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Mitochondrial dysfunction resulting from the absence of mitochondrial porin in *Neurospora crassa*

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ABSTRACT

Porin, the voltage-dependent anion-selective channel (VDAC) in the mitochondrial outer membrane, contributes to metabolism and apoptosis. VDAC function was investigated in *Neurospora*, an obligate aerobe with a single porin. Porinless strains are viable, with cold-sensitive growth, cytochrome deficiencies and overexpression of alternative oxidase. iTRAQ labeling of mitochondria from a porinless strain and its progenitor revealed a small group of proteins with altered expression levels in the mutant organelles. Porinless *Neurospora* appears to compensate not by inducing alternative pores, but by altering electron flow and nucleotide metabolism. Transcriptional and post-transcriptional mechanisms contribute to the response, reflecting the extent of porin influence.
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1. Introduction

Mitochondrial porin, the voltage-dependent anion-selective channel (VDAC) is the most abundant protein in the outer membrane (MOM) of the organelle (Schmitt et al., 2006). The porin channel is dynamic, responding to molecules such as ATP (Rostovtseva and Bezrukov, 1998), and NADH (Zizi et al., 1994) and to Ca^{2+} (Gincel et al., 2001). In addition up to 55 different proteins may interact with porin (Roman et al., 2006), and of these, much attention has been directed to those whose interactions are related to the regulation of certain apoptotic pathways (Baines et al., 2007, Park et al., 2010, reviewed by Kinnally et al., 2010). These roles of porins have been studied extensively in artificial membranes (reviewed in Summers and Court, 2010; Hiller et al., 2010). However, to understand the complex contributions of porin to eukaryotic biology, model systems are required.

Many organisms harbor more than one porin isoform; for example, there are three porins in mammals (reviewed in Young et al., 2007). Unicellular *Saccharomyces* is a simpler system, but expresses two porin isoforms (Blachly-Dyson et al., 1997). Deletion of the yeast *por1* gene leads to a moderate slow-growth phenotype at high temperature on non-fermentable substrates; this mutation can be partially complemented by over-expression of *por2*, which encodes a protein that does not form pores in artificial membranes (Blachly-Dyson et al., 1997). Analysis in *Saccharomyces* is complicated by the fact that this organism is a facultative anaerobe. *Neurospora crassa* is an excellent model system for porin studies due to the combination of a long history of use as an obligately-aerobic model organism for studies of both mitochondrial biology (Mitchell et al., 1953) and porin function (Colombini, 1979), a single porin gene and advances in the genetic manipulation of the organism (Ninomiya et al., 2004). Hence, a porin-deletion strain was created, and analyzed in parallel with a similar strain generated by the *Neurospora* genome deletion project (Colot et al., 2006).

2. Materials and methods

2.1. Strains and growth conditions

Saccharomyces cerevisiae S150 (*ura3-53*, *his3-Δ1*, *trp1-289*, *leu2-3,112*, *MATα*) was used to generate plasmids by homologous recombination, as described in Colot et al. (2006). *N. crassa* strains used in this study are listed in Table 1. General growth and handling of *N. crassa* were as described (Davis and De Serres, 1970); cultures were grown at either 22 °C or 30 °C. ΔPor-1 (Table 1) was generated from FGSC 9718 (Colot et al., 2006) by homologous recombination with a

Abbreviations: DDM, *n*-dodecyl-β-D-maltoside; DG, digitonin; DOC, deoxycholate; iTRAQ, isobaric tag for relative and absolute quantitation; MOM, mitochondrial outer membrane; mtDNA, mitochondrial DNA; SHAM, salicylhydroxamic acid; qPCR, quantitative PCR; VDAC, voltage-dependent anion-selective channel; VMM, Vogel's minimal medium.

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Table 1
Neurospora crassa strains used in this study.

Strain	Genotype (mating type)	Female fertility[a]	Source	Reference
FGSC 6103	*his-3 (A)*	+	FGSC[b]	Colot et al. (2006)
Por-WT (FGSC 9718)	Δ*mus*51::bar$^+$ *(a)*	+	FGSC	Colot et al. (2006)
ΔPor-1 (WS004)	Δ*por*::*hph*$^+$ Δ*mus51*::*bar*$^+$ *(a)*	−	This work	
ΔPor-2 (FGSC 18892)	Δ*por*::*hph*$^+$ *(A)*	+	FGSC	Colot et al. (2006)

[a] Visible protoperithecia within 7 days on Westergaard's medium (Davis and De Serres, 1970).
[b] Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City.

linear DNA fragment comprised of the upstream region of the *por* locus (−320 to −1020 relative to the translation start site), the hygromycin-resistance cassette from pCNS44 (Staben et al., 1989) and 1413 bp of downstream sequence, beginning after the stop codon for *por*. Following selection on hygromycin-containing Vogel's minimal medium (VMM), individual transformants were purified following four rounds of plating for single conidia on VMM containing hygromycin (1800 U/ml, EMD Biosciences, La Jolla, CA), and confirmed by PCR, Southern blot hybridization, and western blot analysis (see Fig. 4B). FGSC 18892 was created as part of the Dartmouth Medical School *Neurospora* deletion project, from either FGSC 9718 or FGSC 9719 (Colot et al., 2006). In this case, a homokaryotic, *mus*$^+$ strain was generated through a cross with a wild-type strain, either FGSC 2489 or *his-3* (FGSC 6103), depending on the starting strain.

2.2. Phenotypic analysis of porin-less strains

Growth rate was determined on solid medium, using "race tubes" (Davis and De Serres, 1970). Cytochrome spectra were obtained and absolute cytochrome concentrations were determined basically as described by Lambowitz and Slayman (1971). Isolated mitochondria (5 mg/ml) were solubilized with 2% deoxycholate (DOC) and oxidized with potassium ferricyanide. Absorbance was measured from 500 nm to 650 nm. The solution was then reduced using sodium dithionite and the wavescan repeated. Difference spectra (reduced–oxidized) were used to calculate absolute cytochrome levels.

Alternative oxidase activity was examined by following oxygen consumption by intact mycelia in VMM using a Gilson oxygraph and Clark oxygen electrode. Cyanide (final concentration 2.5 mM) and salicylhydroxamic acid (SHAM 6.25 mM) were added to block cytochrome oxidase and alternative oxidase, respectively (Nargang et al., 1978). Chemicals were purchased from Fisher Scientific (Nepean, ON, Canada) or Sigma-Aldrich Canada (Oakville, ON, Canada).

2.3. Fluorescence microscopy

N. crassa was grown in VMM at 22 °C or 30 °C and stained with MitoTracker Green FM (Invitrogen). One ml of culture was incubated at growth temperature with 0.5 µl of MitoTracker Green FM (1 µg/ml in DMSO) for 30 min. Hyphae were washed with H_2O and resuspended in 1% low gelling temperature agarose (Nusieve GTG Agarose, Fisher Scientific). Mitochondrial fluorescence was observed using a Zeiss Axio Imager Z1 at a magnification of 1000× with the appropriate filters.

2.4. Protein analysis

Mitochondria for western blot analysis were isolated by differential centrifugation (Harkness et al., 1994) from wild-type (Por-WT) mycelia grown for 16 h at 30 °C or 24 h at 22 °C, or from ΔPor-1 mycelia grown for 36 h at 30 °C or 120 h at 22 °C. Approximately 5 g (wet weight) of mycelia was used per isolation, yielding about 1 mg of mitochondria. Mitochondrial proteins were separated through 4–12% SDS-PAGE. Western blot analysis was carried out using antibodies against residues 7–20 of wild-type porin, and against Tom70, kindly provided by Drs. R. Lill, K. Hell and W. Neupert, Universität München.

For native gel electrophoresis of Tom40-containing complexes, mitochondria were isolated from ΔPor-1 and Por-WT cultures grown at 22 °C for 5 days and 24 h, respectively. Complexes were isolated from digitonin (DG, 1%) and dodecylmaltoside (DDM, 1%) solubilized mitochondria as described by Sherman et al. (2006), and analyzed on non-denaturing 6–18% acrylamide gels containing Ponceau S, using a 6% stacking gel and buffer conditions indicated by Dráb et al. (2011). Electrophoresis was carried out at 4 °C, at 60 V for the initial 30 min, and 130 V until the dye front reached the bottom of the gel. The resulting gel was blotted to nitrocellulose and probed with anti-Tom40 antibodies, generously provided by Drs. Hell and Neupert.

2.5. Proteomic analysis

Mitochondria for proteomic analysis were obtained from two biological replicates each of ΔPor-1 and Por-WT cultures grown at 22 °C (15–20 g of mycelium). These organelles were further purified by flotation gradient centrifugation (Lambowitz, 1979) and solubilized with 700–1500 µl of solubilization buffer (10 mM Tris–Cl, pH 7.4, 3 mM $CaCl_2$, 2 mM $MgCl_2$ and NP40 (0.1% (v/v))). The mixture was subjected to 1 min of sonication with a Fisher Scientific Sonifier Model 300, set to 30% output, on ice. The suspension was incubated on ice for 30 min, with mixing by vortex every 10 min. Insoluble material was removed by centrifugation at 13,000 rpm at 4 °C for 30 min.

Proteomic analysis was carried out at the Manitoba Centre for Proteomics and Systems Biology, using iTRAQ labeling (Ross et al., 2004). Each 100 µg protein sample was adjusted to 100 µl with 100 mM ammonium bicarbonate buffer. Samples were reduced with 10 mM dithiothreitol (DTT) for 40 min at 56 °C followed by alkylation using 50 mM iodoacetamide (IAA) for 30 min at room temperature. Excess IAA was neutralized by the addition of 17 mM DTT. Proteins were digested with sequencing-grade trypsin (Promega, Madison, WI, USA) at a 1:50 enzyme:substrate ratio overnight at 37 °C. Samples were frozen at −80 °C and dried using a speed vacuum. Trypsin-digested peptides were purified using a reversed-phase 100 Å C18 sorbent Luna C18(2) column (Phenomenex, Torrance, CA). Tryptic-digested peptides were labeled with different iTRAQ reporters (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's procedure.

2.6. Liquid chromatography–MS/MS analysis

Labeled samples were mixed in equal proportions and subjected to two dimensional high-performance liquid chromatography (LC)–mass spectrometry (MS) analysis were carried out as described (Dwivedi et al., 2009). Briefly, dried iTRAQ-labeled peptide samples were dissolved in 0.1% trifluoroacetic acid (TFA) and were gradient-fractionated on a C18 X-Terra column (1×100 mm, 5 µm, 100 Å; Waters Corporation, Milford, MA, USA) with an Agilent 1100 Series HPLC system. 40 fractions were collected using a gradient of 1% to 40% of solvent B (20 mM ammonium formate pH 10 in both eluents A and B, 1%acetonitrile/min, 150 µl/min flow rate).

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