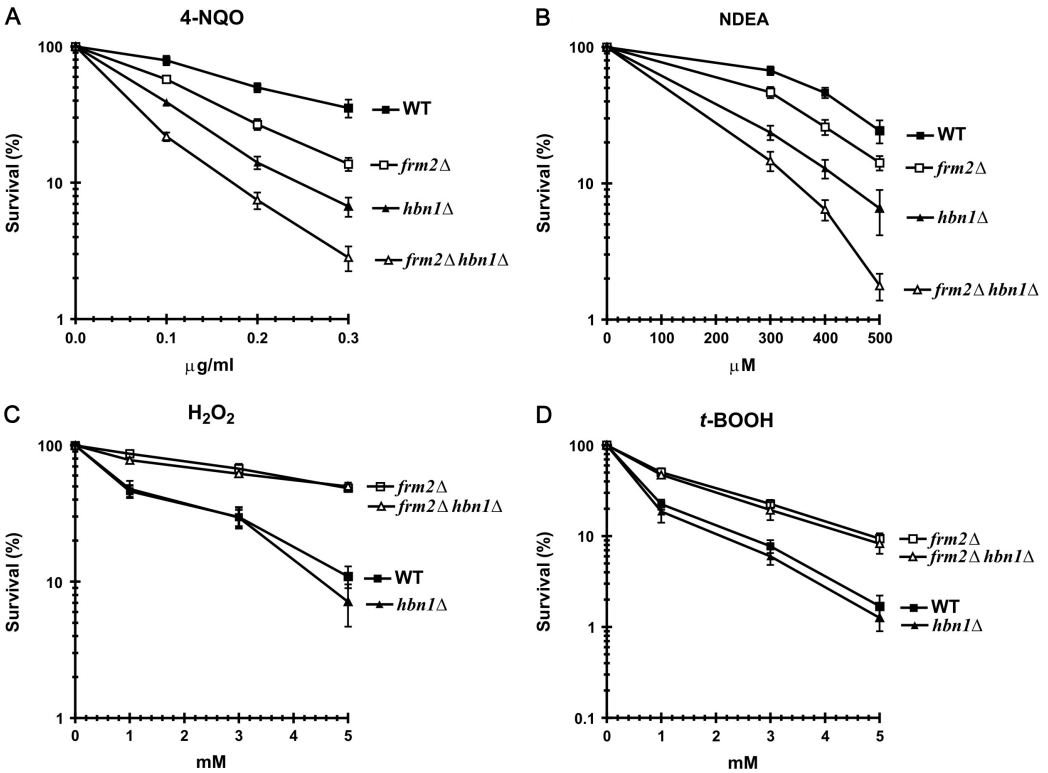

Figure 1. Sensitivity of yeast mutant strains in stationary growth phase to different oxidants. (A) 4-NQO; (B) NDEA; (C) H_2O_2; (D) *t*-BOOH. Cells were treated for 2 h at 30 °C. The data represent an average of three independent experiments. Data are expressed as means ± SD

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NDEA (Figure 1B) and tolerant to the inorganic and organic peroxides (Figure 1C, D). On the other hand, the *hbn1* Δ mutant was sensitive to nitrocompounds (Figure 1A, B) and displayed the same phenotype as WT cells in response to H_2O_2 and *t*-BOOH (Figure 1C, D). The double mutant *frm2* Δ *hbn1* Δ showed increased sensitivity to 4-NQO and NDEA (Figure 1A, B) as compared to the single mutants, but displayed the same peroxide cytotoxicity response (Figure 1C, D).

In turn, the ability of yeast cells to reduce TCC is an important indicator of respiratory competence, which is a parameter that changes in mutant cells when they present disturbances in cellular redox homeostasis. In the present study, the single and double mutant strains presented higher *petite* formation compared to WT (Figure 2), indicating total or partial loss of respiratory capacity.

Frm2p and Hbn1p yeast mutants exhibit altered antioxidant enzyme activity and reduced GSH levels

In order to determine the mechanisms that contribute to the phenotypic differences in the response to oxidative agents in the *frm2* Δ, *hbn1* Δ and *frm2* Δ *hbn1* Δ strains, we measured SOD, CAT, GPx basal enzymatic activity and GSH content. Interestingly, all mutant strains presented lower SOD basal activity than WT cells, in the following decreasing order: WT>*frm2* Δ > *hbn1* Δ > *frm2* Δ *hbn1* Δ (Figure 3A). On the other hand, CAT (Figure 3B) and GPx (Figure 3C) activities, as well as GSH contents (Figure 3D), were increased in the *frm2* Δ and *frm2* Δ *hbn1* Δ mutants.

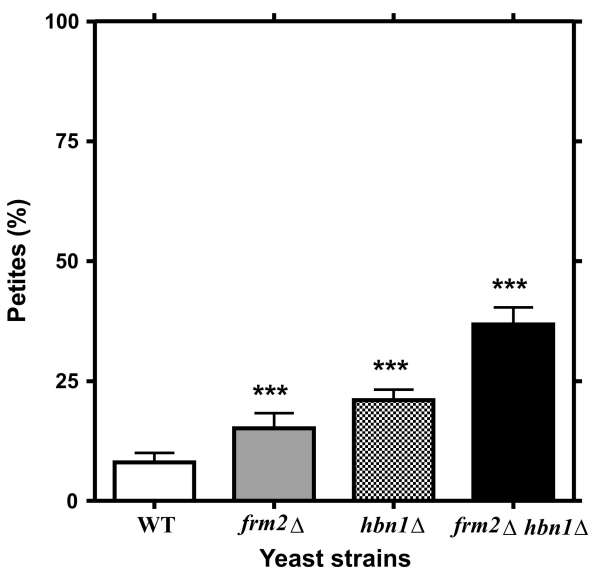

Figure 2. Induction of respiratory deficient colonies in WT and mutant yeast strains during metabolism respiratory growth. Data are expressed as means ± SD. Values shown are the means of at least three independent experiments. $^{***}p < 0.001$ indicates a significant increase in *petite* percentage (ANOVA, Tukey's test)

Different ROS accumulation and lipid peroxidation profiles are evident in single and double mutants challenged with oxidative-generating agents

In order to determine changes in ROS accumulation and oxidative damage in mutant strains, ROS accumulation was measured by DCFH-DA, superoxide by NBT assay and lipid peroxidation index by TBARS (Figure 4A, D). First, in response to the treatment with 4-NQO (Figure 4A) and NDEA (Figure 4B), the single and double mutants *frm2* Δ, *hbn1* Δ and *frm2* Δ *hbn1* Δ accumulated more ROS than isogenic WT cells. Double mutants displayed higher ROS levels as compared to single mutants when exposed to oxidants (Figure 4A, B). In response to H_2O_2 and *t*-BOOH (Figure 4C, D), the single mutant *frm2* Δ and the double mutant both exhibited low ROS accumulation, while the single mutant *hbn1* Δ displayed the same response as the WT strain.

Superoxide accumulation was evaluated using the NBT reduction assay (Figure 5). The mutant strains showed an increased level of superoxide relative to WT when exposed to 4-NQO and NDEA, and the highest levels were observed in the *frm2* Δ *hbn1* Δ mutant. However, in response to H_2O_2 or *t*-BOOH exposures, no changes compared to the basal levels of superoxide were detected (Figure 5C, D).

Concerning lipid peroxidation, single and double mutant strains presented higher TBARS levels when exposed to 4-NQO and NDEA (Figure 6A, B). It should be noted that ROS and TBARS levels seemed to be dose- and strain-dependent. However, when cells were exposed to H_2O_2 or *t*-BOOH, *frm2* Δ and double mutant strains presented lower TBARS levels when compared to treated WT and *hbn1* Δ strains (Figure 6C, D).

No differences in the basal levels of ROS accumulation or in lipid peroxidation were observed

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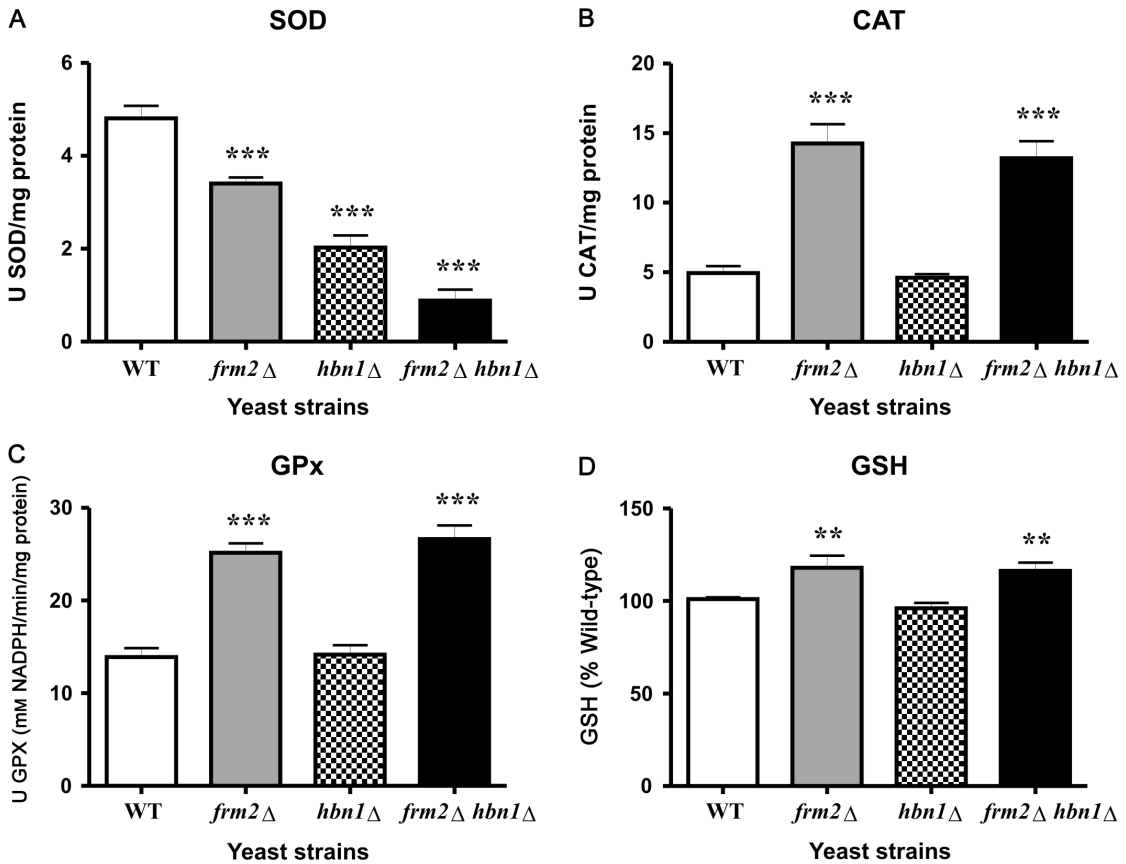

Figure 3. Analysis of SOD (A), CAT (B), GPx (C) activity and quantification of GSH (D) in Frm2p and Hbn1p-proficient and deficient *S. cerevisiae* strains not exposed to oxidizing agents. Data are expressed as means ± SD. Values shown represent the means of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences (ANOVA, Tukey's test)

among the strains in any of the assays (Figures 4–6).

Discussion

The biological function of the nitroreductase family of proteins is largely unknown. It has been postulated that the *E. coli* nitroreductase NfsB may also be able to reduce the 3-nitrotyrosine (3-NT) residues in proteins (Liochev *et al*., 1999). However, a study carried out by Lightfoot *et al*. (2000) indicated that neither NfsA nor NfsB reduced 3-NT. Some studies suggest the possible involvement of nitroreductases in the oxidative stress response (Liochev *et al*., 1999; Paterson *et al*., 2002; Streker *et al*., 2005). For instance, the *E. coli nfsA* gene, which encodes a nitroreductase NfsA, is part of the *SoxRS* regulon and is strongly induced by paraquat (Liochev *et al*., 1999; Paterson *et al*. 2002), a well-known superoxide generator (Hassan and Fridovich, 1978; Bagley *et al*., 1986). In addition, *snrA* in *Salmonella typhimurium* and *nprA* in *Rhodobacter capsulatos* are also induced by paraquat (Nokhbeh *et al*. 2002; Pérez-Reinado *et al*., 2005) and some NfsA-like proteins of *Bacillus subtilis* and *Staphylococcus aureus* help to maintain the cell thiol–disulphide balance (Sterker *et al*., 2005). Taking these data into account and considering the phylogenetic position of Frm2p and Hbn1p as described by de Oliveira *et al*. (2007), the role of these proteins in the oxidative stress response of *Saccharomyces cerevisiae* was investigated.

4-NQO and NDEA, superoxide-generating compounds that divert electrons from NADPH to

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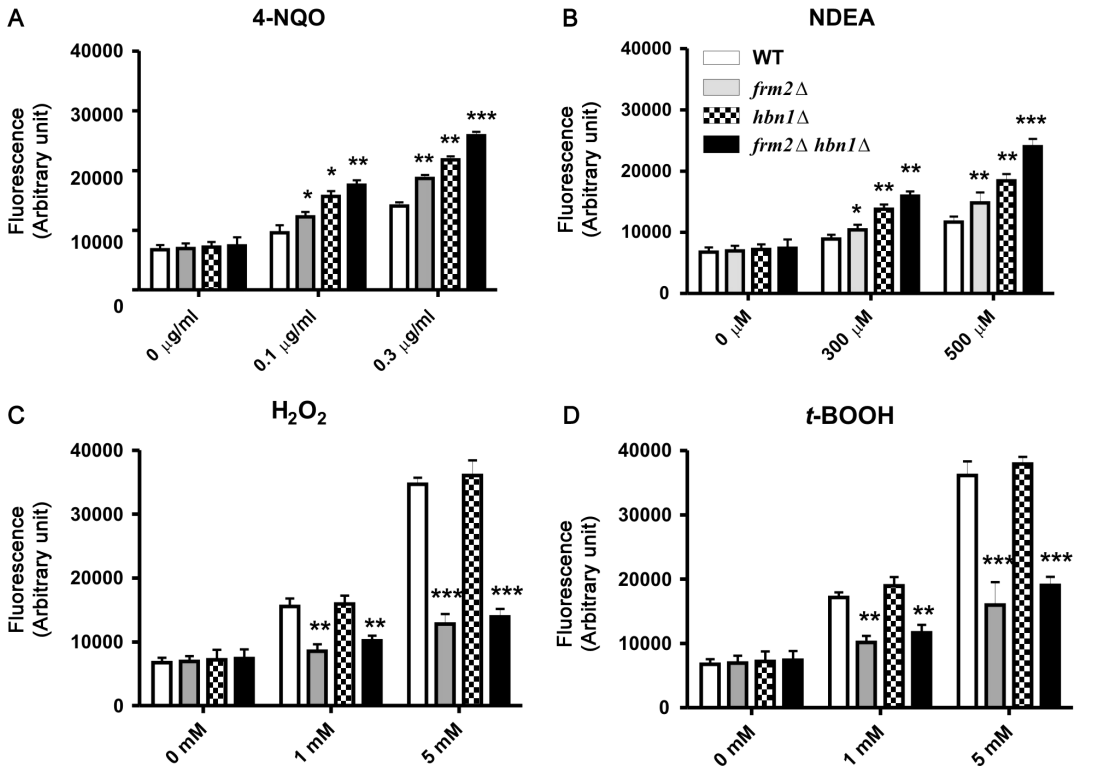

Figure 4. Production of intracellular reactive oxygen species in *S. cerevisiae* strains proficient and deficient in nitroreductases by the DCFH-DA real-time fluorescence assay. (A) 4-NQO; (B) NDEA; (C) H_2O_2; (D) *t*-BOOH. Data are expressed as means ± SD. Values shown are the means of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences (ANOVA, Tukey's test)

O_2 to generate a flux of superoxide, were used to induce oxidative stress (Nunoshiba and Demple, 1993; Fann *et al*., 1999; Aiub *et al*., 2006). H_2O_2 and *t*-BOOH were also tested as peroxide-generating substances. The sensitivity of the mutant strains to superoxide and peroxide was first evaluated by survival assay. Mutants demonstrated higher sensitivity to the oxidant agents 4-NQO and NDEA (Figure 1A, B). Interestingly, higher sensitivity of these strains to other oxidants, such as 1-chloro-2,4-dinitrobenzene (CDNB), pentachlorophenol (PCP) and methyl methane sulphonate (MMS), was also detected (data not shown). This can be partially explained by the reduced SOD activity in mutants (Figure 3A), which is supported by the fact that SOD mutants show higher sensitivity to 4-NQO, PCP, CDNB and MMS in several biological models (Hassan and Fridovich, 1978; Gralla and Kosman, 1992; Nunoshiba and Demple, 1993; Huang *et al*., 1997; Mutoh *et al*., 2005). Interestingly, Gaudu *et al*. (1994) showed that an *E. coli* strain deficient in nitroreductase-like Fre protein has a slight, but significant, lower basal level of *sodA* gene expression.

Thus, an increase in superoxide can generate other ROS, such as $HO_2^{\bullet}$, H_2O_2 and $HO^{\bullet}$, which can initiate autocatalytic lipid peroxidation (Lavine, 2002; Temple *et al*., 2005; Halliwell and Gutteridge, 2007). This fact may partially explain the observed ROS (Figures 4A, B, 5A, B) and TBARS (Figure 6A, B) that increase in the nitroreductase mutant cells, supporting the hypothesis that ROS accumulate in these strains upon exposure to 4-NQO and NDEA. Interestingly, *sod*-deficient yeast cells have been reported to present higher ROS and peroxidation lipid levels under oxidative stress conditions (de Freitas *et al*., 2000; Srinivasan *et al*., 2000; Pereira *et al*., 2003).

The importance of Frm2p and Hbn1p nitroreductases for respiration was also investigated. During respiration, mitochondria are the main sources of superoxide in the cell (Boveris and Chance,

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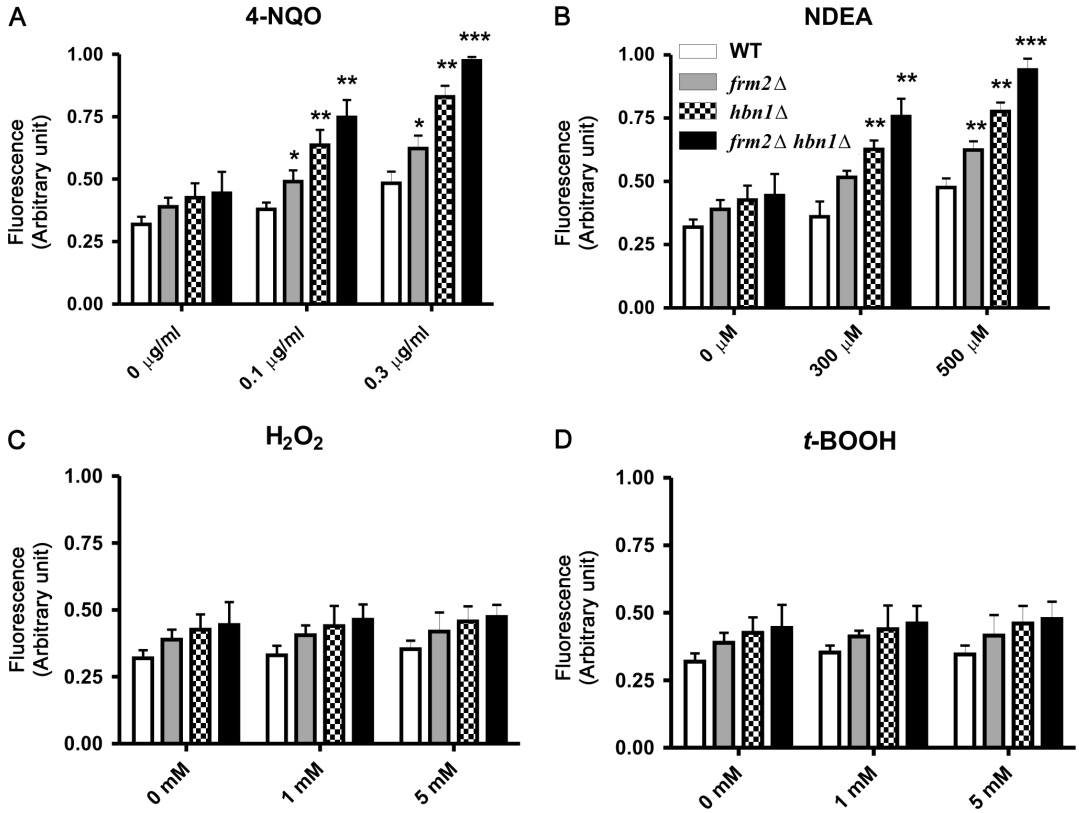

Figure 5. Production of intracellular superoxide in yeast strains proficient and deficient in Frm2p and Hbn1p by the NBT real-time fluorescence assay. (A) 4-NQO; (B) NDEA; (C) H_2O_2; (D) *t*-BOOH. Data are expressed as means ± SD. Values shown represent the means of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences (ANOVA, Tukey's test)

1973; Hassan and Fridovich, 1978; Liu. 1997). Frm2p- and Hbn1p-defective yeast cells induced more *petite* colonies (Figure 2), probably due to reduced SOD activity. It was previously shown that *sod* mutants also present a larger percentage of *petite* induction (Longo *et al*., 1994).

A different pattern of action was observed for the second type of oxidative stress generators utilized in this study: peroxides, H_2O_2 and *t*-BOOH. The *frm2* Δ and *frm2* Δ *hbn1* Δ mutants exhibited higher CAT and GPx enzyme activities, as well as greater GSH content (Figure 3B–D), leading to lower ROS (Figure 4C, D) and lipid peroxidation (Figure 6C, D) levels in response to peroxide exposure. These results are consistent with those of the survival assays (Figure 1). Thus, the absence of Frm2p may be responsible for the higher CAT, GPx and GHS activity levels observed. Curiously, Manfredini *et al*. (2004) found higher GPx activity in the *sod1* Δ *sod2* Δ double mutant, as well as higher GSH levels in the *sod1* Δ mutant, while CAT levels remained unchanged. Unfortunately, the results obtained in the present study cannot be explained by a compensatory mechanism of other antioxidant enzymes, considering that the *hbn1* Δ strain exhibited diminished SOD activity but normal levels of the other enzymes and of GSH.

Another interesting finding is that none of the tested strains showed any differences in basal ROS accumulation or peroxidation, suggesting that the changes in enzyme activities did not affect ROS levels under physiological conditions. In contrast, the nitroreductase-like activity in yeast seems to be important under oxidant exposure situations or when the cell activates the mechanisms required to maintain the intracellular redox balance under such conditions. Manfredini *et al*. (2004) detected higher lipid peroxidation levels in *sod1* Δ and *sod2* Δ single mutants, but in double mutants the level remained the same.

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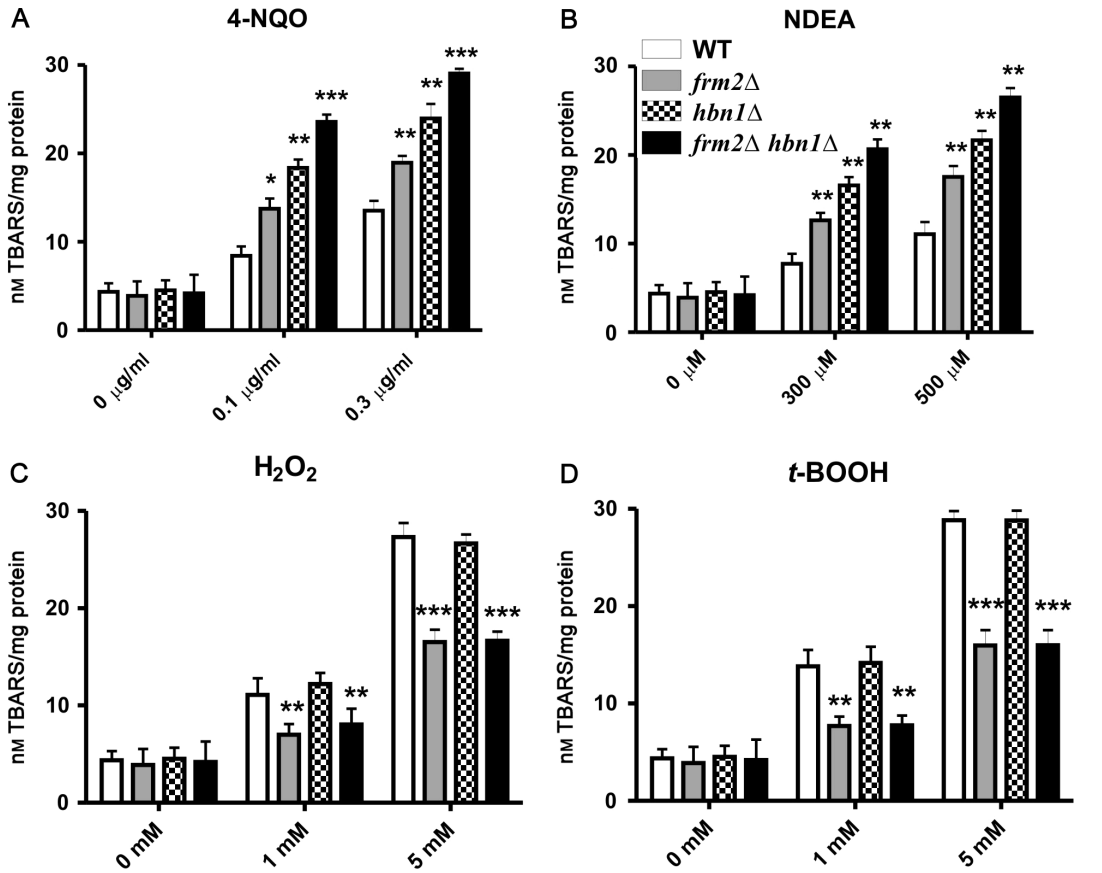

Figure 6. Determination of TBARS in nitroreductase proficient and deficient yeast strains treated for 2 h at 30 °C or not treated with oxidants. (A) 4-NQO; (B) NDEA; (C) H_2O_2; (D) *t*-BOOH. Data are expressed as means ± SD. Values shown are the means of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences (ANOVA, Tukey's test)

These actions of Frm2p and Hbn1p do not depend on metabolic status and showed the same sensitivity response patterns under both stationary and exponential growth conditions, as well as during fermentative and respiratory metabolism (data not shown). These data indicate that expression of these nitroreductases may be constitutive and not dependent on the physiological state of the cell. In fact, a model of action of Frm2p and Hbn1p can be drawn from the data gathered in this work (Figure 7). The model considered that 4-NQO (compound 1, Figure 7) generates a nitro anion radical (compound 2, Figure 7) that under physiological conditions of growth and in the presence of molecular oxygen (O_2) regenerates 4-NQO and form $O_2^{\bullet -}$ and H_2O_2 as by-products, establishing a futile redox cycle (Figure 7; Fann *et al.*, 1999). In this sense, Hbn1 can act nitroreducing 4-NQO to 4-hydroxyaminoquinoline-*N*-oxide (4-HAQO; compound 4; Figure 7), which is a substrate for Frm2p and forming 4-aminoquinoline-*N*-oxide (4-AQO; compound 5; Figure 7), neutralizing the cytotoxic and genotoxic action of 4-NQO in yeast cells in a similar way observed in bacterial nitroreductases (Roldán *et al.*, 2008). Thus, it is expected from the model that deletion of the gene-coding Hbn1p results in an increase in ROS generation by establishing the 4-NQO-associated futile redox cycle, resulting in elevated GSH content and high CAT and GPx activity. In the absence of Frm2p, 4-HAQO can suffer spontaneous oxidation forming a nitroxide radical (compound 6, Figure 7) that generates 4-nitrosoquinoline-*N*-oxide (4-NOQO; compound 3, Figure 7) and H_2O_2 as a by-product. This reaction was reported to occur *in vitro* (Nunoshiba and Demple, 1993) but it is possible

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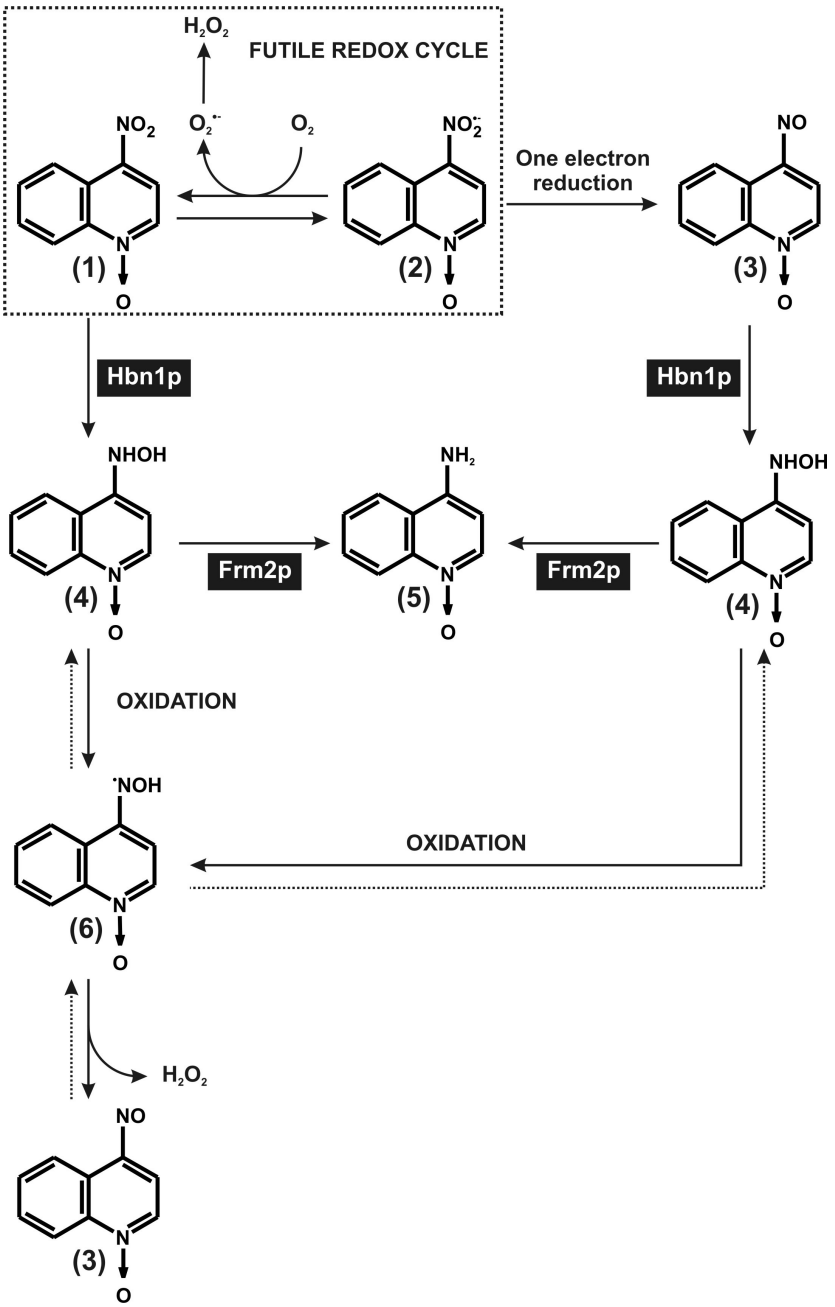

Figure 7. Putative mechanism of action of Hbn1p and Frm2p against 4-NQO (1) in yeast cells. The compound 4-NQO can generate a nitro anion radical (2), which in the presence of molecular oxygen (O_2), regenerates 4-NQO and forms superoxide radical ($O_2^{\bullet-}$) that can be converted in hydrogen peroxide (H_2O_2) and composing a futile redox cycle. However, in the presence of Hbn1p and Frm2p (black boxes), 4-NQO is first reduced to 4-hydroxyaminoquinoline-N-oxide (4-HAQO; 4) and then to 4-aminoquinoline-N-oxide (4-AQO; 5). A second pathway of nitroreduction can be observed after conversion of nitro anion radical (2) to 4-nitrosoquinoline-N-oxide (4-NOQO; 3) by one electron reduction. 4-NOQO (3) is then converted to 4-HAQO (4) by Hbn1p and then to 4-AQO (5) by Frm2p. Absence of Frm2p results in the oxidation of 4-HAQO to a nitroxide radical (6) that spontaneously generates H_2O_2 and 4-NOQO (3). It should be noted that 4-NOQO can also regenerate 4-HAQO in a reversible non-catalysed reaction (dotted arrows)

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that the same reaction take place *in vivo*. Moreover, this reaction could explain why the single and double mutant strains for the *FRM2* gene contain elevated levels of GSH and high activity of CAT and GPx, as observed in our assays. In addition, our model also considered that the nitro anion radical (compound 2, Figure 7) can suffer reduction by catalysed or non-catalysed one-electron transfer (Kano *et al*., 1987; Fann *et al*., 1999) and generate 4-NOQO (compound 3, Figure 7), which is a substrate of Hbn1p to form 4-HAQO. Thus, the absence of Frm2p results in a reversible oxidative pathway (Kano *et al*., 1987) that generate 4-NOQO and ROS, as discussed before. A similar pathway of yeast nitroreduction can be probably observed with NDEA and other nitrocompounds. We are now proceeding with Hbn1p and Frm2p purification in order to study their biochemical mechanisms of action and their roles in nitrocompound detoxification in yeast cells.

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Capítulo V

APRESENTAÇÃO DO CAPÍTULO V

Interações proteína-proteínas das nitrorredutases Frm2p e Hbn1p e suas influências na resposta ao estresse oxidativo em *S. cerevisiae*.

Neste trabalho, foram investigados os mecanismos pelos quais Frm2p e Hbn1p influenciam nas defesas antioxidantes em *S. cerevisiae*. Para tanto, foi analisada a rede de interações dessas nitrorredutases, seguindo a identificação de processos biológicos envolvidos e de importantes proteínas da rede. A presença de processos biológicos relacionados com o metabolismo de RNA foi evidenciada. As proteínas Cad1p (ativador transcricional) e Ski8p (envolvida na via de degradação do RNA no sentido 3'-5') foram selecionadas com base na sua relevância na rede e a significativa resposta de linhagens deficientes nessas proteínas na exposição a oxidantes. Seguiu-se a construção de linhagens duplo e triplo mutantes deficientes nestas proteínas e em Frm2p e Hbn1p com o propósito de avaliar o efeito dessas interações na exposição a oxidantes. Os resultados permitiram a elaboração de um modelo sugerindo que Cad1p estimula a transcrição do gene *FRM2* e a interação Frm2p-Ski8p regula as atividades das enzimas antioxidantes pela degradação do RNAm. A interação Hbn1p-Nab2p (Nab2p é necessária para a exportação do RNAm do núcleo para o citoplasma) controla a atividade da enzima superoxido dismutase (SOD) pela exportação do RNAm. Adicionalmente, Frm2p pode influenciar na suscetibilidade a oxidantes por modular a degradação dos transcritos do gene *OLE1* (Ole1p atua na síntese de ácidos graxos insaturados) e alterar a composição lipídica da membrana plasmática.

Os resultados apresentados a seguir estão sob a forma de um manuscrito que será submetido ao periódico *FEMS Yeast Research*.

Frm2p/Hbn1p nitroreductases protein–protein interactions and their influence in the oxidative stress response in *Saccharomyces cerevisiae*

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Abstract

Nitroreductases catalyze NAD(P)H-dependent reduction of nitrocompounds. Previous studies showed that Frm1p and Hbn1p nitroreductases influence *Saccharomyces cerevisiae* oxidative stress response. To elucidate the mechanisms how nitroreductases regulate antioxidant defenses, we undertook a systems biology approach to identify Frm2p and Hbn1p interactions. A protein-protein interaction (PPI) network was obtained and biological processes related to RNA metabolism were observed. Thus, network centrality analysis was performed, which allows for selection of important proteins of network. A sensitivity screening of yeast strains proficient and deficient in these proteins to oxidants was performed and selected Ski8p (mediates 3'-5' RNA degradation) and Cad1p (transcriptional activator). This information was used to construct double and triple mutants defective for Frm2p, Hbn1p, Cad1p or Ski8p followed by determination of sensitivity, reactive oxygen species (ROS) accumulation, lipid peroxidation following oxidants exposure and basal antioxidant-enzyme activities. The results obtained allow us to draw model suggesting that Cad1p activate *FRM2* following Frm2p-Ski8p interaction influences to oxidative stress response by regulates mRNA degradation of antioxidant-enzyme following their activities or *OLE1* (Ole1p act in unsaturated fatty acid synthesis) transcripts degradation modifying the plasma membrane fatty acid composition. The Hbn1p-Nab2p (Nab2p act in mRNA export) interaction controls superoxide dismutase (SOD) activity by mRNA export.

Keywords**:** *Saccharomyces cerevisiae*; Frm2p; Hbn1p; nitroreductases; systems biology.

Introduction

Nitroreductases comprise a family of proteins with conserved sequences that catalyze the reduction of nitrosubstituted compounds using FMN or FAD as prosthetic group and NADH or NADPH as reducing power agents (Haynes, *et al.*, 2002). These proteins have raised enormous interest because of their central role in mediating nitroaromatic toxicity, their potential use in bioremediation, biocatalysis and chemotherapeutic tumor treatment (Roldan, *et al.*, 2008, de Oliveira, *et al.*, 2010). Nitroreductases are classified into two groups based on their biochemical characteristics. Type I nitroreductases (oxygen-insensitive) catalyze the reduction of nitro groups via sequential two-electron reductions to nitroso and hydroxylamine intermediates, and finally to primary amines (Roldan, *et al.*, 2008). Type II nitroreductases (oxygen-sensitive) catalyze a one-electron reduction of the nitro group to produce a nitro-anion radical that subsequently reacts with oxygen to form a superoxide radical and the original nitroaromatic compound. This ‘‘futile cycle’’ can cause oxidative stress by producing large amounts of superoxides (Peterson, *et al.*, 1979). In addition, type I nitroreductases are known to catalyze the reduction of organic nitroaromatic and nitroheterocyclic compounds, such as nitrofurazone, metronidazole, and nitrofurantoin (Whiteway, *et al.*, 1998, Roldan, *et al.*, 2008). Nitroreductases were originally discovered in eubacteria. Few data exist on the distribution of nitroreductase-like sequences in eukaryotic cells, but recently have been also described in protozoan, fungal and mammalian (de Oliveira, *et al.*, 2007, Thomas, *et al.*, 2009). Although, the biological functions of these enzymes remain unclear, some studies suggest their possible involvement in oxidative stress responses (Liochev, *et al.*, 1999, de Oliveira, *et al.*, 2010).

In the yeast *Saccharomyces cerevisiae*, two genes, *FRM2* (YCL026c-A) and *HBN1* (YCL026c-B), encode Frm2p and Hbn1p nitroreductases; respectively, as we identified by *in silico* analysis (de Oliveira, *et al.*, 2007) and recently have been purified and confirmed as new nitroreductases (Bang, *et al.*, 2012). Although the functions of the *S. cerevisiae* nitroreductases are not fully known, experimental data from McHale *et al.* (1996) indicated that Frm2p may be involved in lipid metabolism. Subsequently, we show that Frm1p and Hbn1p nitroreductases influence *S. cerevisiae* oxidative stress response by modulating glutathione (GSH) contents, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities (de Oliveira, *et al.*, 2010), but the mechanisms are still not fully elucidated. To achieve this goal, we first conducted a systems biology study with the aim of elucidate the significance of the relationship of *S. cerevisiae* nitroreductases with other proteins in oxidative stress response. The data generated by systems biology analyses were applied in a phenotypic screening of the effects of oxidative agents in yeast mutant strains defective in important proteins of protein-protein interaction (PPI) network and proteins that showed higher response to oxidants were selected. Subsequently, double and triple mutant strains defective in *S. cerevisiae* nitroreductases and these important proteins of PPI network were constructed. Thus, we evaluated the sensitivity of single, double and triple mutant yeast strains to oxidative agents. Also, the antioxidant-enzyme activities, intracellular reactive oxygen species (ROS) accumulation and lipid peroxidation were investigated.

Materials and Methods

Protein–protein network design

To design binary protein–protein interaction (PPI) networks related to nitroreductases Frm2p and Hbn1p of *S. cerevisiae*, the metasearch engine STRING 9.0 [http://string.embl.de/] was initially applied by using Frm2p and Hbn1p. The STRING 9.0 software allowed us to visualize the physical connection among proteins related to *S. cerevisiae* nitroreductases. In this sense, the following parameters were used within STRING 9.0: active prediction methods all enabled except text mining; no more than 50 interactions; medium confidence score (0.400); and network depth equal to 2. The iHop [http://www.ihop-net.org/UniPub/iHOP/] search engines were also employed using the default parameters. In addition, a PPI network that contains 4399 nodes and 34,630 edges that describe physical interactions among yeast proteins was downloaded from the *Saccharomyces* Genome Database (http://www.yeastgenome.org). The results gathered from these search engines were subsequently analyzed using Cytoscape 2.8.3 (Shannon, *et al.*, 2003). To select the most relevant protein subnetworks from the interactome, the initial PPI network was analyzed using Molecular Complex Detection (MCODE) (Bader & Hogue, 2003), which is a Cytoscape plug-in freely available at http://baderlab.org/Software/MCODE. The parameters used in MCODE to generate the subnetworks were: loops included; degree cutoff 2; deletion of single connected nodes from cluster (haircut option enabled); expansion of cluster by one neighbor shell allowed (fluff option enabled); node density cutoff 0.1; node score cutoff 0.2; kcore 2; and maximum depth of network 100. A MCODE score was calculated for each protein/small compound present in the interactome networks.

Gene Ontology Analysis

Gene ontology (GO) clustering analysis was performed using Biological Network Gene Ontology (BiNGO) (Maere, *et al.*, 2005) software, a Cytoscape plugin available at [http://chianti.ucsd.edu/cyto_web/plugins/index.php]. The degree of functional enrichment for a given cluster and category was quantitatively assessed (*p* value) by hypergeometric distribution (Rivals, *et al.*, 2007) and a multiple test correction was applied using the false discovery rate (FDR) (Benjamini, *et al.*, 2001) algorithm, fully implemented in BiNGO software. Overrepresented biological process categories were generated after FDR correction, with a significance level of 0.05.

Network Centralities and Local Topological Analyses

Major network centralities (node degree, stress, closeness, and betweenness) were computed from the PPI networks using the Cytoscape plugin CentiScaPe 1.0 (Scardoni, *et al.*, 2009). The local topologies of the network (HB, H-NB, NH-B, and NH-NB) were defined considering the threshold generated by each centrality calculated by CentiScape 1.0. In this sense, HB was defined as a node with a value above the threshold calculated for node degree and betweenness, H-NB is a node with high node degree and low betweenness, NH-B is a node with low node degree and high betweenness, and NH-NB is a node with both low node degree and betweenness. A PPI subnetwork containing the 10 major nodes with the highest HB and NH-B scores was drawn using the Cytoscape plugin Cyto-Hubba (Lin, *et al.*, 2008) and is available at http://hub.iis.sinica.edu.tw/cytoHubba.

Chemicals

The chemicals 4-nitroquinoline-oxide (4-NQO), N-Nitrosodiethylamine (NDEA), hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (*t*-BOOH), reduced

glutathione (GSH), oxidized glutathione (GSSG), NADPH, glutathione reductase, thiobarbituric acid (TBA), trichloroacetic acid (TCA), phenylmethylsulfonyl fluoride (PMSF), 5,5-dithionitrobenzoic acid (DTNB), 2,7-dichlorofluorescein diacetate (DCHFDA), nitroblue tetrazolium (NBT), amino acids (L-histidine, L-threonine, L-methionine, L-tryptophan, L-leucine, and L-lysine) and bases (adenine and uracil) were purchased from Sigma (Saint Louis, MO, USA). Yeast extract, bacto-peptone, and bacto-agar were obtained from Difco Laboratories (Detroit, MI).

Yeast strains and culture media

The *S. cerevisiae* strains used in this study are all isogenic derivatives of the wild-type (WT) strains BY4741 or BY4743 (Table 1). Complete YPD medium containing 0.5% (w/v) yeast extract, 2% (w/v) bacto-peptone, and 2% (w/v) glucose was used for routine growth of yeast cells (yeast-fermenting cells). For plating, the medium was solidified with 2% (w/v) bacto-agar. Minimal medium (MM) containing 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, and 2% (w/v) bacto-agar was supplemented with the appropriate amino acids. Synthetic complete medium (SynCo) was supplemented with 2 mg adenine, 2 mg arginine, 5 mg lysine, 1 mg histidine, 2 mg leucine, 2 mg methionine, 2 mg of uracil, 2 mg of tryptophan, and 24 mg of threonine per 100 mL MM. The SynCo medium without uracil (SynCo-ura) was used for the selection of yeast-transformant cells. The mutant strains were checked periodically in SynCo-ura and SynCo media with addition of 200 μg/mL of geneticin (SynCo+gen).

Yeast growth conditions

Stationary phase cultures were obtained by the inoculation of *S. cerevisiae* strains in liquid YPD. After 48 h at 30°C, cells were harvested by centrifugation (1 min/1,500 *g*), washed twice in phosphate buffer (PBS 0.067 M pH 7.0), and the cell density was determined microscopically using a Neubauer counting chamber. The cells were then resuspended in the same buffer at a final density of 2×10^7 cells mL^{-1} and further tests were carried out.

Disruption of the *FRM2* and *HBN1* genes

S. cerevisiae double e triple mutant strains lacking were generated by one-step gene replacement, as described by (Rothstein, 1983). Briefly, the gene *URA3* was amplified by PCR from YCpLac33 vector (Hou, *et al.*, 2013) using the following sense (S) and antisense (A) oligonucleotides to *HBN1* and *FRM2*, respectively:

HBN1-S (5´atgtctgctgttgcaacttatttgaaaactttaactgctACCGAGGAACTCTTGGTATT 3´)

HBN1-A (5´ttaattgaagatttcaacatcgtttttgatgtaggtcttCGCACATTTCCCCGAAAAGT 3´).

FRM2-S (5´atgtccccaactggaaactacttaaacgctattacaaACCGAGGAACTCTTGGTATT 3´)

FRM2-A-(5´tcagtgataaacgttgattacgttattgataaaagtcCGCACATTTCCCCGAAAAGT 3´)

Lowercase letters indicate the flanking region of the *HBN1* or *FRM2* gene, while uppercase letters represent the first 20 nucleotide residues complementary to the *URA3* sequence. After generation of the disruption cassettes by PCR, single mutant *S. cerevisiae* strains were transformed with PCR product by lithium acetate/ssDNA protocol, as described by (Gietz & Woods, 2006). Transformants were selected in SynCo-ura medium.

Yeast survival curves

Suspension of 2×10^7 cells mL^{-1} cells were treated with 4NQO at exposure concentrations from 0 to 0.3 μg mL^{-1} (v/v), with NDEA at concentrations ranging between 0 and 500 μM, and with varying amounts of H_2O_2 and *t*-BOOH at exposure concentrations ranging from 0-5 mM in phosphate buffer (PBS 0.067 M pH 7.0) for 2 h in a rotary shaker at 30ºC. After the treatments, the suspensions were serially diluted to 2×10^3 cells mL^{-1}, plated on solid medium, and incubated at 30ºC for 3 d. The number of colonies was counted to measure the survival rate.

Preparation of yeast cell-free extracts

For preparation of yeast cell-free extracts, the cells were grown as described before. Yeast extracts were prepared by glass bead lysis as follows: Cells were suspended in lysis buffer (50 mM Tris, 150 mM NaCl, 50 mM EDTA, pH 7.2) with an equal volume of acid-washed 425-600 μm glass beads and PMSF, vortexed for 10-15 cycles (30 s each), followed by 30 s of cooling. The mixture was then microcentrifuged for 5 min at 6,000 *g* to remove the cellular debris and the glass beads (Longo, *et al.*, 1996). The supernatant was kept on ice for immediate use. Protein concentrations were determined by the Bradford assay (Bradford, 1976).

Antioxidant enzyme activity estimations

Antioxidant enzyme activity of WT and mutant strains not exposed to oxidizing agents was determined by using the yeast cell-free extract obtained as described before. In this sense, total SOD activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation by spectrophotometry at 480 nm, as previously described (Misra & Fridovich, 1972). The results obtained for SOD activity are expressed as U SOD/mg protein. CAT activity was assayed by measuring the

decreasing rate of H_2O_2 absorbance at 240 nm (Aebi, 1984). The CAT activity results are expressed as U CAT/mg protein. GPx activity was determined by measuring the rate of NAD(P)H oxidation at 340 nm, as described (Flohe & Gunzler, 1984). The GPx results are expressed as mM NADPH/min/mg protein. Total glutathione (GSH plus GSSG) was measured by photometric determination of 5-thio-2-nitrobenzoate (TNB) produced from DTNB in a kinetic assay, according to Akerboom & Sies (1981). The results are expressed as % GSH in relation the WT.

Determination of yeast intracellular ROS accumulation

Intracellular ROS accumulation was determined by the 2′,7 dichlorodihydrofluorescein diacetate, DCFH-DA-based real-time assay using intact living cells, as described Wang & Joseph, 1999). DCFH-DA enters the cells and predominantly reacts with highly oxidizing ROS to produce the fluorophore dichlorofluorescein (DCF) (LeBel, *et al.*, 1992, Gomes, *et al.*, 2005). The procedure for detection of intracellular superoxide accumulation was carried out by NBT reduction, as described (Durak, *et al.*, 1993).Briefly, suspensions of 6×10^4 cells mL^{-1} were incubated for 30 min with 100 μM DCFH-DA dissolved in PBS to allow cellular incorporation, or incubated with nitroblue tetrazolium (NBT) for 1 h at 30ºC. The cells were then centrifuged, and the pellet was resuspended in PBS containing 4NQO at concentrations from 0.3 μg mL^{-1} (v/v), NDEA at concentration of 500 μM, or H_2O_2 and *t*-BOOH at exposure concentration of 5 mM. DCFH-DA oxidation or NBT reduction were monitored at 5 min intervals at 30ºC based on fluorescence emission intensity in a 96-well plate fluorescence reader with an emission wavelength set at 535 and 560, respectively. Fluorescence values are expressed as arbitrary units.

Thiobarbituric acid reactive species (TBARS)

Formation of the thiobarbituric acid malondialdehyde (TBA-MDA) complex during an acid-heating reaction was used as a ROS accumulation index. This technique is widely used as a sensitive method to measure lipid peroxidation, as previously described (Draper & Hadley, 1990). Briefly, a suspension of 2×10^7 cells mL^{-1} cells were grown as described before and were treated with 0.3 μg mL^{-1} (v/v) of 4-NQO, with NDEA at concentration of 500 μM, and with H_2O_2 and *t*-BOOH at exposure concentration of 5 mM in PBS for 2 h in a rotary shaker at 30ºC. After, crude extracts were prepared as described and the supernatants precipitated with 10% (w/v) TCA and centrifuged for 5 min at 1,000 *g*. The 300 μL supernatants were mixed with 100 μL 10% TCA and 600 μL 0.67% TBA, and then heated in a boiling water bath for 15 min. Thiobarbituric acid reactive species (TBARS) were determined by absorbance in a spectrophotometer at 532 nm. Results are expressed as nmol TBARS/mg protein.

Statistical analysis

All assays were performed at three independent times, each carried out in triplicate. Statistical analyses of the data were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. P-values less than 0.05 were considered as significant. Data were expressed as means ± SD values.

Results

Analyses of interactome networks and subnetworks associated to *S. cerevisiae* nitroreductases

The major PPI network of Frm2p and Hbn1p contains 360 nodes and 2,493 connectors (Fig. 1) and is composed of five heavily interconnected clusters (Fig. 2), each of them comprising different biological processes (Table 2). GO analyses revealed that these biological processes can be classified into the following categories (Table 2): (i) RNA biosynthetic process/ RNA polymerase complex, (ii) nuclear pore/ RNA transport/ RNA export from nucleus, (iii) exosome (RNase complex)/ mRNA catabolic process/ ski complex, (iv) ribosome biogenesis/ lipid metabolism. As expected, proteins that could not be classified into any cluster were also identified in the network (unclustered protein subnetworks) (Table 2). These unclustered proteins, when submitted for GO analyses, belonged to the following group: response to stress.

Identification of important nodes within *S. cerevisiae* nitroreductases associated interactome networks and subnetworks by centrality analyses

Centrality analyses were performed in this work to identify the most important nodes within the PPI networks of Frm2p and Hbn1p. ‘‘Node degree’’ corresponds to the number of nodes directly connected to a given node and highly connected nodes in a network are termed ‘‘hubs’’ (Scardoni, *et al.*, 2009). Stress is a centrality measure that represents how much a node is traversed by a high number of short paths in a network (Scardoni, *et al.*, 2009). Betweenness shows the influence of a node over the spread of information throughout the network. The measure of node degree and betweenness gives rise to a third piece of information called ‘‘node bottleneck,’’ which is defined as all nodes with high betweenness values. ‘‘Bottleneck’’ also indicates all nodes that are ‘‘between’’ highly interconnected subgraph clusters, and removing a bottleneck could divide a network (Yu, *et al.*, 2007).

The measures of betweenness and node degree allow us to define four major groups of nodes within a network: (i) hub-bottleneck (HB), (ii) non-hub-bottleneck (NH-B), (iii) hub-non-bottleneck (H-NB), and non-hub-non-bottleneck (NH-NB) (Yu, *et al.*, 2007). Nodes that belong to the HB group tend to correspond to highly central proteins that connect several complexes or are peripheral members of central complexes, while nodes that belong to the NH-B group correspond to proteins that are important communication points between two complexes (Yu, *et al.*, 2007). By applying specific algorithms to detect bottlenecks, we identified the 10 major bottlenecks in the PPI network (Fig. 3A), which are the proteins Frm2, Hbn1, Cad1 (Yap2), Ski8, Sua7, Nab2, Ole1, Are1, Are2 and Pox1. When stress was combined with the betweenness score calculated from all nodes of the PPI network, the same nodes identified as bottlenecks showed high values of stress and betweenness (Fig. 3B). Thus, to define whether the bottleneck nodes belong to the HB or the NH-B group, an analysis of betweenness and node degree was performed (Fig. 3C). The data indicated that Frm2p and Hbn1p can be defined as NH-B nodes, while Cad1 (Yap2), Ski8, Sua7, Nab2, Ole1, Are1, Are2 and Pox1 as HB nodes (Fig. 3C). Finally, the analysis of betweenness and closeness (indicating the overall importance of the bottleneck nodes to the flow of the information in the network) showed that the 10 selected bottleneck nodes appear to be important for the PPI network (Fig. 3D). The selected HB and NH-B proteins compose a small network formed by 10 nodes (Fig. 3A), which comprise proteins from all subnetworks observed. Interestingly, three HB proteins have experimental records related to the oxidative stress response (Table 3).

Screening of yeast mutant strains in the oxidative stress response

Taking into account the results obtained from our systems biology analysis, to evaluate the possible participation in response to oxidative stress of proteins that appear to be important for the PPI network in centrality analysis, we performed a surviving screening of haploid strains deficient in the respective HB proteins or diploid strains with a single functional gene copy (in case of mutants in essential genes) (Table 1) to oxidants exposure. In this sense, we exposed stationary cultures of yeast mutant strains to increasing concentrations of 4-NQO, NDEA, H_2O_2 and *t*-BOOH. The screening results showed that *cad1Δ* and *ski8*Δ single mutant were sensitive to the nitrocompounds 4-NQO (Fig. 4A) and NDEA (Fig. 4B). The *ski8*Δ strain was tolerant to the inorganic and organic peroxides (Fig. 4C, 4D). In addition, the *ole1*Δ/*OLE1* showed low sensitivity to peroxides (Fig. 4C, 4D) and weakly nitrocompounds tolerance (Fig. 4A, 4B)**.** The *nab2*Δ/*NAB2* strain also showed low sensibility to 4-NQO e NDEA (Fig. 4A, 4B). In contrast, *sua7Δ/SUA7*, *are1*Δ, *are2*Δ and *pox1*Δ strains showed sensitivity to oxidants similar to BY4743 (Fig. 4). Thus, the strains with most significant responses, *cad1Δ* and *ski8*Δ, were selected for double and triple mutant strains construction.

Interactions of *S. cerevisiae* nitroreductases with Cad1p and Ski8p are involved in defense against oxidative stress

Taking into account the results obtained from survival screening, double and triple mutant strains deficient for the *FRM2*, *HBN1*, *CAD1* or *SKI8* genes were generated by single gene disruption (Table 1). As expected, the results in relation to yeast strains deficient in nitroreductases to oxidants exposure were consistent with the results obtained by de Oliveira *et al.* (2010). The survival data showed that yeast strains

deficient in nitroreductases were sensitive to the nitrocompounds 4-NQO and NDEA (Fig. 5 and 6). On the other hand, strains deficient in Frm2p were tolerant to the inorganic and organic peroxides (Fig. 5 and 6). The *hbn1Δ* mutant displayed the same phenotype as WT cells in response to H_2O_2 and *t*-BOOH (Fig. 5 and 6).

The *cad1*Δ showed more sensitivity to 4-NQO e NDEA than the other single mutant strains (Fig. 5A, 5B). The *frm2*Δ *cad1*Δ double mutant showed similar sensitivity to *cad1*Δ single mutant, while *hbn1*Δ *cad1*Δ double mutant showed higher sensitivity than *frm2*Δ *hbn1*Δ (Fig. 5A, 5B). The triple mutant *frm2*Δ *hbn1*Δ *cad1*Δ exhibited similar sensitivity to *hbn1*Δ *cad1*Δ double mutant to nitrocompounds (Fig. 5A, 5B). In contrast, the lack of Cad1p seems not to influence the response to peroxides (Fig. 5C, 5D).

The *ski8*Δ mutant presented similar nitrocompounds sensitivity to *frm2Δ* strain (Fig. 6A, 6B). The *frm2Δ hbn1Δ, hbn1Δ ski8Δ* and *frm2Δ hbn1Δ ski8Δ* strains showed similar response to nitrocompounds (Fig. 6A, 6B). In contrast, the *ski8*Δ strain is more tolerant to peroxides than *frm2Δ* (Fig. 6C, 6D). The double and triple mutant strains deficient in Ski8p displayed the same peroxide cytotoxicity response as *ski8Δ* single mutant (Fig. 6C, 6D).

Interactions of Frm2p and Hbn1p nitroreductases with Cad1p and Ski8p altered antioxidant enzyme activity and GSH levels

In order to determine the mechanisms how *S. cerevisiae* nitroreductases interaction with Cad1p and Ski8p act in the response to oxidative agents, we measured SOD, CAT, GPx basal enzymatic activity and GSH content. In accordance with previously observed by de Oliveira *et al.* (2010), *frm2*Δ, *hbn1*Δ and *frm2*Δ *hbn1*Δ

strains presented lower SOD basal activity than WT cells (Table 4). On the other hand, CAT and GPx activities, as well as GSH contents, were increased in the strains deficient in Frm2p (Table 4). The lack of Hbn1p not altered GSH levels and CAT and GPx activities (Table 4).

The strains deficient in Cad1p also presented decreased SOD basal activity as compared to WT cells. In relation to the other strains, we can observe a decreasing order: WT > *frm2*Δ > *hbn1*Δ > *cad1*Δ = *frm2*Δ *cad1*Δ > *frm2*Δ *hbn1*Δ > *hbn1*Δ *cad1*Δ = *frm2*Δ *hbn1*Δ *cad1*Δ (Table 4). On the other hand, CAT and GPx activities, as well as GSH contents, showed no alterations in the strains deficient in Cad1p in comparison to strains proficient in this protein (Table 4).

The strains deficient in Ski8p showed lower SOD activity following decreasing order: WT > *frm2*Δ = *ski8*Δ = *frm2*Δ *ski8*Δ > *hbn1*Δ > *frm2*Δ *hbn1* = *hbn1*Δ *ski8*Δ = *frm2*Δ *hbn1*Δ *ski8*Δ (Table 4). The double and triple mutant strains deficient in Ski8p have the highest basal CAT and GPx activities, as well as GSH content and that was similar to *ski8*Δ single mutant (Table 4).

Different ROS accumulation and lipid peroxidation profiles are evident in yeast deficient in Cad1p and Ski8p challenged with oxidative-generating agents

In order to determine changes in ROS accumulation and oxidative damage in mutant strains, ROS accumulation was measured by DCFH-DA, superoxide accumulation by NBT assay (Table 5) and lipid peroxidation index by TBARS (Table 6). The results were consistent with the previously showed by de Oliveira *et al.* (2010). In response to the treatment with 4-NQO and NDEA, the yeast nitroreductases mutants accumulated more ROS and superoxide (Table 5), and presented higher TBARS levels

than the isogenic WT cells (Table 6). However, the strains deficient in Frm2p exhibited low ROS accumulation (Table 5) and TBARS levels following exposure to peroxides (Table 6).

The *cad1*Δ showed higher ROS and superoxide accumulation (Table 5) and TBARS levels (Table 6) when exposed to 4-NQO and NDEA than the other single mutant strains. The *frm2*Δ *cad1*Δ presented similar results to *cad1*Δ (Tables 5 and 6). However, *hbn1*Δ *cad1*Δ and *frm2*Δ *hbn1*Δ *cad1*Δ strains showed the highest ROS accumulation (Table 5) and TBARS levels (Table 6). In exposure to peroxides, the strains deficient in Cad1p appear to have no alteration in ROS (Table 5) and TBARS levels compared to strains proficient in this enzyme (Table 6).

The *ski8*Δ mutant presented similar TBARS levels (Table 6), ROS and superoxide accumulation to *frm2Δ* strain in nitrocompounds exposure (Table 5). The *frm2Δ hbn1Δ, hbn1Δ ski8Δ* and *frm2Δ hbn1Δ ski8Δ* strains showed similar TBARS levels (Table 6), accumulation of ROS and superoxide when exposed to 4-NQO and NDEA (Table 5). In contrast, the strains deficient in Ski8p exhibited low ROS accumulation (Table 5) and lower TBARS levels (Table 6) in response to peroxides and double and triple mutant strains showed ROS (Table 5) and TBARS levels (Table 6) similar to *ski8*Δ single mutant.

As expected, in response to H_2O_2 or *t*-BOOH exposures, no changes compared to the basal levels of superoxide were detected (Table 5). In addition, no differences in the basal levels of ROS accumulation or in lipid peroxidation were observed among the strains in any of the assays (Tables 5 and 6).

Alteration in lipid peroxidation mediated by Ole1p and Nab2p influence the SOD activity

In addition, the possible mechanisms by which Ole1p contribute to the sensitivity differences in the response to oxidative agents were investigated. The *ole1*Δ/*OLE1* strain showed lower TBARS levels than BY4743 following 4-NQO exposure (Fig. 7A). In contrast, *ole1*Δ/*OLE1* presented more lipid peroxidation as a result of the H_2O_2 exposure than WT (Fig. 7A).

An interesting aspect gathered of the network is direct relation of Nab2p with Sod enzymes (Fig. 7B). Thus, in order to determine the effect of this interaction in SOD activity, we measured SOD basal activity in *nab2*Δ/*NAB2*. Interestingly, *nab2*Δ/*NAB2* strain presented lower SOD activity (Fig. 7C) and higher superoxide accumulation (Fig. 7D) than BY4743 cell.

Discussion

The largest number of evidences regarding the physiological role of nitroreductases is about their possible involvement in the oxidative stress response (Liochev, *et al.*, 1999, Cortial, *et al.*, 2010, de Oliveira, *et al.*, 2010). In this context, in previous works we show that Frm1p and Hbn1p nitroreductases of *S. cerevisiae* influence the response to oxidative stress by modulating the GSH contents and antioxidant enzymatic activities, such as SOD, CAT and GPx (de Oliveira, *et al.*, 2010). Although the pathways by which *S. cerevisiae* nitroreductases act in the modulation of antioxidant defenses are not yet fully elucidated. In this sense, to gain insights into possible mechanisms underlying the Frm2p and Hbn1p function, we performed unbiased searches for proteins interacting with these nitroreductases by means of systems biology analyzing PPI network, an increasingly emerging method to allow

hypothesis formulation (Barabasi & Oltvai, 2004). Thus, the role of Frm2p and Hbn1p interactions in *S. cerevisiae* oxidative stress response was determined.

Some interesting aspects of the PPI network were generated in this work. The subnetwork 1 shows proteins involved in RNA biosynthetic process and RNA polymerase complex. In this sense, in the subnetwork 1 are presents the RNA polymerase II subunits (Rpb1 to Rpb12 proteins) (Fig. 2A and Table 2). Interestingly, some of these proteins contribute to the resistance to oxidative stress, such as yeast RNA polymerase II subunit Rpb4p that is required for transcription under various stress conditions, including oxidative stress (Farago, *et al.*, 2003). Koyama *et al.* (2010) showed that subunit Rpb9p maintain transcriptional fidelity, which is critical for oxidative stress resistance.

In subnetwork 1 also are included proteins components of general transcription factors (GTFs) (reviewed in Lee & Young, 2000): TFIIA (Toa1p and Toa2p), TFIIB (Sua7p), TFIID (Taf1 to Taf13 proteins), TFIIE (Tfa1p and Tfa2p), TFIIF (Tfg1p and Tfg2p) and TFIIH (Kin28p, Tfb1 to Tfb4 proteins, Ssl1p, Ccl1p and Rad3p) (Fig. 2A and Table 2). The GTFs are important in the response to oxidative stress (Kraemer, *et al.*, 2006, Paes de Faria & Fernandes, 2006). In this sense, TFIIA mutant strains exhibit growth defects under conditions that require a functional response to oxidative stress; TFIIA interacts with Yap1p, which is the key regulator of the oxidative stress response in yeast (Kraemer, *et al.*, 2006). In this context, Frm2p nitroreductase have direct interaction with TFIIB (Sua7p) (Fig 3A), which was classified as a HB in our network analysis (Fig 3C). TFIIB is responsible for transcription start-site selection (reviewed in Lee & Young, 2000). Although TFIIB is a ubiquitous GTF required for transcription initiation by RNA polymerase II, it was suggested to be specific for a subset of genes such as genes linked with mitochondrial activity and hence with oxidative environments

(Paes de Faria & Fernandes, 2006). In addition, *SUA7* expression under oxidative conditions occurs at transcriptional and mRNA stability levels dependent of Yap1p (Paes de Faria & Fernandes, 2006). In contrast, our screening indicated that *sua7*Δ/*SUA7* strain presented no sensitivity to the oxidizing agents tested, since the presence of a single allele is possibly sufficient to regenerate the wild-type phenotype (Fig. 4).

Following synthesis by RNA polymerase II, the nuclear export of the mature RNA represents the culmination of the nuclear phase of the gene expression pathway (reviewed in Stewart, 2010). Thus, subnetwork 2 presented proteins involved in nuclear pore, RNA transport and RNA export from nucleus (Fig. 2B and Table 2). In this context, Nup proteins, which are proteins components of the core of nuclear pore complex, are represented in subnetwork 2 (Fig. 2B). The Nab2p was identified as HB in the network analysis (Fig. 3C) and centrality analyzes suggest that this is an important protein for the PPI network (Fig. 3A and 3B) and interacts directly with Hbn1p (Fig. 7B). The Nab2 protein contributes to the retention of polyadenylated mRNAs during heat shock stress (Stewart, 2010). In this sense, during stress conditions such as heat shock, most polyadenylated mRNAs are retained in the nucleus, whereas the export of specific mRNAs is allowed (Morano, *et al.*, 2012). Interestingly, *NAB2* expression is altered in oxidative stress (Auesukaree, *et al.*, 2009) and indeed *nab2*Δ/*NAB*2 strain showed higher sensitivity than WT to superoxide generate agents (Fig 4A, 4B). Consistently, we can observe the interaction of Nab2p with Sod1p and Sod2p enzymes in PPI network (Fig. 7B). In agreement, *nab2*Δ/*NAB*2 strain presented lower SOD activity, and consequently superoxide radical accumulation, which may explain the sensitivity to superoxide-generating compounds, 4-NQO and NDEA (Nunoshiba & Demple, 1993, Aiub, *et al.*, 2006).

In subnetwork 3 we can observe the presence of proteins involved in the exosome (RNase complex), mRNA catabolic process and ski complex (Fig. 2C and Table 2). In eukaryotes, mRNAs are primarily degraded by two redundant decay pathways, namely the 3'–5' or 5'–3' (reviewed in Parker, 2012). In the 3'–5' pathway, transcripts are further deadenylated and then degraded by a multisubunit 3'–5' exonuclease complex, known as the exosome (Chlebowski, *et al.*, 2013). The exosome is a multimeric complex composed of a ring-shaped core made of three heterodimers (Rrp41p/Rrp45p, Rrp43p/Rrp46p, Rrp42p/ Mtr3p) stabilized by the presence of three other proteins (Ski4p, Rrp4p, Rrp40p) (Balagopal, *et al.*, 2012). Several factors are also required to modulate the exosome activity. Thus, ski complex recruits the exosome to its substrate, where the mRNA is subsequently degraded (Chlebowski, *et al.*, 2013). The ski complex is formed by Ski2p, Ski3p and Ski8p or Ski7p, plays a role in mRNA decay (Synowsky & Heck, 2008). Some of these proteins are represented in subnetwork 3 (Fig. 2C and Table 2). The ski complex is necessary for exosomal degradation of mRNAs subjected to RNA interference (RNAi) and for decay of several kinds of faulty mRNAs: ones with premature termination codons (PTCs), ones lacking termination codons (the non-stop decay (NSD) pathway), ones where ribosomes stall (the no-go decay (NGD) pathway) and the nonsense mediated decay (NMD) (reviewed in Frischmeyer, *et al.*, 2002). Interestingly, NMD also has an important role in regulating transcripts involved in amino acid metabolism and oxidative stress (Rodriguez-Gabriel, *et al.*, 2006, Gardner, 2010), suggesting the targeting of mRNAs that can promote the cellular adaptation to hostile environments. Curiously, *nmd* mutants are more tolerant than WT to some stress conditions, such as toxic copper and others metals levels (Deliz-Aguirre, *et al.*, 2011, Wang, *et al.*, 2013). Our results of network analysis indicated direct interaction of Frm2p with Ski8p (Fig. 3A), which was classified as HB (Fig. 3C).

The Frm2p-Ski8p interaction appears to contribute to the sensitivity phenotype in response to nitrocompounds (Fig. 5A and 5B) by reduction of SOD activity following superoxide accumulation (Table 5) and increase of lipid peroxidation (Table 6). The Frm2p and Ski8p also contributes to peroxide resistance by increasing the activity of enzymes CAT and GPx, as well as by greater GSH contents (Table 4) leading to lower ROS accumulation (Table 5) and lipid peroxidation (Table 6) levels in response to peroxides. These results are consistent with those of the survival assays (Fig. 6C, 6D). Our data suggests that Frm2p and Ski8p act in the same pathway. In agreement with our results, expression analyses conducted by Singh *et al.* (2010) indicated that strains deficient in Lsm1p, a protein involved in the degradation of mRNA, has *CTT1* and *GSH2* genes upregulated and *SOD1* downregulated. In contrast, Ski8p appear to have no interaction with Hbn1p (Fig. 3A). Indeed, the results suggests that Frm2p and Hbn1p act in different pathways in SOD activity modulation and also Hbn1p does not appear to be involved in the response to peroxides (de Oliveira, *et al.*, 2010).

An interesting aspect of the subnetwork 4 is the connection between proteins involved in lipid metabolism with enzymes related to ribosome biogenesis (Fig. 2D and Table 2). This observation is consistent with the fact that changes in lipid metabolism alter the expression of various genes related to ribosome biogenesis (Jesch, *et al.*, 2006). Adaptive responses to oxidative stress also involve alteration in lipid metabolism (Morano, *et al.*, 2012). In this context, the PPI network shows direct interaction of *S. cerevisiae* nitroreductases with proteins belonging cluster and classified as HB in network analysis, such as Ole1p, Pox1p, Are1p and Are2p (Fig. 3A, 3C). The *ole1*Δ/*OLE1* strain showed tolerance to nitrocompounds and sensitivity to peroxides (Fig. 4). *OLE1* encodes a membrane bound Δ9-desaturase in yeast, which produces oleic and palmitoleic unsaturated fatty acids (de Freitas, *et al.*, 2012). Thus, we found

that a higher tolerance to nitrocompounds is probably explained by the fact that an increase in superoxide radical can generate other ROS, such as peroxides, hidroperoxides and hydroxyl radical (Temple, *et al.*, 2005) that possibly attack certain types of lipids produced by Ole1p and initiate autocatalytic lipid peroxidation (Dotan, *et al.*, 2004). Indeed, *ole1*Δ/*OLE1* strain showed higher lipid peroxidation following 4-NQO exposure (Fig. 7A). Consistently with this hiphotesis, *OLE1* was also downregulated as adaptative response to prooxidant herbicide 2,4-dichlorophenoxyacetic acid by decreased percentage of palmitoleic and oleic acids and increase percentage of saturated fatty acids (Viegas, *et al.*, 2005). Interestingly, Horan *et al.* (2006) showed that a collection of genes involved in fatty acid and sterol synthesis, including *OLE1* were repressed during nitrosative stress and *FRM2* was upregulated in this circumstance. In contrast, the *ole1*Δ/*OLE1* strain presented sensitivity to peroxides (Fig. 4). In agreement, Kelley & Ideker (2009) showed that *OLE1* was highly expressed genes following pretreatment with H_2O_2 and suggested that fatty acid biosynthetic pathways may be important during adaptation to oxidative stress. A potential mechanism during adaptation to oxidants is the promotion of an increase in certain types of lipids which decreases the permeability of plasma membrane (Branco, *et al.*, 2004, de Freitas, *et al.*, 2012). Consistently, *ole1*Δ/*OLE1* strain showed lower lipid peroxidation to peroxide exposure (Fig. 7A). Interestingly, *ole1*Δ/*OLE1* strain shows no alterations in the GSH levels and in the basal activity of antioxidant enzymes (data not show). Thus, it is possible that Frm2p may be involved in the oxidative stress response by lipid metabolism. Reinforcing this hypothesis, McHale *et al.* (1996) showed that Frm2p repressed the expression of *OLE1* in response to exogenous fatty acids. Curiously, *OLE1* gene expression is regulated through transcriptional and mRNA stability controls by exosomal 3'- 5'-exonuclease degradation activity (Kandasamy, *et*

al., 2004). Thus, it is possible that Frm2p and Ski8p interaction modulates the *OLE1* expression by mRNA degradation. In contrast, Pox1p, Are1p and Are2p do not appear to be related to the oxidative stress response (Fig. 4). In addition, prominent upregulation of genes involved in ribosome biogenesis was observed in many studies, which may indicate a requirement to replace ribosomal proteins and rRNA that become damaged by oxidative stress (Morano, *et al.*, 2012).

The unclustered proteins subnetwork includes proteins that are associated with response to stress (Table 2). In this subnetwork we can highlight the presence of proteins Yap1 and Cad1 (Table 2). The Yap family proteins are implicated in a variety of stress responses; among these proteins, Yap1 controls the expression of genes encoding most yeast antioxidants and components of the cellular thiol-reducing pathway (Rodrigues-Pousada, *et al.*, 2010). Another member of the Yap family, Yap2p is also known as Cad1p due to the acquisition of cadmium resistance in cells overexpressing the gene and also resistance to stress agents such as 1,10-phenanthroline, cerulenin and cycloheximide (Rodrigues-Pousada, *et al.*, 2004). The strain deficient in Cad1p demonstrated higher sensitivity to 4-NQO and NDEA, superoxide-generating compounds (Fig. 5A, 5B). This can be partially explained by the reduced SOD activity in mutants leads to superoxide accumulation and increase TBARS levels (Tables 4, 5, 6). The Cad1 e Frm2 proteins appear to participate in the same pathway. This evidence is expected once *FRM2* is as a bona fide Yap2 target gene (Azevedo, *et al.*, 2007, Bang, *et al.*, 2013). However, Hbn1p and Cad1p seem not to act in the same pathway and indeed *HBN1* gene expression is not Cad1p dependent (Bang, *et al.*, 2013). Interestingly, Cad1p does not respond to stress caused by peroxides (Vilela, *et al.*, 1998), a fact consistent with our results showing that *cad1*Δ strains have

similar peroxide sensitivity to WT strain (Fig 5B, 5C). This is possibly due to absence of alteration in GSH contents and CAT and GPx activity (Table 4).

In conclusion, a model of action of Frm2p and Hbn1p interaction with others proteins can be drawn from the data gathered in this work (Fig. 8). The model considered that the Hbn1p-Nab2p interaction is important for export of *SOD* transcripts. Thus, it is expected from the model that deletion of the gene-coding these proteins results in a decrease the export of *SOD* transcripts resulting decrease of SOD activity, following increase in superoxide accumulation, consequently in sensitivity to superoxide-generating compounds. In another route, Cad1p regulates the expression of *FRM2* that codify Frm2p, which interaction with Ski8p regulates SOD mRNA degradation. The absence of Frm2p results in higher SOD mRNA degradation following decrease in SOD activity and resulting in sensitivity to superoxide-generating compounds. In contrast, the Frm2p-Ski8p interaction modulates the expression of genes codifying CAT and GPx proteins and proteins of GSH biosynthetic pathway by degradation of mRNA. In absence of Frm2p, occurs decrease in degradation of mRNA for these antioxidant enzymes and increase in their activities result in peroxide resistance. The model also considers that Frm2p-Ski8p interaction can modulate the *OLE1* expression and, consequently, influencing in the lipid profile of the plasma membrane. In the absence of Frm2p, *OLE1* mRNA degradation decreases, leading to production of lipids more susceptible to ROS attack provoked by superoxide. In contrast, in the presence of this protein, the lipid profile of the plasma membrane increases resistance to peroxides, for probably reducing the plasme membrane fluidity.

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Tables

Table 1. *Saccharomyces cerevisiae* strains used in this study.

Strains	Relevant genotypes	Sources
BY4741 (haploid)	wild-type, WT; *MATα; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	EUROSCARF*
BY4743(diploid)	wild-type, WT*; MATa/MATα his3Δ 0/his3Δ 0; leu2Δ /leu2Δ 0; met15Δ 0/MET15; LYS2/lys2Δ 0; ura3Δ 0/ura3Δ 0*	EUROSCARF*
frm2Δ	BY4741; with *frm2::kanMX4*	EUROSCARF*
hbn1Δ	BY4741; with *hbn1::URA3*	de Oliveira *et al.* (2010)
ski8Δ	BY4741; with *ski8::kanMX4*	EUROSCARF*
cad1Δ	BY4741; with *cad1::kanMX4*	EUROSCARF*
are1Δ	BY4741; with *are1::kanMX4*	EUROSCARF*
are2Δ	BY4741; with *are2::kanMX4*	EUROSCARF*
pox1Δ	BY4741; with *pox1::kanMX4*	EUROSCARF*
ole1Δ/OLE1	BY4743; with *ole1*::*kanMX4/OLE1*	EUROSCARF*
nab2Δ/NAB2	BY4743; with *nab2*::*kanMX4/NAB2*	EUROSCARF*
sua7Δ/SUA7	BY4743; with *sua7*::*kanMX4/SUA7*	EUROSCARF*
frm2Δ hbn1Δ	BY4741; with *frm2::kanMX4; hbn1::URA3*	de Oliveira *et al.* (2010)
frm2Δ cad1Δ	BY4741; with *frm2::URA3; cad1:: kanMX4*	This study
hbn1Δ cad1Δ	BY4741; with *hbn1::URA3; cad1:: kanMX4*	This study
frm2Δ ski8Δ	BY4741; with *frm2::URA3; ski8:: kanMX4*	This study
hbn1Δ ski8Δ	BY4741; with *hbn1::URA3; ski8:: kanMX4*	This study
frm2Δ hbn1Δ cad1Δ	BY4741; with *frm2::URA3; hbn1::URA3 cad1:: kanMX4*	This study
frm2Δ hbn1Δ ski8Δ	BY4741; with *frm2::URA3; hbn1::URA3 ski8:: kanMX4*	This study

∗European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF; Johan Wolfgang Goethe-University, Frankfurt, Germany).

Table 2. Specific gene ontology (GO) classes derived from *S. cerevisiae* nitroreductases protein-protein interactions

Subnetwork number	GO biological process category	GO number	*p*-Value[a]	Corrected *p*-Value[b]	*k*[c]	*f*[d]	Proteins
Subnetwork 1	RNA biosynthetic process	32774	1.84×10^{-53}	1.81×10^{-51}	36	230	CCL1;SSL1;SSL2;SPT15;TFA1;TFA2;TAF9;TOA2;TOA1;TAF1;TAF5;RPB9;TFB4;RPB8;RPO21;RPB5;RPO26;TFB1;RPB4;TFB2;RPB7;TFB3;TAF11;TAF10;TAF13;RPB11;RAD3;RPB2;RPB3;TAF12;RPB10;SUA7;TFG1;KIN28;TFG2;RPC10
	RNA polymerase complex	30880	1.53×10^{-21}	2.30×10^{-20}	13	36	RPB9;RPB8;RPO21;RPB5;RPO26;RPB4;RPB7;RPB2;RPB11;RPB3;RPB10;TFG1;RPC10
Subnetwork 2	RNA transport	50658	6.99×10^{-51}	3.93×10^{-49}	28	114	ASM4;NUP145;MEX67;PML39;NSP1;NUP120;SAC3;NUP188;GFD1;NUP53;GLE2;POM34;GLE1;NUP133;NUP170;NUP1;NUP2;NUP15
	RNA export from nucleus	6405	4.30×10^{-44}	1.00×10^{-42}	25	95	ASM4;NUP145;MEX67;NSP1;NUP120;SAC3;NUP188;GFD1;NUP53;GLE2;GLE1;NUP170;NUP133;NUP1;NUP2;NUP159;NUP85;NUP157;NUP82;NUP60;NUP42;NIC96;NAB2;THP1;POM152
Subnetwork 3	exosome (RNase complex)	178	3.26×10^{-38}	4.38×10^{-36}	17	17	MTR3;SKI6;RRP6;RRP4;CSL4;RRP45;RRP46;DIS3;LRP1;RRP40;RRP42;RRP43;YNR024W;SKI8;SKI7;SKI4;SKI2
	mRNA catabolic process	6402	2.15×10^{-9}	2.03×10^{-7}	9	86	PAT1;SKI2;SKI3;DCP1;KEM1;SKI8;LSM1;SKI6;SKI7
	Ski complex	55087	2.05×10^{-8}	1.07×10^{-6}	6	6	SKI2;SKI3;SKI8;SKI6;SKI7;SKI4

Table 2 (continued)

Subnetwork number	GO biological process category	GO number	*p*-Value[a]	Corrected *p*-Value[b]	*k*[c]	*f*[d]	Proteins
Subnetwork 4	ribosome biogenesis	42254	2.96×10^{-7}	3.67×10^{-5}	6	372	TSR1;RRB1;ERB1;RIO2;BFR2;KRE33
	lipid metabolic process	6629	3.81×10^{-11}	1.85×10^{-8}	33	284	FAA3;FAA4;FAA1;ETR1;ERP2;CRM1;ERG6;COG2;PEX5;OAF1;CAT2;ERG2;ERG3;MAG2;ARE2;ARE1;POX1;COG7;FAR8;RML2;LRO1;FAR3;YPK1;UBX2;ERG10;POT1;ARV1;DGA1;SCS3;HDS1;STE20;SCS7;GET2
Unclustered	response to stress	6950	3.33×10^{-6}	7.78×10^{-5}	51	704	VRP1;INO2;CDC37;UBI4;SLX5;DOA1;MLP1;YPK1;EDC1;SRV2;CKA1;SOH1;SPT23;RLR1;SLT2;YAP1;SNF2;GLC7;TIR1;SLA1;SUB1;SUB2;YDJ1;ARP8;RDH54;GEA2;RAD52;HSP82;RPL40A;STM1;AHP1;YRA1;HHT1;EAF1;CAD1;ARF1;BCK1;STE20;HSC82;ACT1;ESA1;TUF1;PHO4;IST2;POL2;PTC1;OGG1;SOD1;SOD2;HYR1;YPD1

[a] *p*-Values calculated by hypergeometric distribution of a given gene ontology cluster observed in the network.
[b] Values calculated from *p*-value after FDR application.
[c] Total number of proteins found in the network that belong to a specific gene ontology.
[d] Total number of proteins belonging to a specific gene ontology.

Table 3. List of the major hub-bottleneck (HB) proteins identified by the topological network analysis

Protein	Systematic name[a]	Biochemical function[b]	Oxidative stress involvement	Referência
Cad1 (Yap2)	YDR423C	AP-1-like basic leucine zipper (bZIP) transcriptional activator; involved in stress responses	cadmium resistance in cells overexpressing *CAD1* gene Cad1p regulates proteins involved in the stabilization and folding of proteins during responses against oxidative stress	(Azevedo, *et al.*, 2007)
Nab2	YGL122C	Nuclear polyadenylated RNA-binding protein; required for nuclear mRNA export and poly(A) tail length control	*NAB2* is upregulated in oxidative stress	(Auesukaree, *et al.*, 2009)
Sua7	YPR086W	Transcription factor TFIIB, a general transcription factor required for transcription initiation and start site selection by RNA polymerase II	suppression of cellular oxidative sensitivity by *SUA7* overexpression	(Paes de Faria & Fernandes, 2006)
Ski8	YGL213C	Ski complex component and WD-repeat protein, mediates 3'-5' RNA degradation by the cytoplasmic exosome	ND	-
Ole1	YGL055W	Delta(9) fatty acid desaturase, required for monounsaturated fatty acid synthesis	*OLE1* is upregulated in H_2O_2 exposure, but downregulated in nitrosative stress	(Kelley & Ideker, 2009)
Are1	YCR048W	Acyl-CoA:sterol acyltransferase; endoplasmic reticulum enzyme that contributes the major sterol esterification activity in the absence of oxygen	ND	-
Are2	YNR019W	Acyl-CoA:sterol acyltransferase; endoplasmic reticulum enzyme that contributes the major sterol esterification activity in the presence of oxygen	ND	-
Pox1	YGL205W	Fatty-acyl coenzyme A oxidase, involved in the fatty acid beta-oxidation pathway	ND	-

[a](SGD) acession number
[b]Biochemical functions were obtained from the Saccharomyces Genome Database (SGD; http://www.yeastgenome.org/).
ND: not described

Table 4. Analysis of SOD, CAT, GPx activity and quantification of GSH in mutant and WT strains not exposed to oxidizing agents.

Strains	SOD[a]	CAT[b]	GPx[c]	GSH[d]
BY4741	5.81±0.27	4.93±0.20	13.91±0.54	100
*frm2*Δ	3.40±0.13***	14.27±0.56***	25.15±0.58***	118±3.78***
*hbn1*Δ	2.03±0.16***	4.77±0.18	14.16±0.59	98±1.73
*frm2*Δ *hbn1*Δ	0.89±0.13***	13.21±0.49***	24.63±0.84***	116.33±2.60***
cad1Δ	1.51±0.37***	5.23±1.17	12.82±1.54	97.33±2.08
*frm2*Δ *cad1*Δ	1.40±0.32***	13.64±1.23***	23.95±1.63***	114.03±2.05***
*hbn1*Δ *cad1*Δ	0.50±0.24***	5.30±1.04	13.04±0.58	96.28±4.04
*frm2*Δ *hbn1*Δ *cad1*Δ	0.40±0.11***	13.39±1.16***	24.10±1.82***	115.41±2.52***
ski8Δ	3.51±0.37***	20.27±1.38***	30.82±1.94***	128.07±4.56***
*frm2*Δ *ski8*Δ	3.55±0.32***	20.64±1.23***	31.45±1.22***	127.12±3.95***
*hbn1*Δ *ski8*Δ	0.90±0.46***	19.32±1.51***	29.69±0.88***	127.23±6.41***
*frm2*Δ *hbn1*Δ *ski8*Δ	0.99±0.31***	19.06±1.08***	30.43±1.27***	129.23±4.93***

Data are expressed as means ± SD. Values shown represent the means of at least three independent experiments. ***$p < 0.001$ indicate significant differences (ANOVA, Tukey's test)

[a]SOD activity was expressed as U SOD/mg protein

[b]CAT activity results were expressed as U CAT/mg protein

[c]GPx results were expressed as mM NADPH/min/mg protein

[d]Results were expressed as percentage GSH in relation the WT (% Wild-type)

Table 5. Determination of intracellular accumulation of reactive oxygen species by DCFH-DA and superoxide by NBT assay to 4-NQO; NDEA; H_2O_2 or *t*-BOOH exposure.

	DCFH-DA[a]					**NBT**[a]				
Strains	**Basal**	**4-NQO**	**NDEA**	**H_2O_2**	***t*-BOOH**	**Basal**	**4-NQO**	**NDEA**	**H_2O_2**	***t*-BOOH**
BY4741	6737±756	11058±621	9664±892	34681±1012	36123±2154	0.22±0.03	0.44±0.05	0.48±0.04	0.33±0.02	0.34±0.02
*frm2*Δ	6947±822	16698±548**	14794±1654**	12771±1569***	15938±3569***	0.29±0.07	0.63±0.02*	0.62±0.05**	0,39±0.05	0,35±0.06
*hbn1*Δ	7181±845	19791±548***	17389±1121**	36042±2365	37873±1112	0.23±0.05	0.72±0.06**	0.74±0.03***	0.34±0.04	0,33±0,05
*frm2*Δ *hbn1*Δ	7374±1451	26833±621***	23987±1254***	13919±1235***	16024±1325***	0.21±0.05	0.88±0.03***	0.88±0.08***	0.37±0.03	0.37±0,09
cad1Δ	6987±845	23791±578***	20938±1569***	33989±1632	35741±1969	0.31±0.11	0.80±0.05***	0.76±0.07***	0.39±0.06	0.36±0.04
*frm2*Δ *cad1*Δ	6737±769	22762±1265***	21879±1356***	13564±1847***	14862±1523***	0.30±0.09	0.79±0.02***	0.77±0.04***	0.32±0.04	0.34±0.03
*hbn1*Δ *cad1*Δ	7447±932	31023±1323***	27365±1045***	32968±1396	34978±1659	0.23±0.08	0.95±0.05***	0.93±0.05***	0.35±0.14	0.33±0.08
*frm2*Δ *hbn1*Δ *cad1*Δ	6123±939	30989±1237***	28966±1587***	13124±1598***	17231±2298***	0.24±0.03	0.97±0.08***	0.97±0.02***	0.34±0.07	0.32±0.05
ski8Δ	5947±1024	17598±1356**	15394±1475**	7771±1061***	10938±1569***	0.33±0.09	0.65±0.05*	0.67±0.03*	0.36±0.11	0.39±0.12
*frm2*Δ *ski8*Δ	6789±1214	16587±1321**	16326±2103**	6762±1265***	9879±1356***	0.26±0.06	0.64±0.11*	0.65±0.09*	0.35±0.06	0.36±0.10
*hbn1*Δ *ski8*Δ	6235±1125	25463±1589***	24632±2506***	8023±1323***	11365±1045***	0.32±0.12	0.87±0.05***	0.86±0.11***	0.32±0.15	0.35±0.02
*frm2*Δ *hbn1*Δ *ski8*Δ	6123±1058	26741±1756***	23687±1425***	6989±1237***	8966±1587***	0.29±0.04	0.88±0.09***	0.89±0.12***	0.35±0.13	0.36±0.09

Data are expressed as means±SD. Values shown are the means of at least three independent experiments. Data significant in relation to the BY4741strain ∗p<0.05, ∗∗p<0.01 and ∗∗∗p<0.001 indicate significant differences (ANOVA, Tukey's test)

Compounds concentrations: 4-NQO: 0.3 μg mL^{-1} (v/v); NDEA: 500 μM; H_2O_2: 5 mM and *t*-BOOH: 5 mM.

[a]Fluorescence values are expressed as arbitrary units

Table 6. Determination of TBARS levels in WT and mutant yeast strains treated for 2h at 30 ^{o}C to oxidants or not treated.

	TBARS[a]				
Strains	**Basal**	**4-NQO**	**NDEA**	**H_2O_2**	***t*-BOOH**
BY4741	4.37±1.94	11.53±1.02	12.07±1.36	26.72±1.51	27.22±0.60
*frm2*Δ	3.90±1.63	17.95±0.78*	17.53±1.24*	14.51±1.28***	14.96±1.57***
*hbn1*Δ	4.52±1.15	21.29±1.37**	20.65±1.06**	26.66±0.94	28.88±1.04***
*frm2*Δ *hbn1*Δ	4.19±2.11	28.03±2.01***	26.52±1.95***	15.05±1.03***	15.98±1.55***
cad1Δ	4.62±1.38	24.27±1.08***	22.27±2.67***	25.87±3.23	28.07±2.24
*frm2*Δ *cad1*Δ	5.61±0.92	23.98±2.40***	23.84±2.59***	15.56±2.06***	15.65±3.97***
*hbn1*Δ *cad1*Δ	5.78±0.66	30.37±0.82***	30.45±1.32***	27.01±3.96	29.43±4.04
*frm2*Δ *hbn1*Δ *cad1*Δ	5.96±1.39	30.05±2.07***	31.42±1.08***	16.23±2.78***	16.22±3.88***
ski8Δ	5.63±1.25	18.02±2.56*	19.02±3.32**	9.94±0.61***	9.27±1.09***
*frm2*Δ *ski8*Δ	4.69±1.56	19±3.21**	28.09±2.14***	9.89±1.21***	9.34±1.02***
*hbn1*Δ *ski8*Δ	5.23±1.63	27.61±3.65***	26.89±3.18***	10.04±1.35***	9.29±1.14***
*frm2*Δ *hbn1*Δ *ski8*Δ	5.41±2.21	26.98±3.48***	28.23±4.11***	10.93±2.07***	9.09±1.94***

Data are expressed as means±SD. Values shown are the means of at least three independent experiments. Data significant in relation to the BY4741strain $*p<0.05$, $**p<0.01$ and $***p<0.001$ indicate significant differences (ANOVA, Tukey's test)

Compounds concentrations: 4-NQO: 0.3 μg mL^{-1} (v/v); NDEA: 500 μM; H_2O_2: 5 mM and *t*-BOOH: 5 mM.

[a]The results were expressed as nM TBARS/mg protein

Figure legends

Fig. 1. A physical protein–protein interaction network of *S.cerevisiae* nitroreductases. Both clustered proteins (composing different subnetworks) and unclustered proteins are represented by nodes of different shapes and colors, as indicated into figure.

Fig. 2. An amplified view of proteins that compose subnetworks from the PPI network. In Panel (A): Proteins that compose subnetwork 1, associated with RNA biosynthetic process and RNA polymerase complex. Panel (B): Proteins that compose subnetwork 2, associated with nuclear pore/ RNA transport and RNA export from nucleus. Panel (C): exosome (RNase complex), mRNA catabolic process and ski complex. Panel (D): ribosome biogenesis and lipid metabolism.

Fig. 3. Centralities analysis of proteins associated with the PPI network. Panel (A): Smaller PPI subnetwork derived from calculated centralities analyses. The ten major hub-bottleneck and non-hub-bottleneck proteins with the highest bottleneck values are indicated. Panel (B to D): Dashed lines represent the threshold value calculated for each centrality. Only those proteins with a high bottleneck score are indicated. Legend: hub-bottleneck (HB); non hub-bottleneck (NH-B).

Fig. 4. Surviving screening of haploids strains deficient in respective HB proteins or diploids strains with a single copy of gene in exposure to different oxidants. (A) 4-NQO; (B) NDEA; (C) H_2O_2; (D) *t*-BOOH. Cells were treated for 2 h at 30 °C. Data represent an average of three independent experiments. Data are expressed as means ± SD.

Fig. 5. Sensitivity of Cad1p yeast mutant strains in stationary growth phase to different oxidants. (A) 4-NQO; (B) NDEA; (C) H_2O_2; (D) *t*-BOOH. Cells were treated for 2 h at 30 ◦C. Data represent an average of three independent experiments. Data are expressed as means ± SD.

Fig. 6. Sensitivity of Ski8p yeast mutant strains in stationary growth phase to different oxidants. (A) 4-NQO; (B) NDEA; (C) H_2O_2; (D) *t*-BOOH. Cells were treated for 2 h at 30 ◦C. Data represent an average of three independent experiments. Data are expressed as means ± SD.

Fig. 7. Ole1p influence in lipid peroxidation and Nab2p interaction with Sod enzymes. Panel (A): Determination of TBARS in diploid strains with or two copies of *OLE1* gene for 2 h at 30 ◦C or not treated with oxidants. Panel (B): An amplified view of interaction of Nab2p with Sod enzymes and Hbn1p gathered of yeast nitroreductases network. Panel (C): Analysis of basal SOD activity in diploid strains with one or two copies of *NAB2* gene. Panel (D): Production of intracellular superoxide in yeast diploid strains with one or two copies of *NAB2* gene. Data represent an average of three independent experiments. Data are expressed as means ± SD. Values shown are the means of at least three independent experiments. $*p < 0.05$ indicate significant differences (Student *t*-test).

Fig. 8. Putative pathways of action of Hbn1p and Frm2p in oxidative stress response in yeast cells.

Fig. 1.

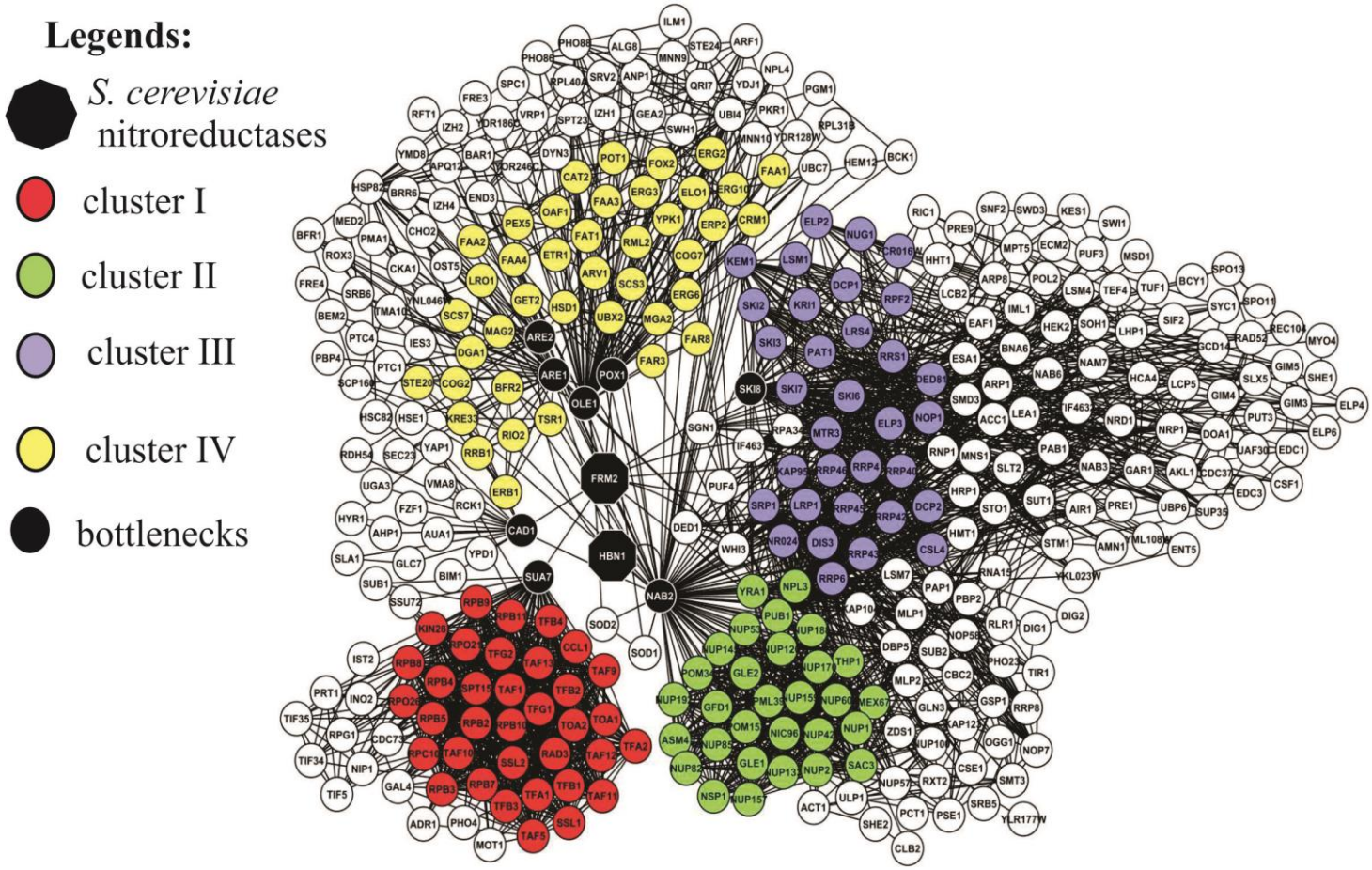

Legends:
S. cerevisiae nitroreductases
cluster I
cluster II
cluster III
cluster IV
bottlenecks

Fig. 2

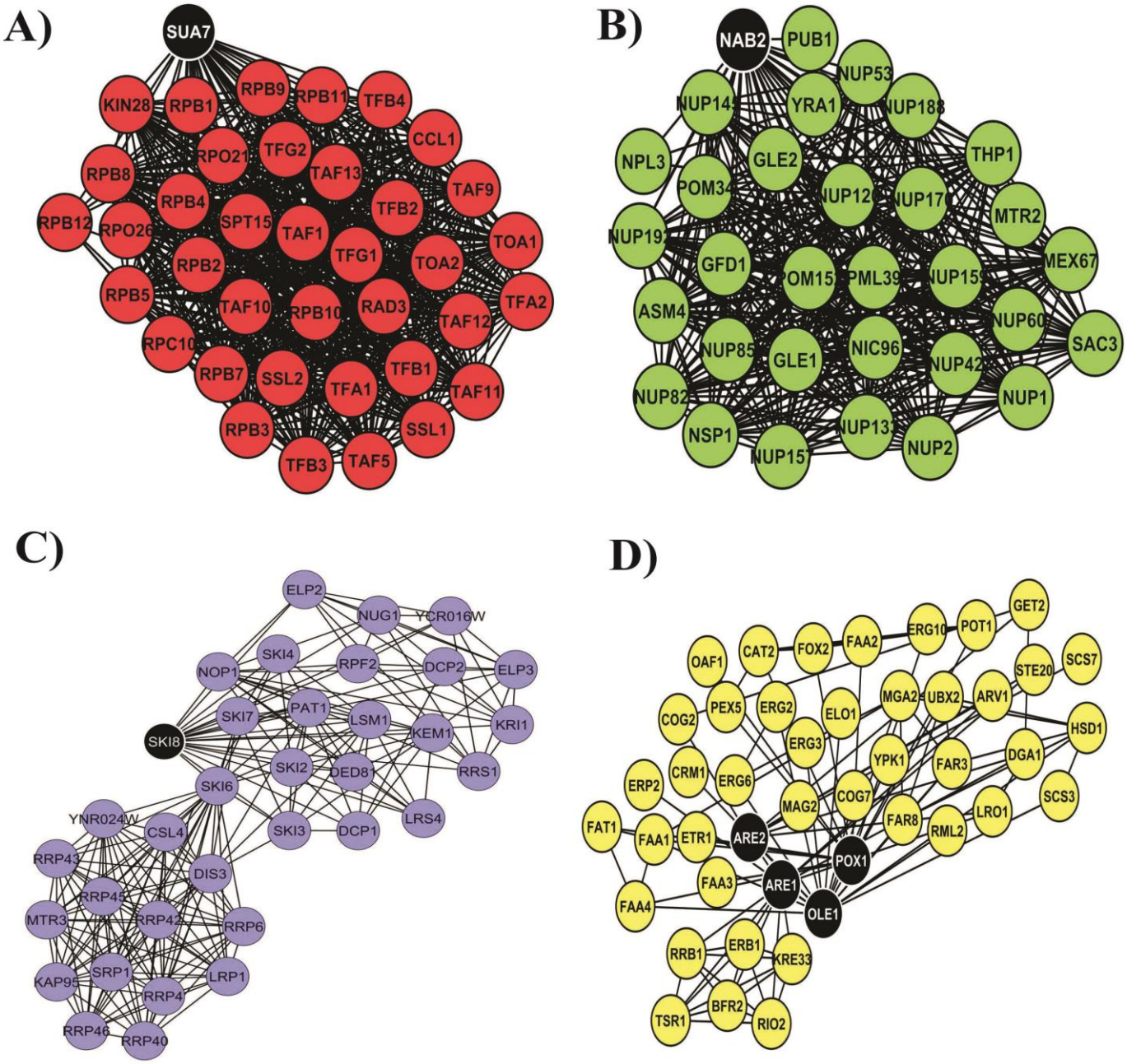

A)
SUA7
KIN28
RPB1
RPB9
RPB11
TFB4
CCL1
RPO21
TFG2
TAF13
RPB8
TAF9
RPB4
TFB2
RPB12
RPO26
SPT15
TAF1
TOA1
TFG1
TOA2
RPB2
RPB5
TAF10
RPB10
RAD3
TFA2
TAF12
RPC10
RPB7
SSL2
TFB1
TFA1
TAF11
RPB3
SSL1
TFB3
TAF5
B)
NAB2
PUB1
NUP53
NUP145
YRA1
NUP188
NPL3
GLE2
THP1
POM34
NUP120
NUP170
MTR2
NUP192
GFD1
MEX67
POM152
PML39
NUP159
ASM4
NUP60
SAC3
NUP85
GLE1
NIC96
NUP42
NUP82
NUP1
NSP1
NUP133
NUP2
NUP157
C)
ELP2
NUG1
YCR016W
SKI4
NOP1
RPF2
DCP2
ELP3
SKI7
PAT1
LSM1
KEM1
KRI1
SKI8
SKI2
DED81
RRS1
SKI6
YNR024W
CSL4
SKI3
DCP1
LRS4
RRP43
DIS3
RRP45
MTR3
RRP42
RRP6
KAP95
SRP1
LRP1
RRP4
RRP46
RRP40
D)
GET2
ERG10
POT1
OAF1
CAT2
FOX2
FAA2
STE20
SCS7
MGA2
UBX2
ARV1
COG2
PEX5
ERG2
ELO1
HSD1
ERG3
YPK1
FAR3
DGA1
CRM1
ERP2
ERG6
COG7
SCS3
MAG2
FAR8
LRO1
FAT1
FAA1
ETR1
ARE2
RML2
POX1
FAA3
ARE1
FAA4
OLE1
RRB1
ERB1
KRE33
TSR1
BFR2
RIO2

Fig. 3.

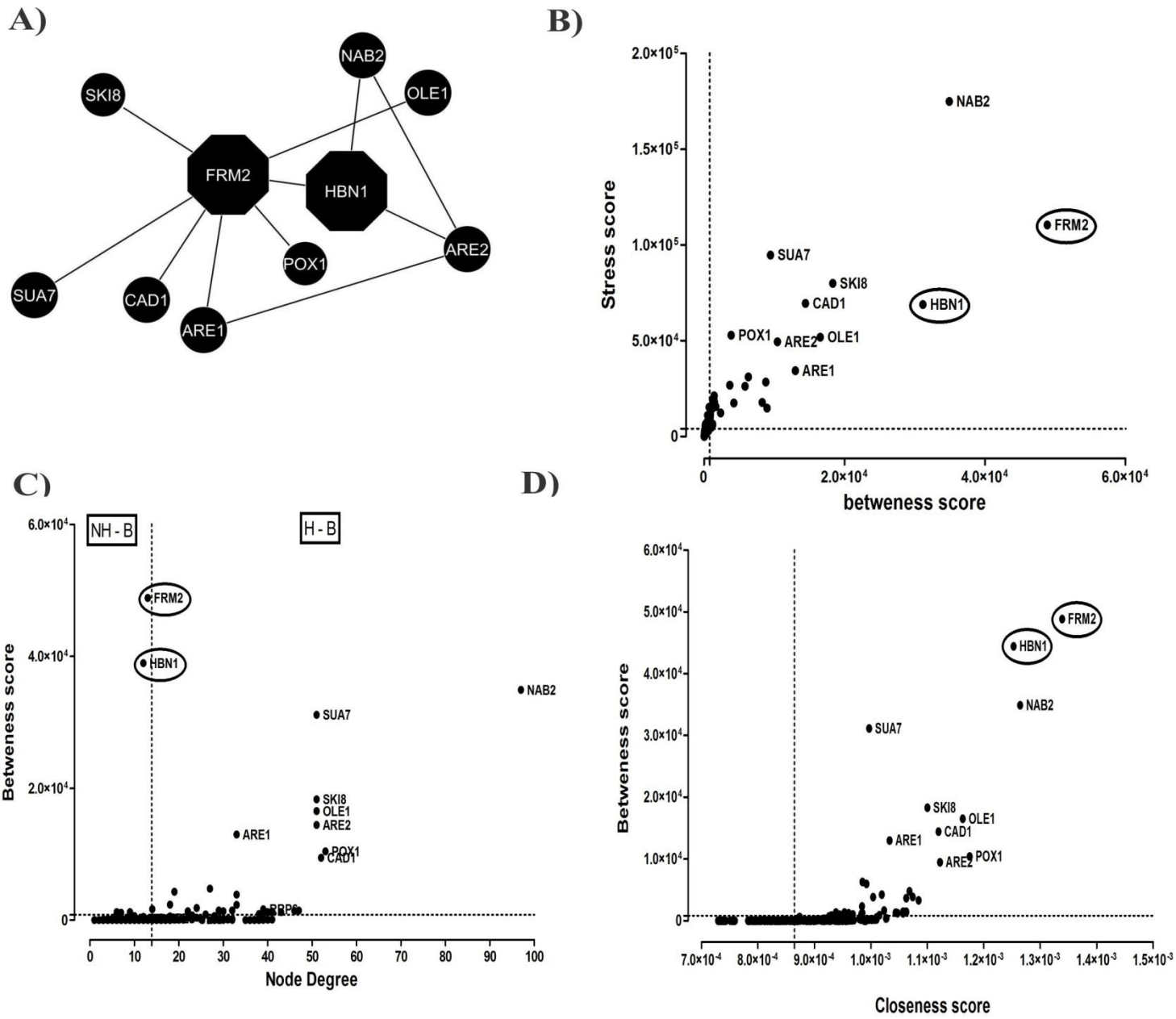

A)
NAB2
SKI8
OLE1
FRM2
HBN1
POX1
ARE2
SUA7
CAD1
ARE1
B)
Stress score
betweness score
NAB2
FRM2
SUA7
SKI8
CAD1
HBN1
POX1
ARE2
OLE1
ARE1
C)
NH - B
H - B
Betweness score
Node Degree
FRM2
HBN1
NAB2
SUA7
SKI8
OLE1
ARE2
ARE1
POX1
CAD1
D)
Betweness score
Closeness score
FRM2
HBN1
NAB2
SUA7
SKI8
OLE1
CAD1
ARE1
ARE2
POX1

Fig. 4.

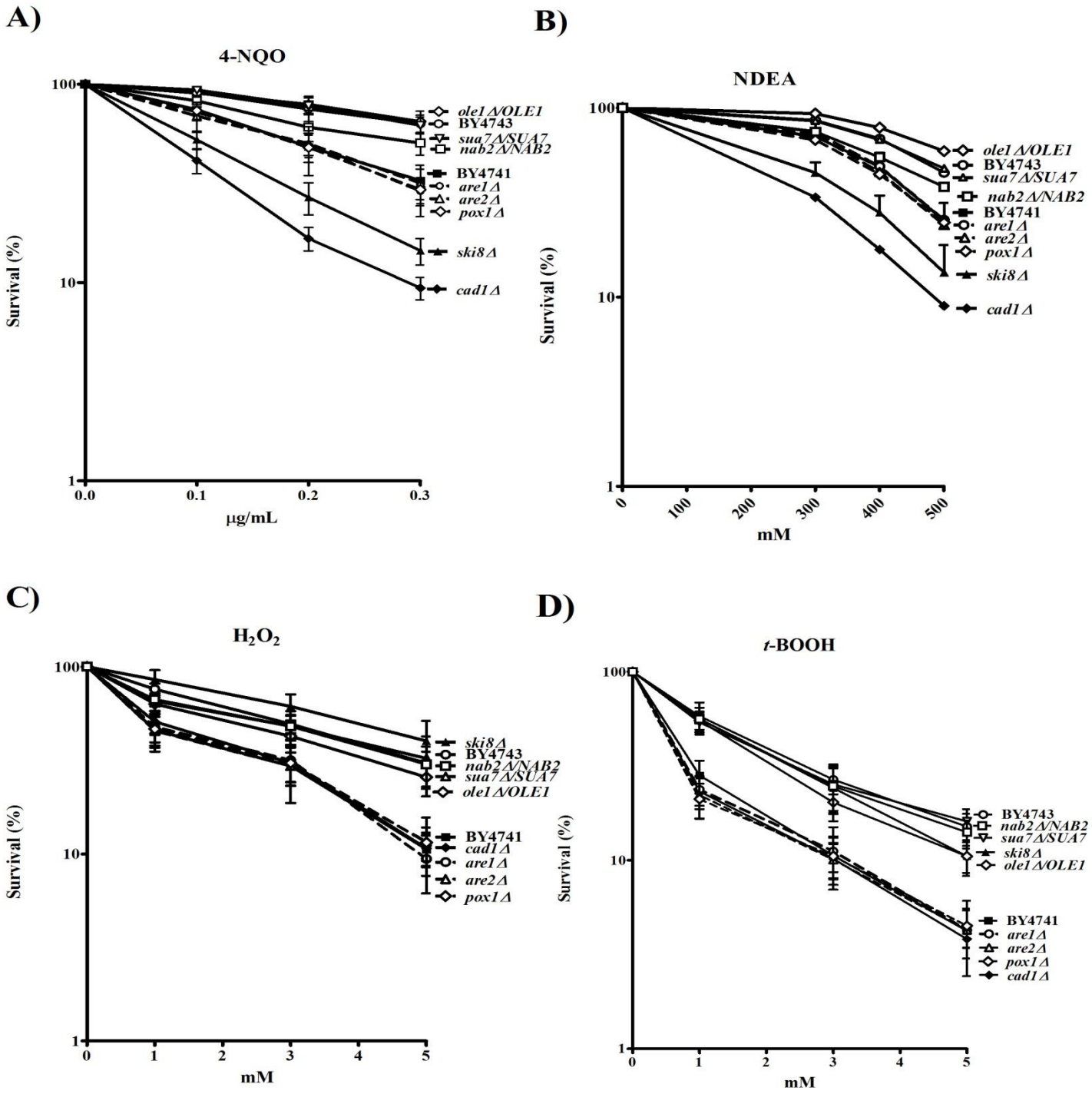

A)
4-NQO
Survival (%)
100
10
1
0.0
0.1
0.2
0.3
μg/mL
ole1Δ/OLE1
BY4743
sua7Δ/SUA7
nab2Δ/NAB2
BY4741
are1Δ
are2Δ
pox1Δ
ski8Δ
cad1Δ
B)
NDEA
Survival (%)
100
10
1
0
100
200
300
400
500
mM
ole1Δ/OLE1
BY4743
sua7Δ/SUA7
nab2Δ/NAB2
BY4741
are1Δ
are2Δ
pox1Δ
ski8Δ
cad1Δ
C)
H2O2
Survival (%)
100
10
1
0
1
2
3
4
5
mM
ski8Δ
BY4743
nab2Δ/NAB2
sua7Δ/SUA7
ole1Δ/OLE1
BY4741
cad1Δ
are1Δ
are2Δ
pox1Δ
D)
t-BOOH
Survival (%)
100
10
1
0
1
2
3
4
5
mM
BY4743
nab2Δ/NAB2
sua7Δ/SUA7
ski8Δ
ole1Δ/OLE1
BY4741
are1Δ
are2Δ
pox1Δ
cad1Δ

Fig. 5.

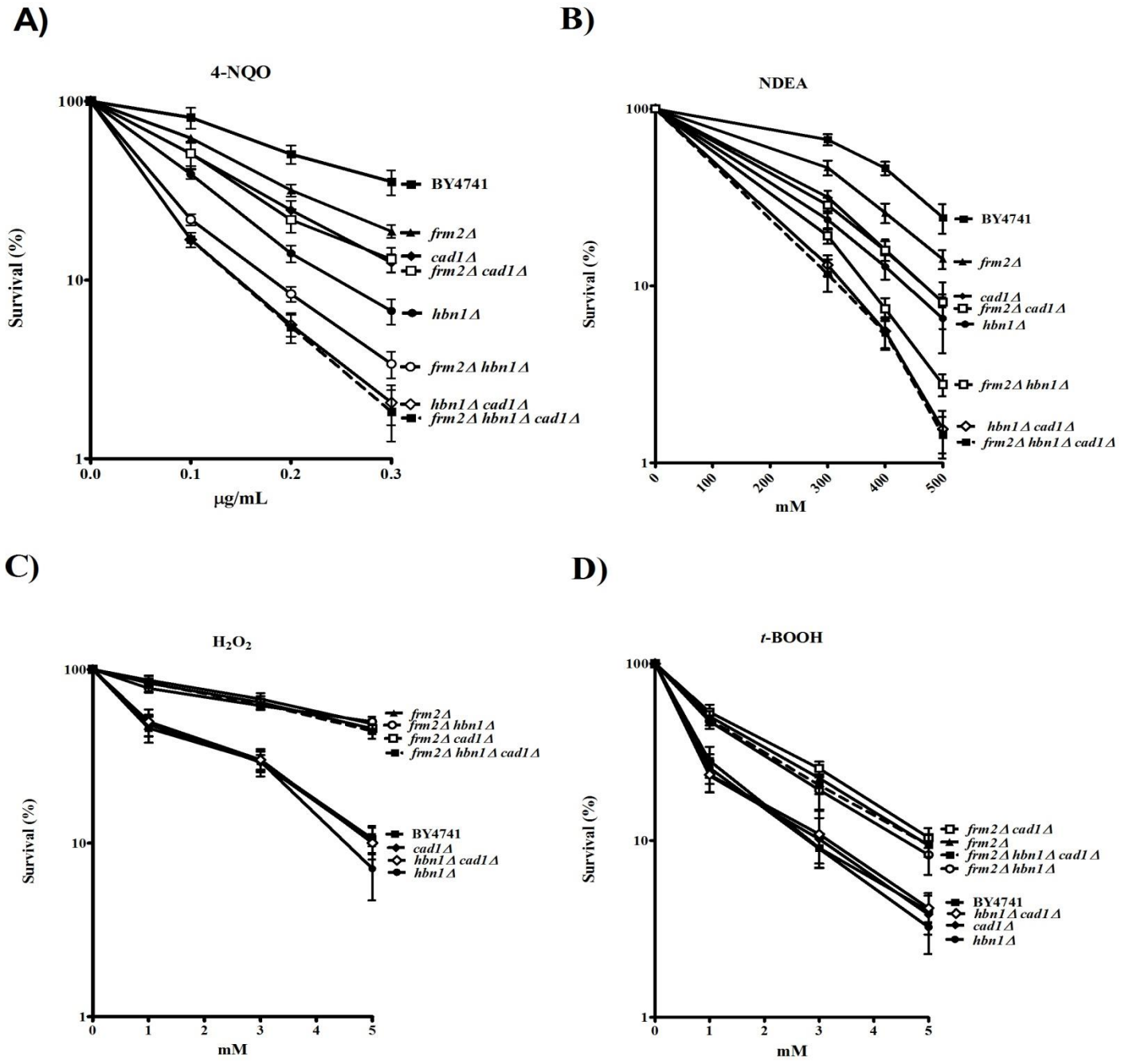

A)
4-NQO
Survival (%)
100
10
1
0.0
0.1
0.2
0.3
μg/mL
BY4741
frm2Δ
cad1Δ
frm2Δ cad1Δ
hbn1Δ
frm2Δ hbn1Δ
hbn1Δ cad1Δ
frm2Δ hbn1Δ cad1Δ
B)
NDEA
Survival (%)
100
10
1
0
100
200
300
400
500
mM
BY4741
frm2Δ
cad1Δ
frm2Δ cad1Δ
hbn1Δ
frm2Δ hbn1Δ
hbn1Δ cad1Δ
frm2Δ hbn1Δ cad1Δ
C)
H2O2
Survival (%)
100
10
1
0
1
2
3
4
5
mM
frm2Δ
frm2Δ hbn1Δ
frm2Δ cad1Δ
frm2Δ hbn1Δ cad1Δ
BY4741
cad1Δ
hbn1Δ cad1Δ
hbn1Δ
D)
t-BOOH
Survival (%)
100
10
1
0
1
2
3
4
5
mM
frm2Δ cad1Δ
frm2Δ
frm2Δ hbn1Δ cad1Δ
frm2Δ hbn1Δ
BY4741
hbn1Δ cad1Δ
cad1Δ
hbn1Δ

Fig. 6.

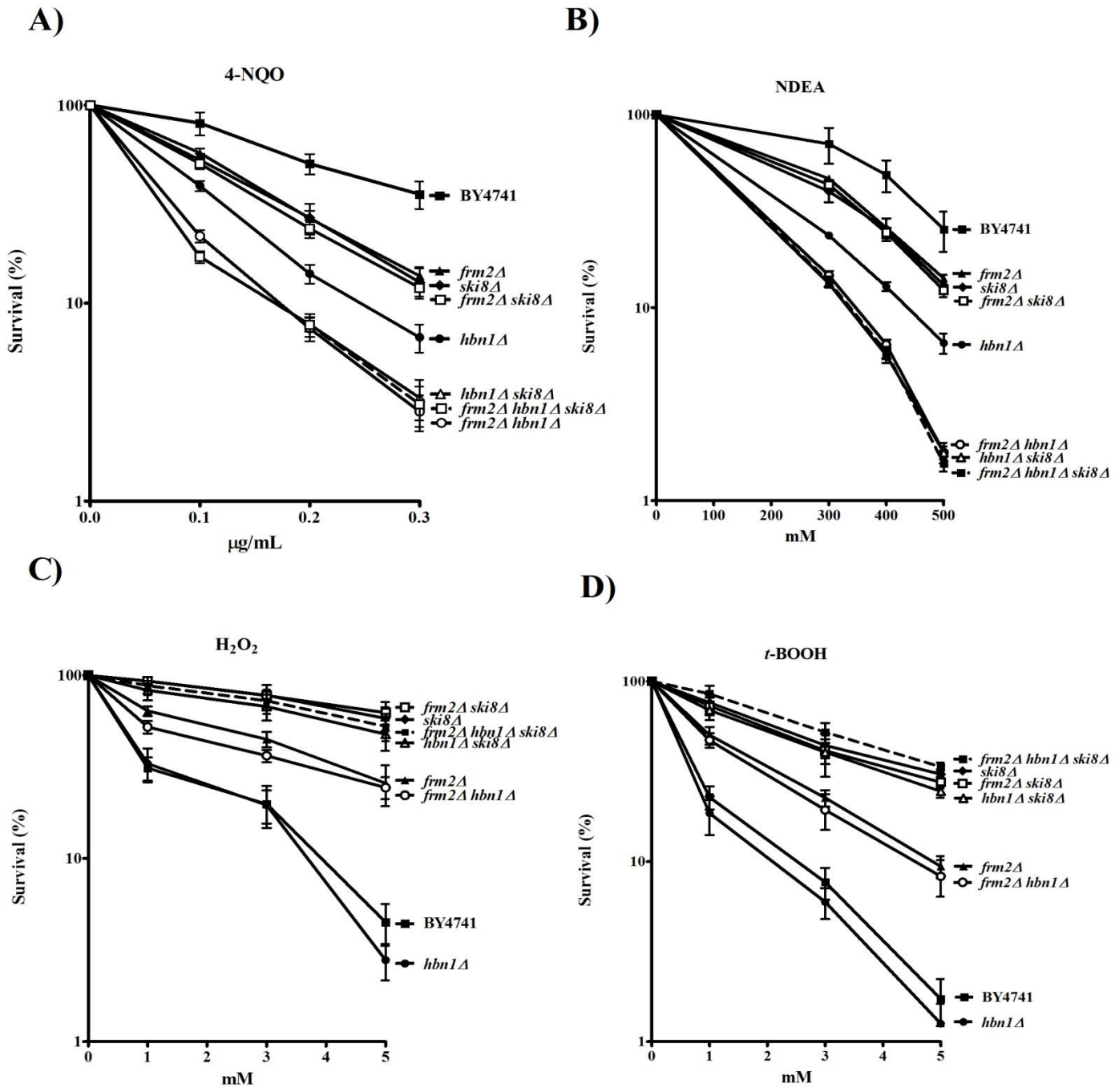

A)
4-NQO
Survival (%)
100
10
1
0.0
0.1
0.2
0.3
μg/mL
BY4741
frm2Δ
ski8Δ
frm2Δ ski8Δ
hbn1Δ
hbn1Δ ski8Δ
frm2Δ hbn1Δ ski8Δ
frm2Δ hbn1Δ
B)
NDEA
Survival (%)
100
10
1
0
100
200
300
400
500
mM
BY4741
frm2Δ
ski8Δ
frm2Δ ski8Δ
hbn1Δ
frm2Δ hbn1Δ
hbn1Δ ski8Δ
frm2Δ hbn1Δ ski8Δ
C)
H2O2
Survival (%)
100
10
1
0
1
2
3
4
5
mM
frm2Δ ski8Δ
ski8Δ
frm2Δ hbn1Δ ski8Δ
hbn1Δ ski8Δ
frm2Δ
frm2Δ hbn1Δ
BY4741
hbn1Δ
D)
t-BOOH
Survival (%)
100
10
1
0
1
2
3
4
5
mM
frm2Δ hbn1Δ ski8Δ
ski8Δ
frm2Δ ski8Δ
hbn1Δ ski8Δ
frm2Δ
frm2Δ hbn1Δ
BY4741
hbn1Δ

Fig. 7.

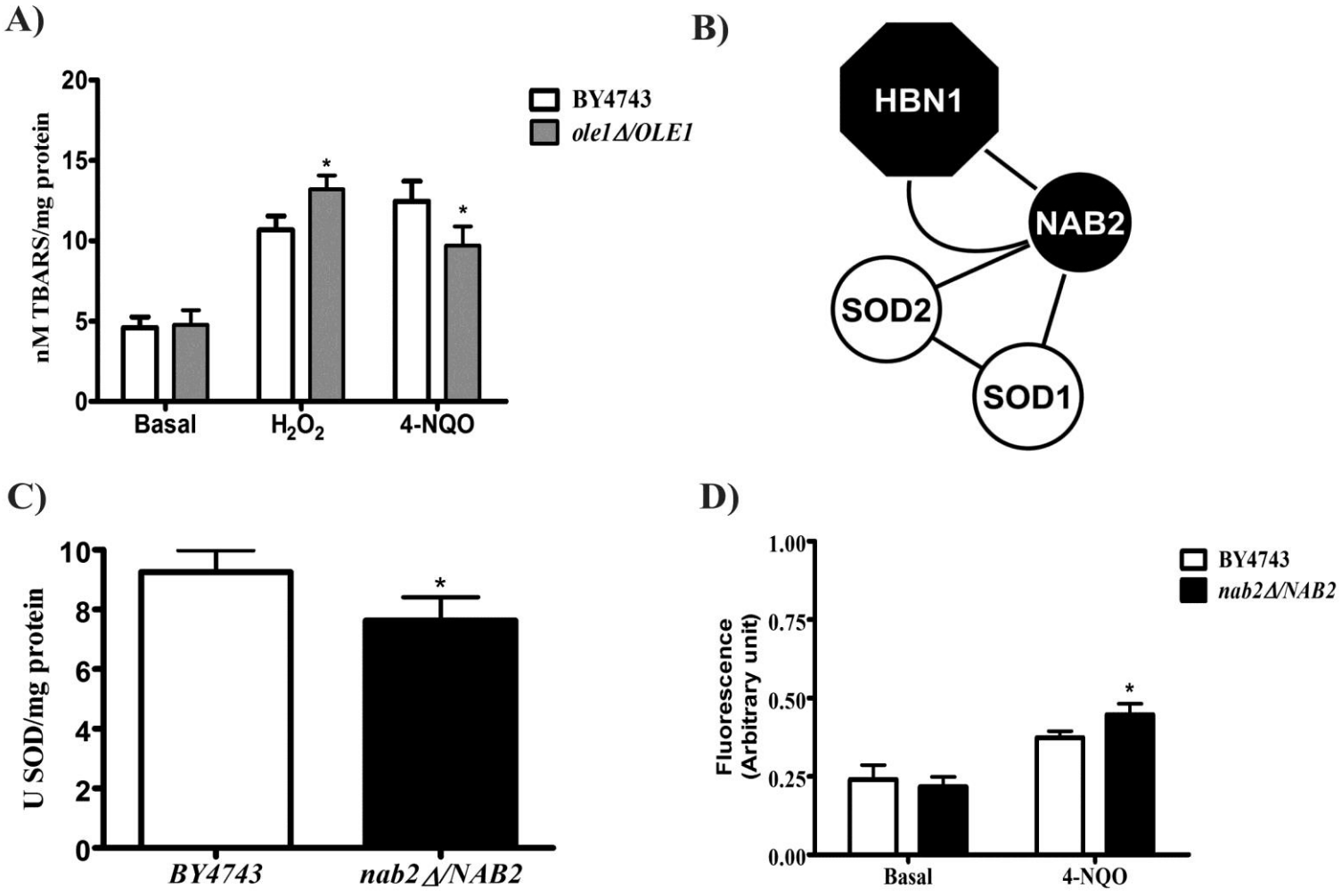
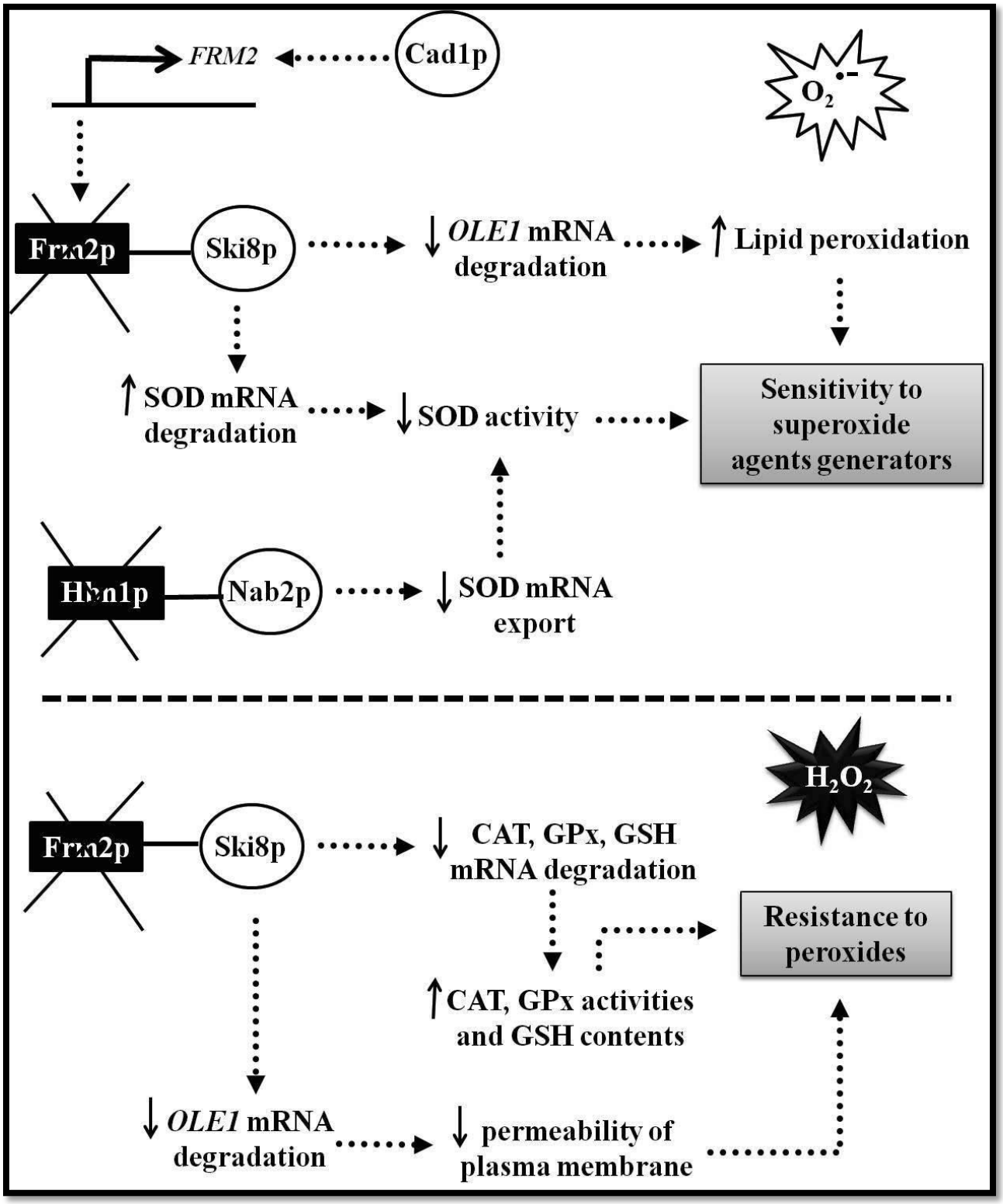

A)
nM TBARS/mg protein
20
15
10
5
0
Basal
H_2O_2
4-NQO
BY4743
ole1Δ/OLE1
B)
HBN1
NAB2
SOD2
SOD1
C)
U SOD/mg protein
10
8
6
4
2
0
BY4743
nab2Δ/NAB2
D)
Fluorescence (Arbitrary unit)
1.00
0.75
0.50
0.25
0.00
Basal
4-NQO
BY4743
nab2Δ/NAB2

Fig. 8.


FRM2
Cad1p
$O_2^{\bullet-}$
Frn2p
Ski8p
↓ OLE1 mRNA degradation
↑ Lipid peroxidation
↑ SOD mRNA degradation
↓ SOD activity
Sensitivity to superoxide agents generators
Hbn1p
Nab2p
↓ SOD mRNA export
H_2O_2
Frn2p
Ski8p
↓ CAT, GPx, GSH mRNA degradation
Resistance to peroxides
↑ CAT, GPx activities and GSH contents
↓ OLE1 mRNA degradation
↓ permeability of plasma membrane

Discussão

Discussão Geral

Por que nitrorredutases?

A grande maioria dos nitrocompostos encontrados no ambiente é oriunda de processos industriais, sendo utilizados na síntese de diversos produtos ou provenientes da combustão incompleta de combustíveis fósseis (MORI *et al.*, 2003; SHEN *et al.*, 2012). Desta forma, considerado um importante grupo de poluentes ambientais (JU & PARALES, 2010; ARORA *et al.*, 2012). Neste contexto, as enzimas nitrorredutases têm atraído um enorme interesse devido ao papel central que desempenham na metabolização de nitrocompostos e, consequentemente, na toxicidade dessas substâncias (PUROHIT & BASU, 2000; PADDA *et al.*, 2003; MAEDA *et al.*, 2007). Este fato influencia diretamente na saúde humana, já que nitrorredutases presentes em bactérias da microbiota intestinal podem metabolizar nitrocompostos exógenos que entram em contato com o organismo humano, como antibióticos ou poluentes resultando em metabólitos tóxicos (RAFII & HANSEN, 1998; DE VRESE & SCHREZENMEIR, 2008).

As nitrorredutases apresentam aplicação clínica devido à sua capacidade de converter pró-drogas não tóxicas, como o composto CB1954 em potentes agentes citotóxicos. Estas enzimas têm sido utilizadas nas terapias *gene-directed enzyme prodrug therapy* (GDEPT) e *antibody-directed enzyme prodrug therapy* (ADEPT) para emprego potencial no tratamento de certos tumores, fazendo com que células alvo expressando nitrorredutases bacterianas sejam capazes de metabolizar o nitrocomposto (Figura 2, Capítulo I) (DENNY, 2002; SEARLE *et al.*, 2004; CHRISTOFFERSON & WILKIE, 2009). Este método também pode ser empregado em diversos estudos quando a intenção é a destruição de uma população de células específicas, como investigação da

recuperação tecidual, mecanismos moleculares da regeneração e desenvolvimento celular (FELMER *et al.*, 2002).

Muitos estudos têm associado as nitrorredutases à suscetibilidade a antibióticos, já que muitos antibióticos necessitam ser metabolizados por estas enzimas (MARTINEZ-JULVEZ *et al.*, 2012). Bactérias *Helicobacter pylori* deficientes em nitrorredutases possuem maior tolerância a antibióticos, como metronidazol, sendo comumente um fator associado às infecções hospitalares (KAAKOUSH *et al.*, 2009; IWANCZAK & IWANCZAK, 2012).

Essas enzimas ainda apresentam um possível uso em biorremediação, sendo empregadas principalmente em solos contaminados com nitrocompostos recalcitrantes como o TNT (SMETS *et al.*, 2007). Para isso, são utilizadas bactérias ou plantas geneticamente modificadas que expressam nitrorredutases bacterianas, metabolizando o composto para formas mais biodegradáveis (SPAIN, 1995; JU & PARALES, 2010). Esse método tem significativa vantagem em relação a métodos tradicionais, como a remoção de TNT por escavação, por reduzir o impacto ambiental (HANNINK *et al.*, 2001; CABALLERO *et al.*, 2005). As nitrorredutases NfsA de *Escherichia coli* e Frp de *Vibrio harveyi* são capazes de reduzir cromato para uma forma menos tóxica, indicando um uso possível na biorremediação deste metal (KWAK *et al.*, 2003; ACKERLEY *et al.*, 2004). Outro emprego dessas enzimas é como biossensor na detecção de nitrocompostos presentem no ambiente. (GWENIN *et al.*, 2007). Este método tem vantagens em relação a procedimentos clássicos como cromatografias, por ser menos dispendioso e mais específico, podendo ser utilizado em detecções *in situ* (SPAIN, 1995). Ainda neste sentido, diversos testes de avaliação da genotoxicidade, como Teste de Ames, vêm sendo realizados utilizando linhagens de *Salmonella typhimurium* deficientes ou que superexpressam estas enzimas, para identificar, avaliar e elucidar o

mecanimo da genotoxicidade de vários compostos nitroheterocíclicos e nitroaromáticos (AIUB *et al.*, 2006).

Classificação, distribuição e estrutura

As nitrorredutases podem ser classificadas funcionalmente pelo mecanismo de redução ou filogeneticamente com base na similaridade da sequência primária. Do ponto de vista funcional, as nitrorredutases são divididas em dois grupos (Figura 1, Capítulo I e Figura 1, Capítulo II): tipo I ou insensíveis ao oxigênio, que realizam uma redução sequencial por transferência de dois elétrons do NAD(P)H para o grupo NO_2^-, formando intermediários nitroso, hidroxilamina e por fim aminas primárias; e tipo II que catalisam, na presença de oxigênio, a transferência de um elétron dos agentes redutores para o grupo NO_2^-, realizando um ciclo redox e produzindo um radical nitro-ânion, o qual regenera o nitrocomposto e origina superóxido (PETERSON *et al.*, 1979; ROLDAN *et al.*, 2008).

Essas enzimas são largamente distribuídas em eubactérias, e, embora de forma mais restrita em eucariotos, como protozoários, fungos e mamíferos (DE OLIVEIRA *et al.*, 2007; THOMAS *et al.*, 2009). As nitrorredutases do tipo I, do ponto de vista filogenético, dividem-se em dois grupos ou famílias: NfsA/Frp (grupo A) e NfsB/Frase I (grupo B) (ROLDAN *et al.*, 2008). Na família NfsA/Frp, são incluídas as nitrorredutases SnrA de *S. typhimurium*, PnrA de *Pseudomonas putida* JLR11 e Frp de *Vibrio. harveyi* (ZENNO *et al.*, 1998a; NOKHBEH *et al.*, 2002; CABALLERO *et al.*, 2005). A família NfsB/Frase I inclui as nitrorredutase NR de *Enterobacter cloacae*, Cnr de *S. typhimurium* e FRaseI de *Vibrio fischeri* (Tabela 1, Capítulo II) (BRYANT & DELUCA, 1991; ZENNO *et al.*, 1996b; WATANABE *et al.*, 1998). Interessantemente, esses dois grupos possuem baixa similaridade entre si (ROLDAN *et al.*, 2008). A nossa

análise filogenética confirmou a presença essas duas famílias de nitrorredutases bacterianas e mais dois grupos de nitrorredutases: o grupo Frm2p/Hbn1p, que reúne as nitrorredutases de *Saccharomyces cerevisiae* e CinD de *Lactococcus lactis* e o grupo IYD/BluB, que reúne as proteínas iodotirosina deiodinase (IYD) de mamíferos e BluB presentes nas bactérias *Rhodobacter capsulatos*, *Sinorhizobium meliloti e Selenomonas ruminantium* (Figura 2, Capítulo II). A presença de sequências de nitrorredutases em genomas eucariotos é coerente com a hipótese de transferência lateral. Este fato já foi descrito por Nixon e colaboradores (2002) que demonstrou a transferência lateral de vários genes de metabolização, incluindo de nitrorredutases, identificando a presença de uma provável nitrorredutase tipo I em *Giardia lamblia*. Adicionalmente, foram também encontramos duas sequências de nitrorredutases bacterianas nos protozoário *Leishmania major* e *Trypanosoma brucei* (Tabela 1, Anexo I).

Apesar das diferenças de similaridade entre os grupos de nitrorredutases, essas enzimas possuem estrutura tridimensional conservada. As nitrorredutases são proteínas com estrutura homodimérica, sem metais ou pontes de dissulfeto, constituídas basicamente por α hélices e estruturas β (Figura 1B, Capítulo II). As duas subunidades se unem formando uma cavidade hidrofóbica entre elas, onde se localiza o sítio ativo da enzima (Figura 3B, Capítulo II) (HAYNES *et al.*, 2002). Suportando a similaridade estrutural dessas enzimas também em eucariotos, os dados obtidos na análise de agrupamentos hidrofóbicos (HCA) apontou um alto nível de semelhança das estruturas secundárias entre nitrorredutases bacterianas e de fungos. São proteínas compostas em grande extensão por α hélices (α1-α8) apresentando dois subdomínios bem característicos (Figura 2, Anexo I). Em adição, a modelagem confirmou os resultados obtidos no HCA, quanto à conservação estrutural das nitrorredutases, bem como apontou um sítio FMN, característico de todas as proteínas pertencentes à família das

nitrorredutases (Figura 3, Anexo I). Ainda neste sentido, Thomas e colaboradores (2009) confirmou a mesma semelhança estrutural da proteína IYD de mamíferos com as nitrorredutases bacterianas.

No sítio ativo há resíduos de aminoácidos conservados responsáveis pela ligação, por pontes de hidrogênio, com o grupo prostético FMN (Figura 3E, Capítulo II) (HAYNES *et al.*, 2002). As nitrorredutases do Grupo A utilizam NADPH como fonte de elétrons, enquanto as nitrorredutases do Grupo B usam tanto NADPH como NADH, possivelmente devido a diferenças de resíduos de aminoácidos no sítio ativo que afetam o tipo de ligação com o cofator (PARKINSON *et al.*, 2000; HAYNES *et al.*, 2002). Um exemplo disso é o resíduo Arg203 em NfsA que estabelece uma ponte de hidrogênio com NADPH. Por outro lado, em NfsB a ligação se dá pelo resíduo Phe124, que interage apenas por uma ponte salina, sugerindo que esse fato reduz a especificidade desse grupo de enzimas em relação ao cofator (MANINA *et al.*, 2010). O mecanismo de redução ocorre por duas reduções sucessivas, onde é transferido, no sítio ativo, um elétron do NAD(P)H para o grupo prostético e deste para o substrato (HAYNES *et al.*, 2002).

Interessantemente, no sítio ativo ainda há resíduos de aminoácidos não conservados no sítio ativo da enzima, que variam entre as diferentes nitrorredutases. Essa porção variável do sítio ativo provavelmente determina as diferenças de especificidade que as nitrorredutases possuem em relação ao substrato (HAYNES *et al.*, 2002). Possivelmente, esse fato também se relacione a variedade de substratos metabolizados pelas nitrorredutases e, consequentemente, com as diferenças nas possíveis funções fisiológicas que as diferentes nitrorredutases possam desempenhar nos organismos (ROLDAN *et al.*, 2008).

Papel fisiológico das nitrorredutases: alguns fatos e muitas possibilidades

Embora as nitrorredutases sejam muito estudadas atualmente, o papel fisiológico real destas enzimas é uma questão a ser refletida. É possível que essas enzimas participem de vias de detoxificação de nitrocompostos, convertendo estes a formas mais solúveis e mais biodegradáveis (SPAIN, 1995). Ainda pode se considerar a utilização de nitrocompostos aromáticos como fontes de nitrogênio ou carbono (SPAIN, 1995; DIAZ *et al.*, 2001). Portanto, essa capacidade de metabolização conferiria vantagens adaptativas às bactérias em ambientes expostos aos nitrocompostos (ROLDAN *et al.*, 2008). Como Roldan e colaboradores (2008) discute em sua revisão, a maior presença de nitrocomposto no ambiente é devido a atividades humanas, principalmente industriais sendo, portanto, eventos recentes na história evolutiva. Desta forma, dificilmente nitrocompostos sejam os substratos originais dessas enzimas e a sua função primordial pode ter sido perdida ou modificada pela recente exposição a estes compostos.

Deve-se salientar ainda que essas enzimas são capazes de reduzir uma ampla variedade de substratos, não somente nitrocompostos, mas também outras substâncias, como metais (KWAK *et al.*, 2003; TAKEDA *et al.*, 2010). Reunindo esses fatos, pode-se considerar a ideia de que as nitrorredutases possuam outras funções, além de participarem da redução de nitrocompostos. Neste contexto, estudos vêm sugerindo possíveis funções para as nitrorredutases no metabolismo celular (Tabela 1, Capítulo II).

A primeira hipótese de função fisiológica para estas enzimas foi a redução de 3-nitrotirosina, um produto gerado no estresse nitrosativo que pode levar à inativação de proteínas (LIGHTFOOT *et al.*, 2000). No entanto, Lightfoot e colaboradores (2000) mostrou que as nitrorredutases NfsA e NfsB de *E. coli* não eram capazes de realizar esta

redução, possivelmente por estas enzimas somente conseguirem abrigar substratos menores em seu sítio ativo.

Algumas evidências sugerem que essas enzimas possam estar envolvidas no metabolismo de íons ferro (TAKEDA *et al.*, 2007; TAKEDA *et al.*, 2010). Por exemplo, as nitrorredutases DrgA de *Synechocystis* e NfsB de *E. coli* podem reduzir o íon Fe^{+3}, tanto livre como complexados com quelantes naturais ou sintéticos (TAKEDA *et al.*, 2010). Esse fato é coerente com os dados de biologia de sistemas, os quais indicaram interação dessas enzimas com proteínas envolvidas com metabolismo de íons ferro (Figura 2A, Capítulo III). Portanto, com base nos nossos resultados, formulamos a hipótese de que essas enzimas possam reduzir o íon Fe^{+3} internalizado, complexado com a enterobactina, liberando na forma de Fe^{+2} (Figura 2B, Capítulo III). Ainda há relação com o sistema SUF que induz a montagem de agrupamentos [4Fe-4S] (OUTTEN *et al.*, 2004). Os dados também sugerem interação com a enzima NrdR que inibe o regulon *nrdIHEF* responsável por codificar proteínas ribonucleotídeo redutases dependentes de Mn^{+2} (TORRENTS *et al.*, 2007). Interessantemente, as ribonucleotídeo redutases após serem oxidadas devem ser novamente reduzidas e a proteína NrdI realiza esta redução. Essa proteína realiza redução dependente de FMN, o qual é oxidado. Consideramos que as nitrorredutases possam participar da redução do FMN "reativando" a proteína NrdI para realizar novas reduções de ribonucleotídeo redutases oxidadas (Figura 2B, Capítulo III).

É importante salientar que a atividade das nitrorredutases de *E. coli* na redução de FMN é baixa em comparação com as nitrorredutases Frp e FRaseI. No entanto, a substituição do resíduo Glu99 por Gly no sítio ativo de NfsA e Phe124 por Ser em NfsB, aumenta a atividade FMN redutase dessas enzimas. Esses resíduos podem estar limitando fisicamente ou quimicamente o acesso de substratos maiores como FMN

ao sítio ativo. A remoção ou substituição desses aminoácidos faz com que esse impedimento espacial seja removido e a molécula de FMN consiga se encaixar no sítio ativo da enzima (ZENNO *et al.*, 1996b; ZENNO *et al.*, 1998a). Esta evidência origina uma questão evolutiva interessante: As nitrorredutases ganharam ou perderam a capacidade de reduzir FMN? É uma questão ainda sem resposta.

É interessante ressaltar que essa atividade de FMN redutase contribui na bioluminescência em *V. fischeri* e *V. harveyi* por fornecer FMN reduzido na reação da luciferase produzindo luz (INOUYE, 1994; CHUNG & TU, 2012). Foi constatado que a nitrorredutase NfrA1 de *Bacillus subtilis* possui interação física com proteínas do sistema lux, envolvido na bioluminescência (ZENNO *et al.*, 1998b). A análise da rede de interações das proteínas NfsA e NfsB revelou a interação dessas enzimas com um membro desse sistema, a proteína LuxS (Figura 3A, Capítulo III). Entretanto, em *E. coli*, LuxS está envolvida com metabolismo de metionina na conversão de S-ribosilhomocisteína para homocisteína, posteriormente convertida a metionina (Figura 3B, Capítulo III). Consequentemente, é possível que as nitrorredutases de *E. coli* participem no metabolismo deste aminoácido. Suportando esta hipótese BluB é capaz de realizar a degradação de FMN para formar 5,6-dimetilbenzimidazol que compõe a molécula de cobalamina, que por sua vez pode ser utilizada na via de síntese de metionina (CAMPBELL *et al.*, 2006; TAGA *et al.*, 2007).

A degradação de FMN também é realizada pela enzima IYD em mamíferos na síntese do hormônio da tireoide (THOMAS *et al.*, 2009). Essa capacidade de degradar FMN é comum entre as enzimas IYD e BluB e coerente com o fato de possuirem maior similaridade na estrutura primária (Figura 2, Capítulo II) e tridimensional entre si (THOMAS *et al.*, 2009).

As nitrorredutase de *E. coli* também podem estar envolvidas no metabolismo de carboidratos (Figura 4B, Capítulo III), mais especificamente no síntese de glicogênio, como é sugerido pela interação com a proteína GlgB (Figura 4A, Capítulo III), que participa deste processo. Nossas análises também sugerem uma possível participação das nitrorredutases NfsA e NfsB na utilização de fontes alternativas de carbono, pela interação com a proteína MhpD (Figura 4A, Capítulo III), a qual faz parte do complexo de degradação do composto aromático ácido 3-hidroxifenilpropiônico (3HPP) (Figura 4C, Capítulo III) (BURLINGAME & CHAPMAN, 198). É conhecida a utilização de compostos aromáticos por bactérias como fonte de carbono, sobretudo em ambientes com baixa disponibilidade de glicose como em solos ou no intestino (DIAZ *et al.*, 2001).

No entanto, a grande maioria dos estudos em relação ao papel fisiológico dessas enzimas tem indicado que as nitrorredutases podem estar envolvidas na resposta a estresse oxidativo. (LIOCHEV *et al.*, 1999; ROLDAN *et al.*, 2008; CORTIAL *et al.*, 2010).

Nitrorredutases e o estresse oxidativo em bactérias

Com relação ao possível envolvimento de nitrorredutases com estresse oxidativo, a primeira evidência neste sentido foi o gene *nfsA* de *E. coli* que está sob o controle do regulon *SoxRS*, havendo, portanto aumento da expressão de *nfsA* na exposição a agentes que geram $O_2^{\bullet-}$, como paraquat (LIOCHEV *et al.*, 1999). O regulon *soxRS* além de responder a presença de superóxido também é induzido pela redução do nível de NADPH (KRAPP *et al.*, 2011; LUSHCHAK, 2011). Na exposição a oxidantes, em um primeiro momento, o nível de NADPH é aumentado, pois diversas enzimas antioxidantes utilizam este cofator, como glutationa redutase, tiorredoxina e glutationa

peroxidase (TEMPLE *et al.*, 2005). Entretanto, em um segundo momento o nível de NADPH deve ser reduzido. Se isto não acontecer pode reagir com íons Fe^{+2} levando a produção do radical $HO^{\bullet}$ e também reduzindo a expressão de *soxRS* (KRAPP *et al.*, 2002). Conforme o modelo gerado pelas nossas análises de biologia de sistemas (Figura 6, Capítulo III) é possível que as nitrorredutases de *E. coli* possam controlar o nível de NADPH, oxidando este cofator. O NADPH é produzido por varias enzimas, incluindo o complexo PtnAB (SAUER *et al.*, 2004), o qual possui direta interação com as nitrorredutases de *E. coli* (Figura 5A, Capítulo III). Adicionalmente, foi mostrado que os genes *snrA* e *nprA* em *S. typhimurium* e em *Rhodobacter capsulatos*, respectivamente, são também induzidos por paraquat (NOKHBEH *et al.*, 2002; PEREZ-REINADO *et al.*, 2005).

Ainda segundo o nosso modelo (Figura 6, Capítulo III), as nitrorredutases podem reduzir o íon Fe^{+3} ligado em fragmentos de enterobactina e libera-los no citosol. Entretanto, sob estresse oxidativo, o regulon *soxRS* quando induzido inibe a síntese de enterobactina (AUESUKAREE *et al.*, 2009), reduzindo a captação de Fe^{+3} do meio extracelular e consequentemente o nível de Fe^{+2} no meio intracelular é diminuído. Com a diminuição do nível de Fe^{+2} no meio intracelular, é inibida a proteína repressora NrdR e induzida a síntese de ribonucleotídeo redutases dependentes de Mn^{+2}, envolvidas na produção de nucleotídeos, sendo importantes na síntese e reparação do DNA (KOLBERG *et al.*, 2004). Em adição, NrdI atua reparando as ribonucleotídeo redutases oxidadas e as nitrorredutases mantêm essa enzima ativa por reduzir o FMN ligado a ela (COTRUVO & STUBBE, 2008). Nesta situação, também é ativado o sistema SUF, montando os agrupamentos [Fe-S] de proteínas importantes na resposta ao estresse como a proteína SoxR, por exemplo (OUTTEN *et al.*, 2004). Estas respostas possivelmente contribuem na limitação do conteúdo intracelular de ferro, evitando a

formação do radical $HO^{\bullet}$ e reduzindo a utilização desse metal, canalizando apenas para proteínas importantes na resposta ao estresse.

Outro aspecto que pode contribuir na resposta ao estresse oxidativo em *E. coli*, é a interação com LuxS, já que essa proteína regula de alguma forma a expressão de enzimas antioxidantes, como catalase, tiol peroxidases e hidroperóxido redutases (HE *et al.*, 2008). É possível que essa interação contribua na manutenção dos níveis de metionina, os quais são marcadamente reduzidos em situações de estresse oxidativo (CHANG & KOSMAN, 1990). Adicionalmente, durante a resposta ao estresse oxidativo o consumo de glicose é aumentado e linhagens de *E. coli* mutantes em enzimas da via glicolítica são mais sensíveis a oxidantes (VALDIVIA-GONZALEZ *et al.*, 2012). Assim, a interação das nitrorredutases de *E. coli* com GlgB pode ser importante no controle do estoque de glicose na forma de glicogênio. Na presença de glicose, NfsA e NfsB estimulam de alguma forma para GlgB sintetizar glicogênio, sendo importante para célula se adaptar mais adequadamente a uma futura situação de estresse. Coerentemente com esta hipótese, Grundel e colaboradores (2012) mostrou que células da cianobactéria *Synechocystis elongatus* deficientes na síntese de glicogênio foram incapazes de se adaptar ao estresse oxidativo. Entretanto, em uma situação de estresse oxidativo, possivelmente as nitrorredutases inibam a atividade de GlgB, reduzindo a síntese de glicogênio, já que a célula está utilizando a glicose no metabolismo energético.

Outras evidências relacionando nitrorredutases e estresse oxidativo englobam as proteína NfrA1 de *Bacillus subtilis* e NfrA de *Staphylococcus aureus* que podem estar atuando no balanço tiólico celular (STREKER *et al.*, 2005; TAVARES *et al.*, 2009). A nitrorredutase NfrA1 de *B. subtilis* tem ainda atividade de NADH oxidase, produzindo altas concentrações de H_2O_2. Em contraste, NfrA1 pode destoxificar o

H_2O_2, provavelmente pela conversão dos resíduos de metionina a sulfóxidos de metionina (CORTIAL *et al.*, 2010).

A nitrorredutase CinD de *L. lactis* apresenta atividade de catalase. Coerentemente, linhagens de *L. lactis* deficiente nessa proteína são mais sensíveis a cobre e a 4-NQO (MAGNANI *et al.*, 2008; MERMOD *et al.*, 2010). Também, a proteína CinD apresenta similaridade com as nitrorredutases Frm2p e Hbn1p de *S. cerevisiae* (Figura 2, Capítulo II).

Nitrorredutases e o estresse oxidativo em *S. cerevisiae*

Tendo em vista a forte indicação da relação das nitrorredutases bacterianas com o metabolismo redox da célula e o fato de que as funções das nitrorredutases de *S. serevisiae* ainda são muito pouco entendidas. O Capítulo IV consistiu em explorar a possível participação das proteínas Frm2 e Hbn1 na resposta ao estresse oxidativo. Para isso, foi realizada uma caracterização fenotípica das linhagens mutantes quanto à sensibilidade a agentes oxidantes e competência respiratória. Buscando uma abordagem bioquímica para explicar os dados observados, foram realizados ensaios para avaliar defesas antioxidantes, bem como a produção intracelular de EROs e peroxidação lipídica.

Para este propósito, foram realizadas exposições das linhagens *frm2*Δ, *hbn1*Δ e *frm2*Δ *hbn1*Δ a oxidantes conhecidamente geradores de $O_2^{\bullet-}$, como 4-NQO e NDEA, que produzem este radical pela transferência de elétrons do NADPH para o O_2 formando um fluxo de $O_2^{\bullet-}$ (NUNOSHIBA & DEMPLE, 1993; AIUB *et al.*, 2006). Além disso, as linhagens também foram expostas aos peróxidos H_2O_2 e *t*-BOOH.

Os resultados apresentados no Capítulo IV definem claramente a participação das proteínas Frm2 e Hbn1 na resistência aos oxidantes 4-NQO e NDEA, fato

constatado pela alta sensibilidade observada nas linhagens mutantes expostas a estes agentes (Figuras 1A e 1B, Capítulo IV). Esses resultados podem ser explicados pela menor atividade basal de SOD encontrada (Figura 3A, Capítulo IV). O trabalho desenvolvido por Gaudu e colaboradores (1994) mostrou que linhagens de *E. coli* deficiente em Fre, uma proteína similar à nitrorredutases, tem o nível basal de expressão de *sodA* altamente reduzido. Consistente com estes resultados, linhagens mutantes de SOD são extremamente sensíveis a 4-NQO e a NDEA em vários modelos biológicos (HASSAN & FRIDOVICH, 1978; GRALLA & KOSMAN, 1992). Adicionalmente, para reforçar os resultados obtidos, outros agentes como pentaclorofenol (PCP) e 1-cloro-2,4-dinitrobenzeno (CDNB), metil-metano sulfonato (MMS) e radiação ultravioleta a 280 nm (UVC) também foram testados, uma vez que é conhecida a maior sensibilidade de mutantes SOD a estes compostos (MUTOH *et al.*, 2005; SANTOS *et al.*, 2013). Novamente, as mutantes *frm2*Δ, *hbn1*Δ e *frm2*Δ *hbn1*Δ, se mostraram mais sensíveis a esses agentes (Figura 17, Anexo II). Já foi constatado o aumento da expressão da proteína Hbn1p em resposta a menadiona, um conhecido agente gerador de superóxido (KIM *et al.*, 2007).

Com objetivo de avaliar a situação do estado redox celular após tratamento com os oxidantes, realizou-se a medida dos níveis de produção intracelular de EROs e de peroxidação lipídica pela determinação dos níveis de substâncias reativas ao ácido tiobarbitúrico.

A determinação de ERO foi realizada por dois métodos fluorescentes, utilizando a sonda DCFH-DA (2',7'-diclorofluoresceína diacetato) e determinada a produção de superóxido com NBT (*nitroblue tetrazolium*). A sonda DCFH-DA penetra na célula e é, então, hidrolisada por esterases, liberando a molécula DCFH que, por sua vez, é oxidada como resultado da reação de peróxidos com peroxidases ou ainda da

ação do citocromo c ou do íon Fe^{+2}, produzindo o fluoróforo diclorofluoresceína (DCF). No entanto, esta sonda não é recomendada para determinar a produção de $O_2^{\bullet-}$, já que este radical não reage com esta sonda diretamente e a oxidação desta leva a geração de $O_2^{\bullet-}$, acarretando um erro na estimativa (ROTA *et al.*, 1999). Por este motivo, para determinação de superóxido foi utilizada a redução de NBT. Um aspecto positivo de ambas as metodologias é o monitoramento da produção ERO em tempo real, utilizando células vivas (WANG & JOSEPH, 1999).

As linhagens mutantes deficientes nas nitrorredutases Frm2p e Hbn1p apresentaram maior produção intracelular de ERO quando expostas a 4-NQO e NDEA, visto pelos métodos tanto de oxidação de DCFH-DA (Figuras 4A e 4B, Capítulo IV) como pelo método de redução de NBT (Figuras 5A e 5B, Capítulo IV). Essas alterações podem ser explicadas pela redução na atividade de SOD levando consequentemente a uma maior produção de ERO pelas linhagens mutantes quando expostas aos agentes oxidantes. Sendo esta explicação suportada pelos resultados que mostram uma grande produção de $O_2^{\bullet-}$ por estes nitrocompostos (Figuras 5A e 5B, Capítulo IV).

O outro marcador do estado redox celular foi a determinação da peroxidação lipídica. Os resultados da dosagem do acúmulo de ERO e da determinação da peroxidação lipídica suportam as curvas de sobrevivência sugerindo fortemente que a maior sensibilidade das mutantes seja pelo aumento da formação de ERO e consequentemente de danos oxidativos como peroxidação lipídica, durante a exposição aos agentes 4-NQO e NDEA, possivelmente devido a menor atividade de SOD nestas linhagens mutantes.

A importância das nitrorredutases Frm2p e Hbn1p na capacidade respiratória também foi determinada. Nesta determinação, verificou-se que as linhagens mutantes para Frm2p e Hbn1p apresentam uma alta indução de *petites* (Figura 2, Capítulo IV).

Essa elevada indução de mutantes citoplasmáticos poderia sugerir uma possível participação da Frm2p e Hbn1p na mitocôndria. A sequência da nitrorredutase de *Magnaporthea grisea* filogeneticamente relacionada à Frm2p e Hbn1p apresenta sinal mitocondrial (Figura 2, Anexo I), indicando um possível papel desta proteína na organela. Entretanto, as nitrorredutases estudadas não apresentam esta sequência sinal verificada na análise *in silico* (Figura 2, Anexo I). Isso não impede que as proteínas Frm2 e Hbn1 não desempenhem um papel, mesmo que indireto, no funcionamento da mitocôndria via regulação do metabolismo redox, por exemplo. Portanto, provavelmente esse fato seja devido às reduzidas atividades de SOD, de maneira que a célula não consegue eliminar o $O_2^{\bullet-}$ produzido durante o metabolismo respiratório, uma vez que durante este metabolismo, a mitocôndria é a maior fonte desse radical (DROSE & BRANDT, 2012). Também é bem conhecido que os mutantes *sod* de leveduras apresentam maior formação de *petites* (LONGO *et al.*, 1996). Isso pode ser devida à oxidação que o $O_2^{\bullet-}$ causa nos agrupamentos [2Fe-2S], com consequente inativação de enzimas importantes, seguido à liberação do íon Fe^{+2} e aumento da ocorrência da reação de Fenton com produção de $HO^{\bullet}$ bem como outros radicais, causando diversos danos oxidativos e por consequência o fenótipo *petite* (LONGO *et al.*, 1996; GARDNER, 1997).

Na exposição aos peróxidos H_2O_2 e *t*-BOOH, um padrão diferente de resposta foi encontrado em relação ao obtido no tratamento com agentes geradores de $O_2^{\bullet-}$. As linhagens mutantes *frm2*Δ e *frm2*Δ *hbn1*Δ apresentam níveis aumentados na atividade das enzimas CAT, GPx e no conteúdo de GSH (Figuras 3B, 3C e 3D, Capítulo IV). Isso é refletido na menor produção de ERO (Figuras 4C e 4D Capítulo IV) e de TBARS (Figuras 6C e 6D, Capítulo IV) quando essas linhagens foram expostas a peróxidos. Também, a sobrevivência foi aumentada nessas linhagens quando expostas a estes

agentes. Entretanto, não foi vista diferença na sensibilidade do mutante *hbn1*Δ a estes peróxidos em relação à linhagem selvagem (Figuras 1C e 1D, Capítulo II). Suportando este fato, as linhagens mutantes *frm2*Δ e *frm2*Δ *hbn1*Δ apresentaram menor produção intracelular de ERO quando expostas a H_2O_2 e *t*-BOOH e consequentemente menor peroxidação lipídica.

Um aspecto interessante é que Manfredini e colaboradores (2004) constaram que duplo mutantes *sod1*Δ *sod2*Δ de *S. cerevisiae* apresentam maior atividade de GPx e um aumento no conteúdo de GSH em mutantes *sod1*Δ. Entretanto, a atividade de CAT permaneceu inalterada. Os resultados obtidos neste trabalho, não podem ser explicados por um possível mecanismo compensatório entre as enzimas antioxidantes, considerando o fato que a mutante *hbn1*Δ possui uma reduzida atividade de SOD, bem como as demais mutantes, mas apresenta níveis normais na atividade das demais enzimas e de GSH.

Os níveis basais de produção de ERO e de TBARS não apresentaram diferenças significativas, sugerindo que as alterações nas atividades enzimáticas não influenciam no balanço redox em condições fisiológicas. Outra possibilidade é que a célula tenha mecanismos compensatórios para que sejam mantidos os níveis basais de ERO e por consequência de TBARS, considerando que níveis controlados de ERO são importantes fisiologicamente, uma vez que estas desempenham diversas funções celulares (CAP *et al.*, 2012; SCHIPPERS *et al.*, 2012).

Outro aspecto interessante é que a ação dessas nitrorredutases não é dependente da condição metabólica, não apresentando diferenças no padrão de resposta, no que diz respeito à sensibilidade aos agentes oxidantes. Porquanto, apresentam esse mesmo padrão na fase estacionária e exponencial (Tabela 3, Anexo III) e em metabolismo fermentativo e respiratório (Tabela 4, Anexo IV).

Com base nos resultados obtidos um primeiro modelo do possível mecanismo de ação dessas enzimas foi proposto (Figura 7, Capítulo IV). O modelo considera que as nitrorredutases Frm2p e Hbn1p estejam envolvidas na metabolização de nitrocompostos. O composto 4-NQO pode participar de um ciclo fútil com geração de $O_2^{\bullet-}$. A enzima Hbn1p pode reduzir 4-NQO a 4-hidroxiaminoquinolina-N-oxido (4-HAQO) ou alternativamente o nitro-ânion pode ser convertido a 4-nitrosoquinolina (4-NOQO), então Hbn1p reduz esse composto a 4-HAQO. O intermediário 4-HAQO pode ser convertido a 4-aminoquinolina-N-oxido (4-AQO) por Frm2p. Na ausência de Frm2p, o intermediário 4-HAQO é oxidado espontaneamente formando o radical nitroxido que gera peroxido e 4-NOQO. Desta forma, essa formação de peróxido pode contribuir no aumento das defesas antioxidantes como nível de GSH, atividade de CAT e GPx, vista nas linhagens deficientes em Frm2p. Corroborando esse modelo, alguns dos intermediários sugeridos foram confirmados experimentalmente no estudo realizado por Bang e colaboradores (2012).

Entretanto, mais experimentos foram realizados para elucidar os mecanismos moleculares pelos quais Frm2p e Hbn1p possam atuar na modulação das defesas antioxidantes. Alguns aspectos como a alteração na atividade basal das enzimas antioxidantes e conteúdo de GSH, bem como a capacidade de repressão da expressão do gene *OLE1* constatado por McHale e colaboradores (1996), podem sugerir um possível envolvimento das nitrorredutases em mecanismos de controle da expressão gênica. Para esta investigação, relatada no Capítulo V, foi realizado um estudo das interações de Frm2p e Hbn1p com outras proteínas e a influência na resposta ao estresse oxidativo.

Primeiramente, foi realizada a construção da rede de interações de Frm2p e Hbn1p (Figura 1, Capítulo V) em seguida foram identificadas as proteínas mais importante da rede pelas análises de centralidade (Figura 3, Capítulo V). As 10

proteínas consideradas *bottleneck* foram: Frm2p, Hbn1p, Cad1p (ou Yap2p), Ski8p, Are1p, Are2p, Nab2p, Ole1p, Sua7p e Pox1p (Figura 3A, Capítulo V). É válido ressaltar que 4 das 8 proteínas (excetuando as nitrorredutases) possuem evidências na literatura que indicam relação com resposta ao estresse oxidativo (Tabela 2, Capítulo V). Tendo em vista esses resultados, foi realizada uma triagem de linhagens mutantes deficientes nestas proteínas classificadas como *bottleneck*. É importante salientar que as proteínas consideradas *bottleneck* tendem a ser essenciais para célula (YU *et al.*, 2007), como no caso as proteínas Nab2p, Ole1p e Sua7p. Nestes casos, foram utilizadas linhagens diploides, contendo uma cópia mutada do gene e a outra cópia normal. Para as proteínas não *bottleneck* foram utilizadas linhagens haplóides apenas com o gene mutado (Tabela 1, Capítulo V). Na triagem, foram identificadas as linhagens *ski8Δ* e *cad1Δ* com respostas mais evidentes ao estresse oxidativo (Figura 4, Capítulo V). A ausência de Ski8p resultou em resistência a peróxidos e sensibilidade aos nitrocompostos enquanto a deficiência em Cad1p conferiu sensibilidade a 4-NQO e NDEA, mas não afetou a resposta a peróxidos quando comparada à linhagem selvagem (Figura 4, Capítulo V). As linhagens diploides *nab2Δ/NAB2* apresentaram baixa sensibilidade aos nitrocompostos e não apresentaram alteração na resposta a peróxidos, enquanto a linhagem *ole1Δ/OLE1* mostrou leve tolerância a 4-NQO e NDEA e baixa sensibilidade a peróxidos (Figura 4, Capítulo V). Portanto, foram construídas para as análises subsequentes, as linhagens duplo e triplo mutantes nos genes *FRM2*, *HBN1*, *SKI8* ou *CAD1* (Tabela 1, Capítulo V).

Os fenótipos de sensibilidade indicaram que a interação das nitrorredutases com a proteína Ski8p está envolvida na resposta a superóxido, tendo em vista a sensibilidade das linhagens mutantes aos nitrocompostos (Figuras 5A e 5B, Capítulo V). Os resultados sugerem que as proteínas Frm2p e Ski8p atuem na mesma via de resposta,

já que o fenótipo do duplo mutante foi igual ao dos simples mutantes. O mesmo padrão foi visto na atividade de SOD, sendo menor nas linhagens mutantes (Tabela 3, Capítulo V). Também foi observada a maior peroxidação lipídica e acúmulo de ERO (Tabela 4, Capítulo V), mais especificamente de superóxido (Tabela 5, Capítulo V), justificando a maior sensibilidade encontrada. Enquanto, Ski8p e Hbn1p parecem não atuar na mesma via de resposta, Cad1p e Frm2p também parecem atuar na mesma via na resposta a superóxido. Por outro lado, os resultados sugerem que Hbn1p atue numa via diferente na resposta a 4-NQO e NDEA (Figuras 5A e 5B, Capítulo V). Corroborando essas observações, a rede de interações não apresenta interação de Hbn1p com as proteínas Ski8 e Cad1 (Fig. 3A, Capítulo V).

Quanto à resposta a H_2O_2 e *t*-BOOH, a interação de Frm2p e Ski8p parece ser relevante, apresentando as linhagens mutantes maior tolerância aos peróxidos testados (Figuras 6C e 6D, Capítulo V). Isso se deve provavelmente ao fato das linhagens deficientes nessas proteínas apresentarem maiores níveis de GSH, atividades de CAT e GPx (Tabela 3, Capítulo V), menor peroxidação lipídica e acúmulo de ERO em relação à selvagem (Tabela 4, Capítulo V). De fato, essas proteínas parecem atuar na mesma via de resposta, visto que o duplo mutante apresenta fenótipos similares ao simples mutante *ski8Δ.*

É interessante citar que a interação de Frm2p com Cad1p já foi descrita na literatura por AZEVEDO e colaboradores (2007) que demonstraram a existência de um sítio de ligação a Cad1p no gene *FRM2*. A proteína Cad1 é um fator de transcrição envolvido na resposta a Cd^{+2} e a drogas, como 1,10-fenantrolina, o qual é um quelador de ferro. Portanto, a expressão de *FRM2* é dependente de Cad1p. Análises de expressão também indicaram que Cad1p estaria envolvido na expressão de um conjunto de genes envolvidos na estabilização de proteínas e metabolismo de glicose (BANG *et al.*, 2013).

Curiosamente, foi constatado que Cad1p não apresenta resposta a peróxidos (VILELA *et al.*, 1998), o que é consistente com os nossos resultados.

A proteína Ski8p faz parte do complexo protético ski, responsável por recrutar o complexo do exossomo, que por sua vez é responsável pela degradação de RNAm no sentido 3'-5' (ARAKI *et al.*, 2001). Essa proteína foi primeiramente identificada na degradação de RNAm não poliadenilados (SEARFOSS & WICKNER, 2000). A degradação de RNAm é um forte controle na expressão de genes e consequentemente um ponto importante na resposta a estresse (PARKER, 2012). Já foi demonstrado que linhagens de levedura mutantes em vias de degradação de RNAm, como na via de degradação de RNAm truncado, são mais resistentes a alguns tipos de estresse como causado por metais (GARDNER, 2010; AGUIRRE & CULOTTA, 2012). Foi observado que linhagens deficientes na proteína Lsm1p, também envolvida na degradação de RNAm, apresenta maior transcrição de vários genes incluindo *GSH2* e *CTT1*, bem como menor expressão do gene *SOD1* (SINGH *et al.*, 2010). Em adição, linhagens deficientes na proteína Ski2, outro componente do complexo ski, são mais sensíveis a MMS (SINGH *et al.*, 2010), de modo semelhante a *frm2Δ* (Anexo II, Figura C).

Um aspecto interessante a ser considerado é que a expressão do gene *OLE1* é regulada pela estabilidade do RNAm e a degradação é realizada na via do exossomo no sentido 3'-5'(KANDASAMY *et al.*, 2004). A proteína Ole1p realiza a insaturação de ácidos graxos de cadeia longa formando os ácidos oleico e palmitoléico (DE FREITAS *et al.*, 2012). A linhagem *ole1Δ/OLE1* apresentou menor peroxidação lipídica na exposição ao 4-NQO (Figura 7A, Capítulo V) e consequente menor sensibilidade aos nitrocompostos (Figuras 4A e 4B, Capítulo V). Já foi demonstrado que a linhagem mutante *frm2*Δ, não é capaz de reprimir a transcrição de *OLE1* (MCHALE *et al.*, 1996).

Sendo assim, é possível que Frm2p controle a expressão de *OLE1* pela interação com Ski8p. Já foi demonstrada a redução na expressão desse gene como resposta adaptativa ao ácido 2,4-diclorofenoxiacetico, um herbicida pro-oxidante (VIEGAS *et al.*, 2005). A expressão de *OLE1* também é reduzida sob estresse nitrosativo, enquanto a expressão de *CAD1* e *FRM2* é aumentada (HORAN *et al.*, 2006). Entretanto, Ole1p parece contribuir na resposta a peróxidos, já que a linhagem apresentou maior sensibilidade na exposição a H_2O_2 e *t*-BOOH (Figuras 4C e 4D, Capítulo V) e maior peroxidação lipídica (Figura 7A, Capítulo V). Neste sentido, Kelley & Ideker (2009 mostrou que sob exposição a peróxidos há uma maior expressão do gene *OLE1*, possivelmente por modificar o perfil lipídico da membrana, diminuindo a sua permeabilidade. Um mecanismo semelhante acontece com ergosterol na resposta a peróxidos (BRANCO *et al.*, 2004).

A proteína Hbn1p apresenta interação com Nab2p, cuja linhagem *nab2Δ/NAB2* também apresenta sensibilidade a superóxido (Figuras 4A e 4B, Capítulo V), possivelmente devido ao fato de apresentar menor atividade de SOD (Fig. 7C, Capítulo V) e consequente maior acúmulo de superóxido (Figura 7D, Capítulo V). É importante ressaltar que Nab2p apresentou interação com as enzimas Sod1p e Sod2p (Figura 7B, Capítulo V). É possível que a interação de Hbn1p com Nab2p seja importante na resposta a superóxido, por modular a atividade de SOD. A proteína Nab2p contribui no transporte do RNAm poliadenilado do núcleo para o citoplasma (STEWART, 2010). Já foi visto que Nab2p esta envolvida em situações de choque térmico, pela retenção de RNAm de proteínas não essenciais na resposta ao estresse e provavelmente favorecendo a exportação de RNAm de proteínas que participem na resposta ao choque térmico (MORANO *et al.*, 2012). A expressão do gene *NAB2* é aumentada durante estresse oxidativo (AUESUKAREE *et al.*, 2009). Outro aspecto a ser salientado é que,

aparentemente, Nab2p não participa na resposta a peróxidos (Figura 4B e 4C, Capítulo V).

Outro aspecto detectado na rede de interações foi a ligação com Sua7p (TFIIB), um fator de transcrição que já foi mostrado ser aparentemente necessário para a expressão de um conjunto de genes que codificam proteínas participantes na atividade mitocondrial, bem como na resposta a ambientes oxidativos (PAES DE FARIA & FERNANDES, 2006). O aumento na expressão do gene *SUA7* parece compensar a ausência da proteína Yap1 na exposição a H_2O_2 (PAES DE FARIA & FERNANDES, 2006). Ainda pode ser citada a interação com Are1p, Are2p e Pox1p, embora as linhagens mutantes deficientes nessas proteínas não apresentem sensibilidade aos oxidantes testados, sugerindo que não participam na resposta a estresse oxidativo (Figura 4, Capítulo V).

Os resultados gerados no Capítulo V permitiram a construção de um modelo hipotético do possível mecanismo de ação na resposta ao estresse oxidativo das nitrorredutases Frm2p e Hbn1p e suas interações (Figura 8, Capítulo V e Figura 15). O modelo considera que Cad1p controla a transcrição de *FRM2* codificando Frm2p que interage com Ski8p controlando a degradação do RNAm das enzimas antioxidantes e de proteínas envolvidas na biossíntese de GSH etambém dos transcritos de *OLE1*. Em outra via, a interação de Hbn1p com Nab2p possivelmente controla a exportação do RNAm de SOD. Na ausência de Frm2p, possivelmente, o RNAm de SOD seja mais degradado e na ausência de Hbn1p, a exportação desse RNA seja reduzida. Durante a exposição a agentes geradores de $O_2^{\bullet-}$, esses fatores levam a uma menor atividade de SOD com consequente maior acúmulo de superóxido e maior nível de peroxidação lipídica, seguindo maior sensibilidade. A ausência de Frm2p aumenta a expressão de *OLE1* já que reduz a degradação dos transcritos desse gene. No entanto, é possível que a

modificação no perfil lipídico da membrana favoreça a peroxidação lipídica pelas espécies derivadas do superóxido. Por outro lado, na exposição a peróxidos , a ausência de Frm2p reduz a degradação de transcritos relativos a genes das enzimas CAT, GPx e de síntese de GSH, levando a uma maior atividade destas enzimas, seguido de um menor acúmulo de ERO, nível de peroxidação lipídica e consequentemente da sensibilidade a peróxidos. A menor degradação do transcrito do gene que codifica Ole1p aumenta a atividade desta enzima, modificando o perfil lipídico da membrana de maneira a reduzir a permeabilidade, reduzindo a entrada de peróxidos na célula, diminuindo a peroxidação lipídica e, logo, contribuindo com a maior resistência observada.

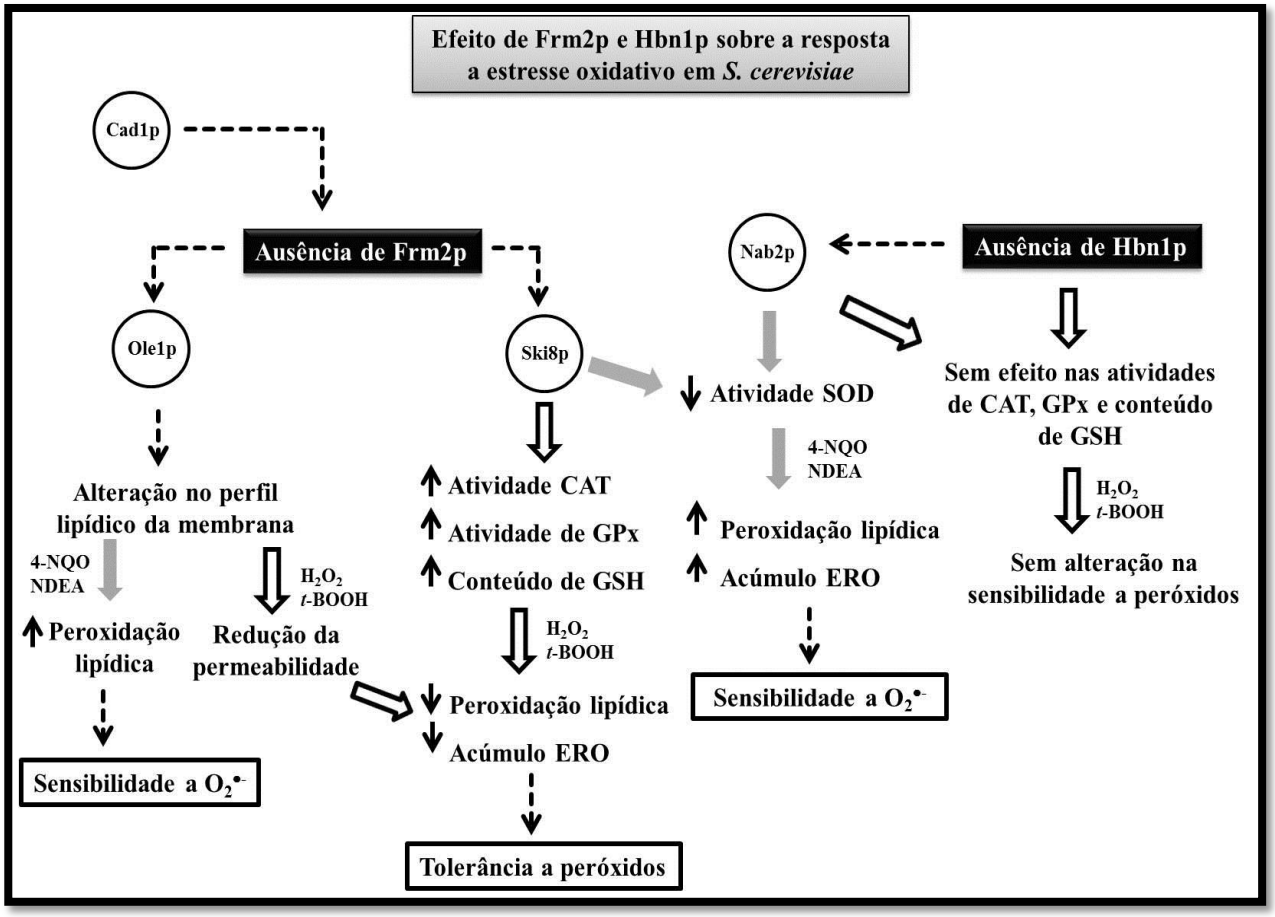


Figura 15: Esquema simplificado mostrando o efeito da ausência das proteínas Frm2 e Hbn1 e as interações que exercem na modulação da resposta a estresse oxidativo em *S. cerevisiae*.

Conclusões

Conclusão geral

O presente estudo mostrou evidências a respeito da participação das nitrorredutases na homeostase redox. Possivelmente essas enzimas influenciem na resposta ao estresse oxidativo atuando em diferentes vias do metabolismo celular de *Escherichia coli* e *Saccharomyces cerevisiae*.

Conclusões específicas

- Podem ser identificados 4 grupos filogeneticamente distintos de nitrorredutases: NfsA/Frp, NfsB/FRaseI, Frm2p/Hbn1p e IYD/BluB;
- As nitrorredutases de *E. coli* podem participar em diferentes vias do metabolismo, como a homeostase de ferro, manutenção dos níveis de NADPH, metabolismo de compostos aromáticos e na síntese de glicogênio. Estas vias podem contribuir na respostas a estresse oxidativo e na limitação de nutrientes.
- As linhagens mutantes *frm2*Δ, *hbn1*Δ e *frm2*Δ *hbn1*Δ apresentaram maior sensibilidade a 4-NQO e NDEA. Entretanto, as mutantes *frm2*Δ e *frm2*Δ *hbn1*Δ mostraram uma maior resistência a peróxidos, H_2O_2 e *t*-BOOH;
- Há uma maior indução na formação de mutantes citoplasmáticos "petites" nas linhagens mutantes em relação à selvagem;
- Há uma reduzida atividade basal de superóxido dismutase nas linhagens *frm2*Δ, *hbn1*Δ e *frm2*Δ *hbn1*Δ. As linhagens *frm2*Δ e *frm2*Δ *hbn1*Δ apresentaram uma maior atividade basal de catalase, glutationa peroxidase e conteúdo de glutationa, mas a mutante *hbn1*Δ mostrou níveis similares aos encontrados na selvagem;

- Houve um maior acúmulo intracelular de ERO e peroxidação lipídica nas linhagens mutantes quando expostas a 4-NQO e NDEA. No entanto, as mutantes *frm2*Δ e *frm2*Δ *hbn1*Δ apresentaram um menor acúmulo intracelular de ERO e TBARS quando expostas a peróxidos;
- A análise da ontologia gênica dos agrupamentos da rede de interações de Frm2p e Hbn1p revela uma relação predominante dessas enzimas com proteínas envolvidas no metabolismo de RNA;
- As análises de centralidade indicaram que as proteínas Frm2, Hbn1, Cad1, Ski8, Are1, Are2, Nab2, Ole1, Sua7 e Pox1 são *bottleneck* na rede de interações;
- As interações de Frm2p com as proteínas Cad1p, Ski8p e Ole1p contribuem na resposta a oxidantes;
- A interação de Hbn1p com Nab2p afeta a resposta a agentes geradores de superóxido por alterar a atividade da enzima superóxido dismutase.

Perspectivas

PERSPECTIVAS

Seria de importância para a continuação do trabalho, complementação dos resultados e progresso do conhecimento a respeito das proteínas Frm2 e Hbn1, buscar o entendimento dos mecanismos moleculares de como essas proteínas estão atuando na modulação das atividades das enzimas antioxidantes, bem como explorar outras possíveis vias do metabolismo em que essas proteínas possam estar envolvidas na resposta ao estresse oxidativo. Para tanto, algumas abordagens são propostas:

- Comparação da rede de interação das nitrorredutases de *E. coli* com outras nitrorredutases bacterianas observando aspectos evolutivos;

- Confirmar experimentalmente os modelos propostos para as nitrorredutases de *Escherichia coli*;

- Analisar a expressão dos genes *FRM2*, *HBN1*, *SKI8*, *CAD1*, *OLE1*, *NAB2*, *SOD1*, *SOD2*, *CTT1*, *CTA1*, *GPX3*, *GSH1*, sob condições normais e em condições de estresse oxidativo;

- Avaliar o decaimento do RNAm das enzimas antioxidantes nas linhagens selvagens e deficientes em Frm2p e Hbn1p, bem como nas proteínas que indicaram interação com essas nitrorredutases;

- Analisar uma possível relação de Frm2p e Hbn1p com ciclo celular.

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Anexos

ANEXO I

Identificação *in silico* de um novo grupo de prováveis nitrorredutases bacterianas e fúngicas.

Neste trabalho foram identificadas por análise *in silico* duas proteínas Frm2p e Hbn1p de *Saccharomyces cerevisiae* como possíveis nitrorredutases. Os resultados da análise filogenética mostram a presença de sequências homólogas às proteínas Frm2p/Hbn1p em espécies de bactérias e fungos, formando dois clados distintos dentro da família. Análises de agrupamentos hidrofóbicos (HCA) e modelagem tri-dimensional foram realizadas para comparar regiões conservadas entre nitrorredutases bacterianas e Frm2p/Hbn1p. Ainda são discutidas possíveis funções fisdiológicas dessas proteínas. É importante reassaltar, que foi a primeira identificação de sequências de nitrorredutases em eucariotos.

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In silico identification of a new group of specific bacterial and fungal nitroreductases-like proteins

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Abstract

The nitroreductase family comprises a group of FMN- or FAD-dependent and NAD(P)H-dependent enzymes able to metabolize nitrosubstituted compounds. The nitroreductases are found within bacterial and some eukaryotic species. In eukaryotes, there is little information concerning the phylogenetic position and biochemical functions of nitroreductases. The yeast *Saccharomyces cerevisiae* has two nitroreductase proteins: Frm2p and Hbn1p. While Frm2p acts in lipid signaling pathway, the function of Hbn1p is unknown. In order to elucidate the function of Frm2p/Hbn1p and the presence of homologous sequences in other prokaryotic and eukaryotic species, we performed an in-depth phylogenetic analysis of these proteins. The results showed that bacterial cells have Frm2p/Hbn1p-like sequences (termed NrlAp) forming a distinct clade within the fungal Frm2p/Hbn1p family. Hydrophobic cluster analysis and three-dimensional protein modeling allowed us to compare conserved regions among NrlAp and Frm2/Hbn1p proteins. In addition, the possible functions of bacterial NrlAp and fungal Frm2p/Hbn1p are discussed.

Keywords: Nitroreductases; Type I nitroreductase; Yeast Frm2p/Hbn1p; Bacterial NrlAp; Reactive nitrogen oxide species

A large number of nitroaromatic and nitroheterocyclic derivatives (nitrosubstituted compounds) are present in environmental samples [1,2] as a consequence of manufacturing processes and as antimicrobial agents [3]. In addition, nitropolyaromatic compounds have been identified as by-products of a variety of combustion processes [4,5]. These nitrosubstituted compounds have attracted a considerable health concern because their metabolization leads to the formation of potent genotoxic and/or mutagenic metabolites [6]. In addition, many nitrosusbtituted compounds are able to generate reactive nitrogen oxide species (RNOxS), which readily react with biological macromolecules [7]. The metabolic pathways that result in the activation of nitrosubstituted compounds are complex, and the nitroreductase proteins have a central role on the activation of nitroheterocyclic and nitroaromatic compounds. The nitroreductases are also biotechnologically attractive, showing potential applications for bioremediation, biocatalysis, and chemotherapeutic tumor treatment [8–11].

The nitroreductases comprise a family of eubacterial conserved sequences that catalyze the reduction of nitrosubstituted compounds using FMN or FAD as prosthetic group and NADH or NADPH as reducing power agents [12,13]. Until now, two types of bacterial nitroreductases have been biochemically characterized: type I (oxygen-insensitive) and type II (oxygen-sensitive) [13]. In *Escherichia coli*, type I nitroreductases are known to catalyze the reduction of organic nitroaromatic and nitroheterocyclic compounds such as nitrofurazone and nitrofurantoin. Two type I genes, *nfsA* and *nfsB*, were cloned and their enzymatic activities have been characterized [14,15]. It has been shown that paraquat strongly induces *nfsA* whereas *nfsB* levels remain essentially unchanged [16].

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Interestingly, the genes that codify for bacterial type II nitroreductases have not been cloned until now [17].

The data available about the distribution of nitroreductase-like sequences in eukaryotic cells are very restricted, but a mammalian iodotyrosine deiodinase that contains a nitroreductase domain was described [18]. In the yeast *Saccharomyces cerevisiae* two genes named *FRM2* (YCL026c-A) and *HBN1* (YCL026c-B) codifies for nitroreductase-like proteins. The functions of Frm2 and Hbn1 proteins are not completely known, but experimental data of McHale et al. [19] indicated that Frm2p is possibly involved in lipid signaling pathway and cellular homeostasis. Considering that the phylogenetic position of both proteins is little known as well as their presence in other prokaryotic and eukaryotic organisms, we identified and characterized *in silico* a new bacterial and fungal Frm2p/Hbn1p nitroreductase-like family. This new family was identified by searching in bacterial and eukaryotic genomic databases using sensitive methods of phylogenetic analysis. Additional hydrophobic cluster analysis (HCA) and three-dimensional protein modeling allowed us to refine the results obtained from phylogeny and to map conserved domains within these new nitroreductase-like proteins.

Experimental procedures

Protein data mining and sequence analyses. Sixty-one protein sequences were obtained directly from GenBank hosted in the National Center for Biotechnological Information (NCBI) web page (http://www.ncbi.nlm.nih.gov/) (Tables 1 and 2). BLAST and PSI-BLAST programs were used for initial domain screening and comparison [20]. Moreover, the *Sce*Frm2p sequence (Table 1) was used as template in the Genolevures Database (http://cbi.labri.fr/Genolevures/index.php) in order to identify possible nitroreductase homologous sequences in unconventional yeast species. All searches were made to saturation. Wise2 program at European Bioinformatics Institute (http://www.ebi.ac.uk/wise2/) was used to identify Hbn1/Frm2 proteins from the unfinished genomic sequences of fungal species. The parameters for prediction were: local mode; no intron bias; splice site modeled; synchronous model; and GeneWise623 algorithm. The theoretical p*I* and molecular weight of selected bacterial NrlA and fungal Hbn1/Frm2 proteins (complete sequences) were calculated with COMPUTE p*I*/Mw program hosted in ExPASy Molecular Biology Server at Swiss Institute of Bioinformatics (http://us.expasy.org/tools/pitool.html). The presence of mitochondrial target signals in selected fungal Hbn1/Frm2 proteins was analysed by MitoProt software [21].

Algorithms for sequence comparison and phylogenetic inference. Global pair-wise multiple-alignment of members of prokaryotic NrlAp and eukaryotic Hbn1p/Frm2p groups was performed in the CLUSTALX 1.8 program [22]. The following alignment parameters were used: gap open penalty 10.00; gap extension 0.20; sequences >10% diverged delayed; BLOSUM series matrix; residue-specific penalties on; and hydrophilic penalties on. When necessary, the alignments were manually adjusted using the BioEdit program [23].

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [24]. Neighbor-Joining (NJ) method was used for phylogenetic tree searching and inference. The statistical reliability of the phylogenetic trees was tested by interior branch analysis with 1000 replications. Moreover, the Poisson correction was applied in NJ for distance estimation. The complete deletion option was used in handling gaps or missing data obtained from the alignment.

Hydrophobic cluster analysis (HCA). HCA of selected protein sequences was performed as previously published [25]. Briefly, it consisted in displaying the primary protein structure on a duplicated α-helical net, where the hydrophobic residues are automatically contoured. The hydrophobic clusters observed in an HCA plot are not distributed in a random manner, but correspond highly with the secondary protein structures associated with conserved globular domains. Thus, HCA plots are extremely valuable for phylogenetic inferences when protein sequences have a weak homology (<25% of identity/similarity) or to define conserved domains and sequence signatures in a set of homologous proteins [25]. It should be noted that HCA, when compared with 'linear' or one-dimensional methods of database screening, e.g., BLAST and PSI-BLAST (which need, at least, >30% of aa identity/similarity over a sufficient length), is a more effective tool to detect sequence similarity that reflects a true three-dimensional relationship between two or more proteins [26]. Moreover, HCA has the advantage that sequence conservation can be detected in a group of proteins without any previous one-dimensional alignment [26,27]. Additionally, an homology score can be calculated from HCA data as previously described by Gaboriaud et al. [27]. The program DRAWHCA, used in such analysis, is available as a freeware at http://bioserv.rpbs.jussieu.fr/RPBS/.

Algorithms for three-dimensional modeling. *In silico* three-dimensional (3-D) modeling of *Sce*Hbn1p sequence was performed with 3D-JIGSAW [28] threading algorithm using as template the *Smu*NrlAp sequence of *Streptococcus mutans* (Table 1), which belongs to the bacterial NrlAp family (Protein Data Bank Accession No. 1YW3). Three-D comparative models were generated by UCSF Chimera software [29].

Results and discussion

Phylogenetic analysis of bacterial and fungal nitroreductase-like proteins

With the aim of identifying new Frm2p/Hbn1p-like sequences in bacterial and fungal species, the *S. cerevisiae* Frm2p sequence (*Sce*Frm2p) was used to query in BLAST and PSI-BLAST programs. Our data mining of bacterial and fungal genomes revealed new homologous proteins similar to *Sce*Frm2p (Table 1). We named the new bacterial Frm2p/Hbn1p-like sequences as NrlA (*NitroReductase-Like A* sequence; Table 1). Interestingly, these new NrlAp sequences have low similarity level when compared to type I nitroreductases of *Bacillus* generum (data not shown).

NfsA, the major type I nitroreductase from *E. coli* has been purified and biochemically characterized. It can reduce nitrofurazone generating a two electron transfer product and has a tightly associated FMN group [15]. Purification and characterization of NfsB, a minor oxygen-insensitive nitroreductase from *E. coli* has been reported [14]. The NfsA and NfsB nitroreductases have also been shown to function as lawsone (2-hydroxy-1,4-napthoquinone)-dependent azo reductases under anaerobic conditions [30]. Moreover, it was reported that some NfsA-like proteins from *Bacillus subtilis* and *Staphylococcus aureus* act in the oxidative stress response, keeping the cellular thiol-dissulfide balance [31].

These new NrlAp/Frm2p/Hbn1p sequences were subjected to a global sequence comparison followed by a phylogenetic analysis (Fig. 1). The phylogenetic data indicated that both bacterial NrlAp and fungal Frm2p/Hbn1p sequences share a recent last universal common ancestor (LUCA), highly supported by interior branch analysis

Table 1
Bacterial and fungal nitroreductase-like proteins used in this study

Protein Group	Species	Protein name	Accession No. (GenBank)
Bacterial nitroreductase-like			
	Acidovorax sp.	*Asp*NrlAp	gi|121594458|
	Acinetobacter sp.	*Aci*NrlAp	gi|50085393|
	Aeromonas hydrophila	*Ahy*NrlAp	gi|117617740|
	Agrobacterium tumefaciens	*Atu*NrlAp	gi|16119431|
	Bacillus anthracis	*Ban*NrlAp	gi|65319266|
	Bacillus cereus	*Bce*NrlAp	gi|52142142|
	Bacillus thuringiensis	*Bth*NrlAp	gi|49478207|
	Bacteroides fragilis	*Bfr*NrlAp	gi|53711523|
	Bacteroides thetaiotaomicron	*Bat*NrlAp	gi|29348801|
	Brucella melitensis	*Bme*NrlAp	gi|17989117|
	Clostridium acetobutylicum	*Cac*NrlAp	gi|15896557|
	Clostridium cellulolyticum	*Cce*lNrlAp	gi|118725480|
	Enterococcus faecalis	*Efa*NrlAp	gi|29375254|
	Enterococcus faecium	*Enf*NrlAp	gi|69246358|
	Erwinia carotovora	*Eca*NrlAp	gi|50121762|
	Lactobacillus brevis	*Lbr*NrlAp	gi|116333485|
	Lactobacillus casei	*Lca*NrlAp	gi|116494949|
	Leishmania major[a]	*Lma*NrlAp	gi|68130245|
	Leuconostoc mesenteroides	*Lme*NrlAp	gi|116618834|
	Listeria innocua	*Lin*NrlAp	gi|16802020|
	Listeria monocytogenes	*Lmo*NrlAp	gi|16804866|
	Listeria welshimeri	*Lwe*NrlAp	gi|116874175|
	Neisseria meningitidis	*Nme*NrlAp	gi|15795064|
	Paracoccus denitrificans	*Pde*NrlAp	gi|69937297|
	Pediococcus pentosaceus	*Ppe*NrlAp	gi|116492034|
	Pelodictyon luteolum	*Plu*NrlAp	gi|78186978|
	Prosthecochloris vibrioformis	*Pvi*NrlAp	gi|71482181|
	Pseudomonas aeruginosa	*Pae*NrlAp	gi|15597771|
	Psychrobacter arcticus	*Par*NrlAp	gi|71066332|
	Psychrobacter cryohalolentis	*Pcr*NrlAp	gi|93006371|
	Staphylococcus aureus	*Sau*NrlAp	gi|82751635|
	Staphylococcus haemolyticus	*Sha*NrlAp	gi|70725999|
	Streptococcus agalactiae	*Sag*NrlAp	gi|22538047|
	Streptococcus mutans	*Smu*NrlAp	gi|24378768|
	Thermosinus carboxydivorans	*Tca*NrlAp	gi|121533904|
	Trypanosoma brucei[a]	*Tbr*NrlAp	gi|72391262|
Fungal nitroreductase-like			
	Aspergillus clavatus	*Acl*Frm2p A	gi|121707539|
	Aspergillus clavatus	*Acl*Frm2p B	gi|121713376|
	Aspergillus fumigatus	*Afu*Frm2p A	gi|70986358|
	Aspergillus fumigatus	*Afu*Frm2p B	gi|70993974|
	Aspergillus fumigatus	*Afu*Frm2p C	gi|70997834|
	Aspergillus nidulans	*Ani*Frm2p A	gi|67523147|
	Aspergillus nidulans	*Ani*Frm2p B	gi|67522793|
	Aspergillus nidulans	*Ani*Frm2p C	gi|67523775|
	Aspergillus oryzae	*Aor*Frm2p A	gi|83776359|
	Aspergillus oryzae	*Aor*Frm2p B	gi|83774315|
	Aspergillus oryzae	*Aor*Frm2p C	gi|83776268|
	Aspergillus terreus	*Ate*Frm2p A	gi|115492421|
	Aspergillus terreus	*Ate*Frm2p B	gi|115401210|
	Candida glabrata	*Cgl*Hbn1p	gi|50285171|
	Chaetomium globosum	*Chg*Frm2p	gi|116198311|
	Cryptococcus neoformans	*Cne*Frm2p	gi|58260498|
	Gibberella zeae	*Gze*Frm2p	gi|46109968|
	Kluyveromyces lactis	*Kla*Hbn1p	gi|50304619|
	Magnaporthe grisea	*Mgr*Frm2p	gi|39957916|
	Neosartorya fischeri	*Nfi*Frm2p	gi|119479343|
	Neurospora crassa	*Ncr*Frm2p	gi|85103226|
	Saccharomyces cerevisiae	*Sce*Hbn1p	gi|10383764|
	Saccharomyces cerevisiae	*Sce*Frm2p	gi|10383765|
	Ustilago maydis	*Uma*Frm2p	gi|71015252|

[a] Both protozoan sequences probably were acquired by genetic lateral transfer.

Table 2
Physico-chemical analyses of bacterial NrlA and fungal Frm2/Hbn1 proteins

Protein Group	Protein name	Length	p*I*	M.W. (kDa)
Bacterial NrlAp				
	*Cce*lNrlAp	205	6.32	23700.9
	*Lca*NrlAp	200	4.98	22827.7
	*Eca*NrlAp	199	5.59	22211.2
	*Lma*NrlAp	233	8.78	25429.7
	*Par*NrlAp	202	5.89	22916.0
	*Sau*NrlAp	208	5.27	24034.0
	*Smu*NrlAp	200	4.95	22384.3
	*Tbr*NrlAp	190	6.84	21208.4
Fungal Frm2p/Hbn1p				
	*Ani*Frm2p A	208	6.31	23275.5
	*Ani*Frm2p B	242	9.94	27067.2
	*Ani*Frm2p C	222	5.45	24518.0
	*Cgl*Hbn1p	193	6.19	20741.3
	*Kla*Hbn1p	196	5.56	21080.8
	*Ncr*Frm2p	208	5.15	22886.9
	*Sce*Frm2p	193	6.51	21232.1
	*Sce*Hbn1p	193	6.43	20993.8

(Fig. 1). Interestingly, two bacterial NrlAp sequences were found in the genomes of the protozoan species *Leishmania major* and *Trypanosoma brucei*, respectively (Fig. 1). We hypothesized that these two protozoan NrlAp sequences were acquired by lateral gene transfer. Supporting this idea, it was reported that the anaerobic protozoan *Giardia lamblia* also has an oxygen-insensitive nitroreductase with high similarity to *Clostridium acetobutylicum* nitroreductase sequence [32]. Moreover, we could observe the presence of paralogous Frm2p-like sequences in the genomes of different *Aspergillus* species (Table 1 and Fig. 1). The *Aspergillus* paralogous Frm2p-like sequences are an indicative that these proteins could have an important and possible distinct physiological roles within this genera.

The phylogenetic results obtained from the primary structures led us to study the theoretical physico-chemical properties of these new proteins (Table 2). The physico-chemical data showed that bacterial NrlA and fungal Frm2/Hbn1 proteins have an acidic p*I* (average p*I* of 6.08 and 6,44, respectively). The polypeptide chains lengths vary from 190 to 233 aa [molecular weight (M.W.) from 21.21 to 25.43 kDa; Table 2] for bacterial NrlAp, while the fungal Frm2p/Hbn1p sequences showed polypeptide chains in the range of 193–242 aa (M.W. from 20.74 to 27.07 kDa; Table 2).

HCA and three-dimensional protein modeling of nitroreductase-like sequences

An HCA comparison between bacterial NrlAp (*Smu*NrlAp and *Lca*NrlAp, respectively; Table 1) and fungal Frm2/Hbn1 proteins (*Sce*Frm2p, *Sce*Hbn1p, and *Mgr*Frm2p, respectively; Table 1) indicated a high degree of similarity of secondary structures among the proteins, being composed in a greater extent by α-helices (α1–α8;

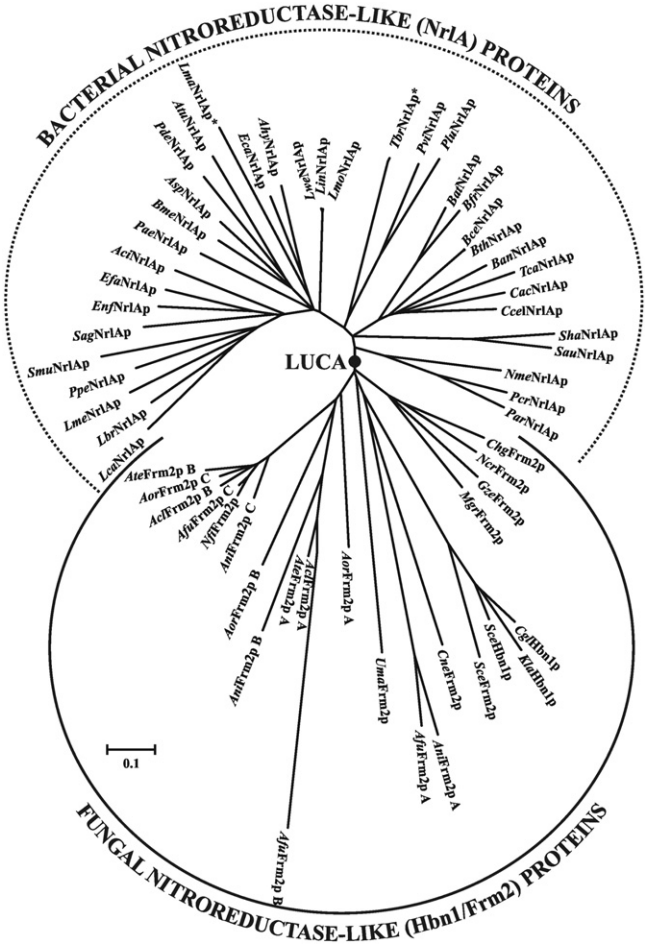

Fig. 1. Unrooted phylogenetic tree obtained from the global alignment of all proteins listed in Table 1. The bacterial nitroreductase-like (NrlA) proteins compose a phylogenetically distinct group (dotted line) when compared to fungal Frm2p/Hbn1p sequences (solid line). An asterisk on *Tbr*NrlAp and *Lma*NrlAp indicate two protozoan sequences that were acquired from bacteria by lateral gene transfer. The unrooted tree is supported by an internal branch analysis value of >90% and the horizontal bar represents a distance of 0.1 substitutions per site. LUCA: last universal common ancestor.

Fig. 2). Two subdomains (subdomains 1 and 2; Fig. 2) separated by a hinge region (Fig. 2) could be clearly indentified in all proteins analysed. Both subdomains 1 and 2 show an average similarity score of 68% and 63%, respectively. In addition, the Frm2p sequence of *Magnaporthea grisea* (*Mgr*Frm2p; Table 1) contains a signal peptide for mitochondrial location (Fig. 2), indicating a possible role of *Mgr*Frm2p within this organelle. We could not identify mitochondrial targeting sequences in the yeast Frm2p/Hbn1p, but experimental data from our laboratory shows that yeast cells defective for Frm2p and/or Hbn1p have a high induction of *petite* colonies (data not shown).

Three-dimensional modeling of *Sce*Hbn1p and *Smu*NrlAp using threading (3D-JIGSAW) algorithm was done to corroborate the results obtained by HCA (Fig. 3A–C). It was possible to model *Sce*Hbn1p (Fig. 3A) using the atomic coordinates of crystalized *Smu*NrlAp complexed

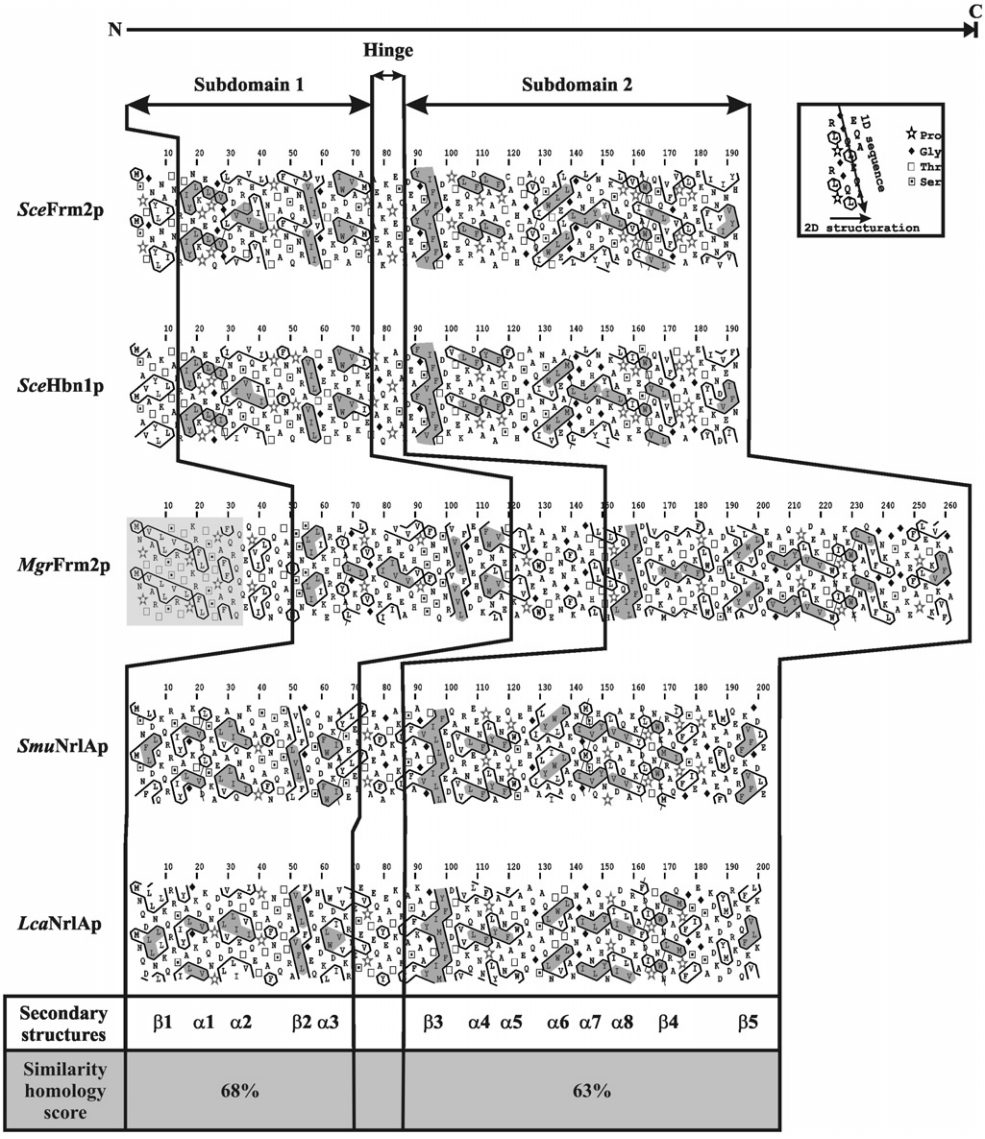

Fig. 2. HCA of selected bacterial NrlAp and fungal Frm2p/Hbn1p sequences. Solid vertical lines separate subdomains 1 and 2 within the nitroreductase domain. Conserved hydrophobic nucleotides as well as the mitochondrial targeting sequence in *Mgr*Frm2p are shaded in gray. Secondary structures (α or β) of subdomains 1 and 2 derived from HCA are shown. An average similarity score (%) calculated from HCA for each region is given at the bottom. An arrow indicates N-terminus to C-terminus directionality. A legend for sequences and special HCA symbols is provided in the inset.

with a FMN molecule (Fig. 3B). The superimposition of both models allowed to identify the FMN binding site in *Sce*Hbn1p model (*E*-value of 2×10^{-71}; Fig. 3C). One interesting aspect of type I nitroreductases is that they are either monomeric or homodimeric FMN-containing proteins with a subunit size of approximately 25 kDa and use NAD(P)H as a reductant [13]. The crystallographic data of *Smu*NrlAp (Protein Data Bank Accession No. 1YW3) also indicated that this protein occurs as a homodimeric protein and probably the same is true for fungal Frm2p/Hbn1p.

The functions of Frm2p/Hbn1p are little understood, and yeast cells mutated for *FRM2* gene are sensitive to polyunsaturated fatty acids, e.g., arachidonic acid [19]. It was reported recently that the *FRM2* gene promoter contains a Yap2p binding site [33], a transcriptional factor associated with cadmium ion (Cd^{2+}) and drug resistance [34,35]. In this sense, the metabolism of polyunsaturated fatty acids and Cd^{2+} generate large amounts of reactive species, including reactive oxygen and nitrogen oxides species (ROS and RNOxS, respectively), able to induce damage or loss-of-function in biological macromolecules [36,37]. Some RNOxS, including peroxynitrite ($ONOO^-$) and nitryl chloride (NO_2Cl), are capable of nitrating virtually all classes of biomolecules. One of the major nitrated products formed in the cell is 3-nitrotyrosine (3-NT) that is generated by $ONOO^-$ and is also found in mammalian inflamed tissues, being implicated in the promotional stage of carcinogenesis and in many degenerative diseases [38]. Moreover, 3-NT induces protein modification, which leads to alteration of

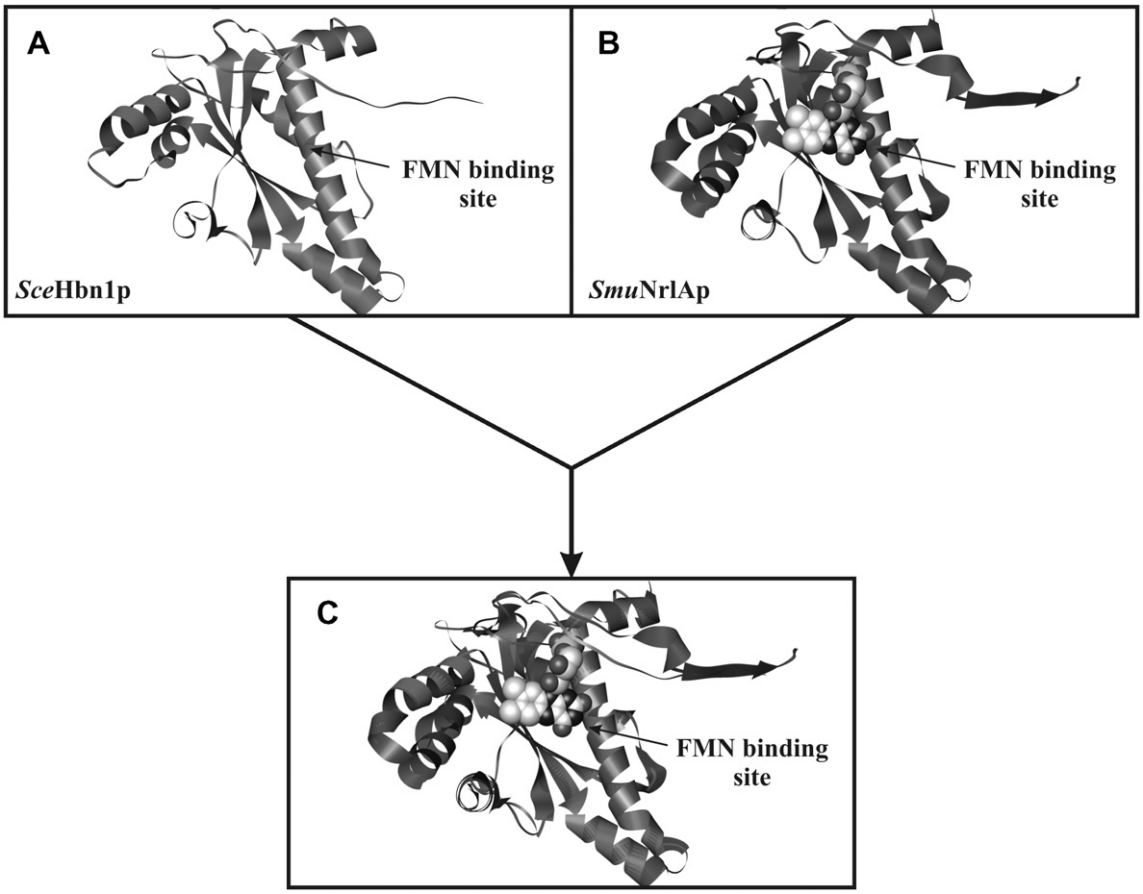

Fig. 3. Threading three-dimensional model of *Sce*Hbn1p (A). The *Smu*NrlAp 3-D model (B) was used for structure comparison with *Sce*Hbn1p (C). The FMN-binding site is indicated by an arrow in (A), (B), and (C). A molecule of FMN is shown in (B) and (C).

protein functions [38]. Besides protein modification by RNOxS, DNA is also a RNOxS-target molecule, generating nitrated DNA adducts, like 8-nitroxanthine (8-NX). 8-NX is the major nitrated DNA adduct in NO_2Cl-treated DNA [38,39]. Once DNA is nitrated, the glycosidic bonds of these nitrated DNA adducts are labile and tend to form the mutagenic apurinic sites [38,39].

Considering the presence of a nitro group in biological macromolecules, it has been postulated that NfsB may also be able to reduce the 3-NT residues in proteins [16]. However, a study performed by Lightfoot et al. [40] indicated that both NfsA and NfsB of *E. coli* do not reduce 3-NT. Interestingly, *E. coli* does not possess a NrlAp-like sequence, and accumulate nitrated proteins within the cell [40]. On the other hand, there are evidences that eukaryotic cells are capable to degrade and/or repair nitrated proteins and DNA at higher rates than unmodified molecules [41]. Considering the data gathered about bacterial NrlA and fungal Frm2/Hbn1 proteins in this work, we could think that these proteins can act keeping the oxidative and/or nitrosative balance within these cells. Experimental data from our laboratory have indicated that yeast cells single or double mutant for *FRM2* and *HBN1* genes are extremely sensitive to nitrosative substances, like 4-NQO (manuscript in preparation), corroborating our sequence analyses data.

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ANEXO II

Os gráficos seguir apresentam os resultasdos referentes a exposição de linhagens de linhagens de *Saccharomyces cerevisiae* proficientes e deficientes nas nitrorredutases Frm2p e Hbn1p os agentes 1-cloro-2,4-dinitrobenzeno (CDNB), pentaclorofenol (PCP), metilmetanosulfonato (MMS) e radiação UVC. As curvas de sobrevivência foram realizadas como descrito nos Capítulos IV e V. Os resultados indicam maior sensibilidade das linhagens simples mutantes *frm2Δ* e *hbn1Δ* aos agentes testados em relação à linhagem selvagem, sendo que a linhagem duplo mutante apresentou a maior sensibilidade.

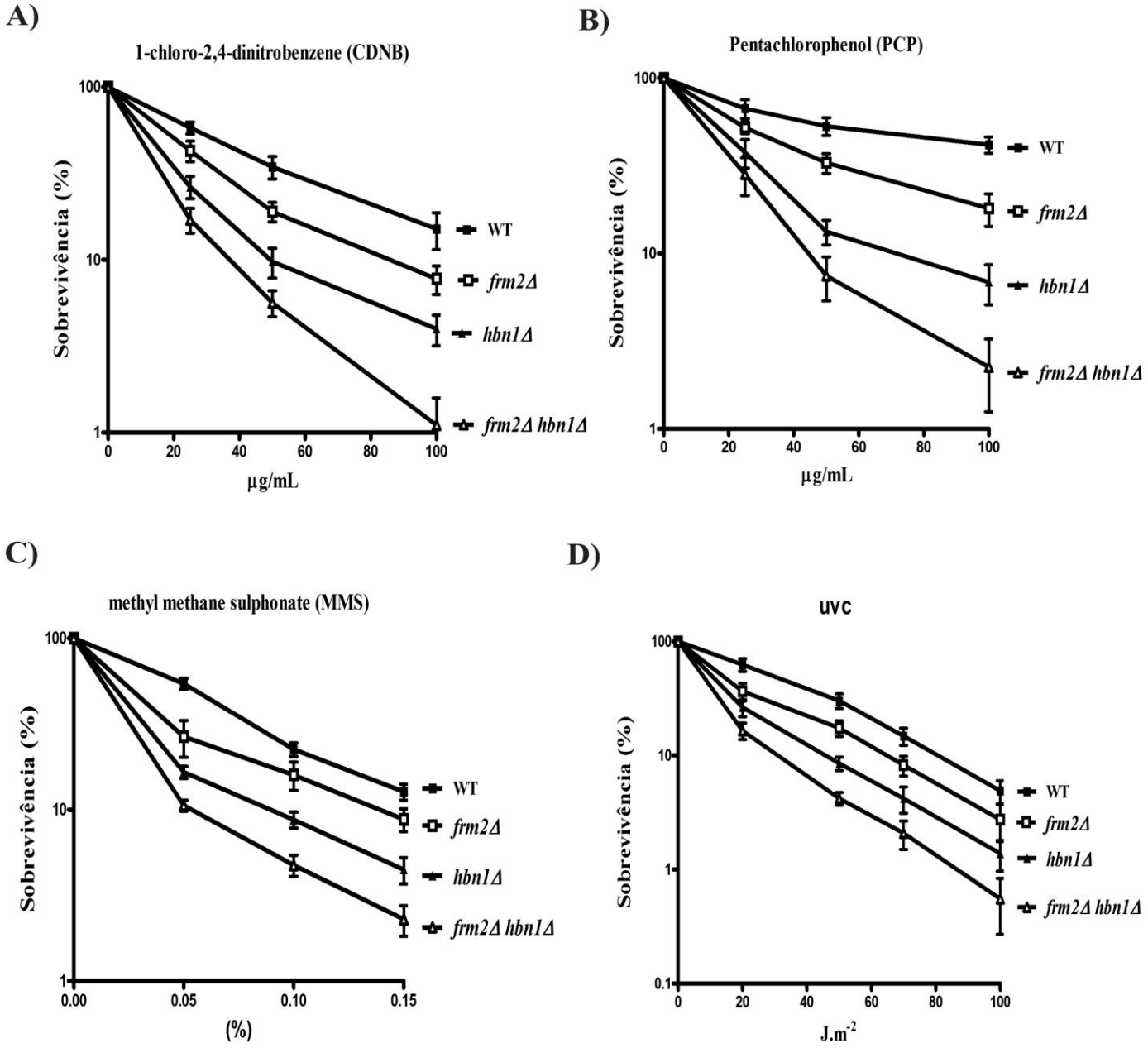

Figura 16: Sensibilidade de linhagens mutantes de levedura em fase estacionária de crescimento a diferentes agentes oxidantes e mutagênicos. (A) CDNB; (B) PCP; (C) MMS; (D) UVC. Os dados representam a média de três experimentos independentes. As células foram expostas por 2 h aos agents à 30 ^{o}C.

ANEXO III

A tabela a seguir apresenta uma comparação dos resultados relativos à sobrevivência de linhagens de *Saccharomyces cerevisiae* proficientes e deficientes nas nitrorredutases Frm2p e Hbn1p em fase estacionária e exponencial (20-30% de brotos) de crescimento expostas a oxidantes. As curvas de sobrevivência foram realizadas como descrito nos Capítulos IV e V. Os dados indicam que não há influência do estado de crescimento no padrão de resposta das linhagens mutantes em relação à selvagem.

Tabela 3 - Comparação das respostas em fase estacionária e exponencial entre linhagens de S. cerevisiae proficientes e deficientes nas nitrorredutases Frm2p e Hbn1p expostas a oxidantes.

		BY4741 (WT)		***frm2Δ***		***hbn1Δ***		***frm2Δ hbn1Δ***	
		Estacionária	**Exponencial**	**Estacionária**	**Exponencial**	**Estacionária**	**Exponencial**	**Estacionária**	**Exponencial**
4-NQO	**0 μg/mL**	100	100	100	100	100	100	100	100
	0,1 μg/mL	79,06±6,23***	33,10±3,69***	38,51±3,72***	20,79±3,24***	43,97±4,81***	19,82±3,52***	41,20±4,29***	17,68±2,71***
	0,2 μg/mL	49,97±3,8***	21,81±2,44***	13,78±2,57***	8,10±1,57***	16,29±2,77***	8,38±1,89***	15,12±1,92***	8,08±1,56***
	0,3 μg/mL	35,36±5,33***	8,42±1,01***	5,49±1,41**	2,37±0,95**	5,74±1,72**	2,82±0,91**	4,57±0,92**	2,32±0,86**
NDEA	**0**	100	100	100	100	100	100	100	100
	300 μM	67,17±4,90***	15,72±1,36***	23,67±2,88***	10,88±1,52***	46,64±4,47***	7,54±0,54***	14,67±2,42***	3,41±0,59***
	400 μM	46,32±4,08***	12,54±1,37***	12,86±2,02***	7,50±0,86***	25,91±3,32***	5,24±0,58***	6,44±1,09***	1,81±0,42***
	500 μM	24,35±4,66***	5,45±0,58***	6,55±2,39**	2,54±0,51**	14,17±1,73***	1,56±0,38***	1,77±0,39**	0,43±0,25**
H_2O_2	**0 mM**	100	100	100	100	100	100	100	100
	1 mM	46,21±4,59***	18,07±1,14***	86,75±4,84***	34,06±1,68***	47,98±6,89***	14,53±0,97***	77,81±4,28***	28,16±1,27***
	3 mM	29,83±5,14***	13,17±1,17***	67,57±5,47***	27,31±1,28***	29,55±4,01***	10,90±1,21***	61,95±2,23***	23,32±1,40***
	5 mM	10,94±1,99***	5,47±0,77***	48,57±2,92***	12,07±1,06***	7,12±2,44***	4,54±0,54***	49,97±3,33***	11,72±1,15***
***t*-BOOH**	**0 mM**	100	100	100	100	100	100	100	100
	1 mM	28,29±3,62***	10,62±1,25***	61,52±6,72***	23,85±1,39***	18,53±4,49***	8,42±0,99***	47,15±4,37***	22,50±5,18***
	3 mM	9,82±1,59***	5,15±0,56***	27,59±2,91***	12,44±1,93***	5,97±0,16*	4,57±0,35*	19,30±4,32***	10,79±0,98***
	5 mM	2,14±0,66*	1,15±0,25*	11,50±1,64***	2,47±0,49***	0,82±0,17*	0,72±0,15*	8,29±1,90***	2,32±0,41***

*$P < 0.05$, **$P < 0.01$ ***P < 0.001

ANEXO IV

A tabela a seguir apresenta uma comparação dos resultados relativos à sobrevivência de linhagens de *Saccharomyces cerevisiae* proficientes e deficientes nas nitrorredutases Frm2p e Hbn1p expostas a oxidantes nos meios fermentativo (Glicose 2%) e respiratório (Glicerol 3%). As curvas de sobrevivência foram realizadas como descrito nos Capítulos IV e V. Os dados indicam que não há influência do estado metabólico no padrão de resposta das linhagens mutantes em relação à selvagem.

Tabela 4 - Comparação das respostas de linhagens de S. cerevisiae proficientes e deficientes nas nitrorredutases Frm2p e Hbn1p expostas a oxidantes nos metabolismos fermentativo e respiratório.

		BY4741 (WT)		***frm2Δ***		***hbn1Δ***		***frm2Δ hbn1Δ***	
		Fermentativo	**Respiratório**	**Fermentativo**	**Respiratório**	**Fermentativo**	**Respiratório**	**Fermentativo**	**Respiratório**
4-NQO	**0 μg/mL**	100	100	100	100	100	100	100	100
	0,1 μg/mL	79,06±6,23***	43,68±4,51***	38,51±3,72***	21,35±2,75***	43,97±4,81***	20,09±3,24***	41,20±4,29***	17,66±2,64***
	0,2 μg/mL	49,97±3,8***	29,96±3,64***	13,78±2,57**	7,85±1,51**	16,29±2,77***	7,99±1,53***	15,12±1,92***	7,65±1,20***
	0,3 μg/mL	35,36±5,33***	12,48±2,36***	5,49±1,41*	2,25±0,79*	5,74±1,72*	2,64±1,00*	4,57±0,92*	2,30±0,85*
NDEA	**0**	100	100	100	100	100	100	100	100
	300 μM	67,17±4,90***	49,65±3,92***	23,67±2,88***	11,28±1,69***	46,64±4,47***	18,16±3,11***	14,67±2,42***	6,69±0,88***
	400 μM	46,32±4,08***	24,45±2,92***	12,86±2,02***	6,17±1,05***	25,91±3,32***	9,31±2,13***	6,44±1,09*	2,92±0,78*
	500 μM	24,35±4,66***	11,47±1,82***	6,55±2,39*	2,81±0,65*	14,17±1,73**	4,28±0,83**	1,77±0,39*	0,71±0,35*
H_2O_2	**0 mM**	100	100	100	100	100	100	100	100
	1 mM	46,21±4,59***	63,36±6,46***	86,75±4,84***	96,66±5,87***	47,98±6,89***	58,74±7,72***	77,81±4,28***	92,27±5,21***
	3 mM	29,83±5,14*	32,66±6,60*	67,57±5,47*	75,31±6,61*	29,55±4,01*	26,33±4,41*	61,95±2,23***	73,46±2,68***
	5 mM	10,94±1,99*	15,01±2,85*	48,57±2,92*	54,09±3,11*	7,12±2,44*	9,43±1,09*	49,97±3,33**	59,26±4,07**
***t*-BOOH**	**0 mM**	100	100	100	100	100	100	100	100
	1 mM	28,29±3,62***	40,08±4,28***	61,52±6,72***	80,64±7,96***	18,53±4,49***	28,25±5,33***	47,15±4,37***	61,06±5,18***
	3 mM	9,82±1,59***	18,07±1,95***	27,59±2,91***	39,88±3,56***	5,97±1,17**	13,34±1,44**	19,30±4,32***	28,40±5,07***
	5 mM	2,14±0,66**	8,93±0,83**	11,50±1,64***	20,54±2,06***	0,82±0,37*	7,22±0,46*	8,29±1,90**	15,48±2,21**

*$P < 0.05$, **$P < 0.01$ ***P < 0.001

Curriculum Vitae

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Produções

Produção bibliográfica

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Artigos completos publicados em periódicos

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12. VILLELA, I. V. ; **de OLIVEIRA IM** ; SILVEIRA, J. ; SILVA, J. ; HENRIQUES, J. A. P. . Biomonitoramento da Atividade Genotóxica na Bacia do Lago Guaíba (Porto Alegre, RS). In: VIIl Congresso Brasileiro de Ecotoxicologia, 2004, Florianópolis - SC. Livro de Resumos, 2004. p. 195.

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2. I Workshop de Invertebrados Límnicos Invasores na América do Sul.O Mexilhão Dourado como Biomonitor. 2010. (Simpósio).

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Orientações

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