Dtr1p, a Multidrug Resistance Transporter of the Major Facilitator Superfamily, Plays an Essential Role in Spore Wall Maturation in Saccharomyces cerevisiae

Authors' GOAL: clarify mechanism of spore wall formation

Spore wall synthesis:
- Prospore membrane (vesicle-derived) starts to form at spindle pole bodies during the second meiotic division
- Extends along outer surface of the nuclear envelope
- Each nucleus covered by prospore membrane by end meiosis II
- Spore wall material deposited in lumenal space of prospore membrane

Mature spore wall: 4 layers with outermost consisting of dityrosine macromolecule

Elucidating the mechanism of spore wall formation: library screen & dityrosine analysis

HCL liberates LL- & DL-dityrosine (+ precursors) from sporulated yeast
- Can be separated by Reverse Phase (RP)-HPLC (dityrosine fluorescent) & determine ratio by peak integration
- WT strains have fixed ratio of LL- & DL-dityrosine (3:2)
- Dityrosine epimerized (DL-) in spore wall & soluble precursors are LL-
- Thus, deviations in ratio serve as markers for perturbations in spore wall formation

Yeast deletion library screen
- Assayed for change in LL:DL
- 191 with altered ratio; 1 with strong phenotype = YBR180w ORF deletion in several strains (DL/LL ratio 0.2 vs. 0.6 in WT)
- YBR180w encodes predicted 63.4kDa protein with 12 TM spans & is homologous to family of drug:H+ antiporters (DHA12)

Disruption of YBR180w (DTR1) leads to decrease in DL-dityrosine levels in asci

DTR1 disruption causes decrease of DL- in sporulated total cell hydrolysates
- Chromatograms show DL/LL ratios
  - A. dtr1-Δ DL/LL ratio 0.2
  - B. WT 0.6
  - C. complementation with DTR1 restores WT ratio

Defect in epimerization or accumulation unincorporated LL-dityrosine in cytoplasm?

dtr1-Δ spores accumulate soluble, unincorporated bisformyl dityrosine

Analysis of cytoplasmic & wall fractions of sporulated cells
- A. dityrosine levels similar in WT & dtr1-Δ cells (not a problem with synthesis)
- B. dityrosine levels in WT wall fraction high
- C. levels in dtr1-Δ cytoplasm rise throughout sporulation (2X > wall)
- D. DL/LL in dtr1-Δ wall fraction same as WT, 60% less dityrosine (not problem with epimerization)
- E. no dityrosine in dtr1-Δ cytoplasm, but DL/LL in cytoplasmic fraction < than wall
Hypothesis: Dtr1p is a dityrosine transporter

Accumulation of dityrosine-containing compounds in the cytoplasm of dtr1-∆ spores

- **Hypothesis:** Dtr1p is a dityrosine transporter
- **Used gradient RP-HPLC:**
  - **d** = free dityrosine
  - **mdf** = N-monoformyl dityrosine
  - **bfd** = N,N’-bisformyl dityrosine

**dtr1-∆ spores have an aberrant spore surface**

- **A. Fluorescence microscopy:** fluorescent dye (Calcofluor white) binds 2nd layer (chitosan) = disrupted outer spore layer
  - **WT** = no fluorescence
  - **dtr1-∆** = mix of WT & aberrant spore surfaces (arrow)
- **B. EM (OsO4 stain):** dityrosine layer (s) electron-dense & chitosan diffuse
  - (I) 25% ascis with WT-like spore wall
  - (II) ascus with 1-WT & 1-lacking outer layer (15% all ascis had 1 or more)
  - (III & IV) 60% had less electron-dense surface layer
- Confirmed with biochemical tests
- Dityrosine outer layer altered by deletion of DTR1

**DTR1 expression sporulation-specific**

- A. Northern analysis of total RNA (SK1) from different stages of sporulation
  - compared with marker genes: NDT80 (delayed early); SPS1 (mid-late); DIT1 (mid-late); SPS100 (late)
  - **DTR1** (early-middle); peak @ 6h
- B. Onset of dityrosine synthesis correlates with max accumulation DTR1
  - dityrosine synthesis
  - densitometry DTR1 expression
- C. DTR1 expression among MATa/a, a/a, & a/a (northern blot)
  - NOT expressed in asporogenous cells

**Dtr1p localizes to prospore membrane**

- 1st Step:
  - free L-tyrosine modified into N-formyl tyrosine (Dit1p)
- 2nd Step:
  - 2 molecules N-formyl tyrosine cross-linked by Dit2p forming LL-N,N’ - bisformyl dityrosine

- **B. Confirmation that Dtr1p localizes to prospore membrane,** which form at ends of microtubule bundles (@ meiosis II)
  - Dtr1p-GFP co-localizes with microtubules (anti-tubulin = red)
- **C. Untagged DTR1-transformed cells @ different stages of meiosis (control)**

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- Dtr1p transports dityrosine to the outer layer of the spore wall
Dtr1p is a dityrosine transporter

A. Dityrosine transport in sporulating cells (cells vs. media)
- *cda1,cda2-Δ* strain (lacks outer 2 layers) = media has 85% dityrosine
- WT, *dtr1-Δ* = trace amounts in media
- *cda1,cda2,dtr1-Δ* = 85% in cells

B. C. Dityrosine transport in vegetative cells, which normally lack dityrosine (*DIT1/2* not expressed)
- Switched promoter to express *DIT1/2* and/or ectopically expressed *DTR1*
- □ = *DIT1/2 & DTR1*, ● = *DIT1/2*
- RP-HPLC: bisformyl dityrosine peaks

Substrate specificity of Dtr1p & other MDRPs

A. Is dityrosine transport restricted to Dtrp?
- Dtr1p & MDR proteins from MFS & ABC families expressed with *DIT1/2* in vegetative cells
- Measured dityrosine in media by RP-HPLC
- 2 other MFS-MDR (same cluster) proteins transported w. 50% efficiency

B. Is Dtr1p a drug R determinant?
- b & c dilutions of cell suspension a
- Measured susceptibility of WT & isogenic *dtr1-Δ* strains transformed with *DTR1*- or empty-vectors
- Dtr1p confers R to propionic, benzoic, & butyric acids (preservatives) & quinine & quindine

Summary

- Spore wall deficient *cda1/2-Δ* strain (dityrosine not epimerized into surface layer): Dtr1p pumps dityrosine into media (C)
- Dityrosine in cells in *cda1/2,dtr-Δ* strains: Dtr1p absent thus not pumped into media (D)
- *DTR1* mRNA sporulation-specific & max accumulation coincides with prospore membrane formation prior to dityrosine synthesis
- Dtr1p localizes to prospore membrane through spore wall formation
- Dtr1p confers resistance to some toxic compounds

*DTR1* 1st MFS-MDR protein w/ described normal physiological role