# Analysis of cell surface glycans of *Δspf1* strain involved in resistance to yeast killer toxin SMKT

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**Research objective**

Killer toxins (KT) are proteins produced by yeasts that kill other yeasts. The structure, mechanism of action, and localization of the gene encoding KT (on chromosome and plasmid) differ significantly between species and strains. Our research group clarified the subunit and crystal structure of SMKT, the KT of the salt-tolerant yeast *Pichia farinosa* (now *Millerozyma farinosa*), and demonstrated that it exerts its lethal effects by disrupting the cell membrane of susceptible yeasts.[1)] Furthermore, we identified a *Saccharomyces cerevisiae* gene essential for SMKT sensitivity and, which we named it *SPF1*, derived from "**S**ensitivity of ***P. f****arinosa* killer toxin." Since the labeled SMKT localizes to the cell surface of *spf1*-deficient (*Δspf1*) strains, we speculate that the cell surface of the *Δspf1* strain traps SMKT and, preventing its action on the cell membrane, and thus conferring resistance[1)]. Spf1 protein (Spf1p) has long been considered an orphan P-type ATPase but was recently shown to be responsible for regulating the orientation of membrane proteins in the endoplasmic reticulum by expelling misfolded transmembrane helices as substrates[2)]. In general, the action of KT has been reported to occur via binding to the cell wall of susceptible yeast, the plasma membrane, and intracellular targets; however, the pathway from the cell wall to the final target has not yet been elucidated. In this study, I aimed to clarify the role of Spf1p in secretory glycan synthesis by analyzing the cell wall glycan structure of spf1-deficient strains, and to elucidate the mechanism of SMKT action by examning the interaction of SMKT with cell wall glycans and cell membrane.

**Methods**

(1) Generation of *spf1gpi10* double-mutant strains

The outermost layer of the yeast cell wall is comprised of a mannoprotein (MP) layer. The *gpi10* mutant strain, which releases MP into the culture supernatant, has a T mutation at base C1493 of the *GPI10* gene. A double disruption strain of the *gpi10* mutant and *Δspf1* strain was generated and used it to obtain MP from the wild type and *Δspf1* strain and planned to analyze the glycan structure of MP.

(2) Purification of MP

Yeast cells were washed with 0.2 M saline, autoclaved twice at 120°C for 25 min, and the supernatant was dialyzed. Four volumes (4 vol. mL) of ethanol containing 0.1% sodium acetate was added, and the precipitate was collected. The precipitate was dissolved in saline,

and the amount of protein was determined by BCA (bicinchoninic acid) protein assay kit.

(3) Electron microscopy of yeast cell walls

Wild-type strain and *Δspf1* strains were analyzed by electron microscopy. Yeast cells were prepared using the quick-freezing and freeze-substitution method. Cell wall and MP layer thicknesses were also analyzed. Cells of wild-type, *Δspf1*, *Δpmr1*, and *Δspf1Δpmr1* strains were also analyzed.

(4) Comparative analysis of *SMK1*, the killer gene encoding SMKT, and *SMK1*-like genes from the Genome Database.

**Results**

(1) Generation of *spf1gpi10* double-mutant strains

An attempt was made to generate *spf1gpi10* double-mutant strains, and it became clear that the double mutant strain (LEU2 + (*Δspf1*) with mutation T in *gpi10*) was susceptible to SMKT after four-spore isolation (Table 1). This is consistent with the release of *Δspf1*-type MPs from the cell wall that contribute to resistance. However, they died during storage at -80°C as a glycerol stock. Random spore isolation was attempted but was stopped because the *gpi10* mutation partially reverted to the wild type.

Table 1. Results of tetrad analysis

| No. | 5 | | | 6 | | | 7 | | | 10 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Leu[a] | Killer[b] | *gpi10*[c] | Leu | killer | *gpi10* | Leu | killer | *gpi10* | Leu | killer | *gpi10* |
| A | - | s | | - | s | | - | s | | - | s | |
| B | + | s | T | - | s | | + | R | C | + | R | C |
| C | + | s | T | - | s | | - | s | | - | s | |
| D | - | s | | + | R | C | + | s | T | + | s | T |

[a] Growth on medium without leucine (LEU2+ phenotype) (*spf1::LEU2*); [b] Sensitivity (s) and resistance (R) to SMKT; [c] Sequence of the *gpi10* mutation site, T is mutation, C is wild-type.

(2) Purification of MP

Autoclave treatment was used as an MP preparation of the method. As a result, 4.5 mg of MP fraction was obtained from 6 g of 500 mL of the wild-type strain. However, only 96 μg of MP was obtained from 5.1 g of the *Δspf1* strain, suggesting that the MP fraction itself was extremely small in the *Δspf1* strain.

(3) Electron microscopic analysis of yeast cell walls

Transmission electron microscopy images of ultrathin sections revealed that the MP layers of the wild-type and *Δspf1* strains (both diploid) were 70 nm and 53 nm, respectively, with the *Δspf1* strain being significantly thinner. Furthermore, the MP layer was significantly thinner

in the *Δspf1*, *Δpmr1*, and *Δspf1Δpmr1* strains than in the wild-type strain, and the cell wall was also significantly thinner in the *Δspf1Δpmr1* strain (Table 2).

Table 2. Thickness of mannan protein (MP) layer and cell wall (CW) of gene disruption strains

| | wt (nm) | *spf1* (nm) | *pmr1* (nm) | *spf1pmr1* (nm) |
|---|---|---|---|---|
| MP | 69.6 ± 11.5 [a] | 52.4 ± 10.9 [b] | 46.4 ± 7.0 [b] | 46.4 ± 6.0 [b] |
| CW | 109.6 ± 14.7 [a] | 107.9 ± 19.2 [a] | 114.7 ± 13.1 [a] | 85.9 ± 10.7 [b] |

* Different letters indicate significant differences between strains in the MP and CW.

(4) Comparative analysis

A database search revealed SMK1-like genes in four strains of *Metschnikowia* sp. yeast, including *Metschnikowia* sp. JCM 33374. The precursor of SMKT is post-translationally processed by Kex2-like enzymes at the C-terminal side of the α-subunit and the N-terminal side of the β-subunit, and the intermediate γ-peptide is removed and secreted as a dimer composed of αβ-subunits. The SMKT-like protein from *Metschnikowia* sp. also has a conserved basic amino acid sequence that serves as a Kex2-like enzyme cleavage site on the precursor, suggesting an αβ dimeric structure (Fig. 1). The AlphaFold2 analysis of the peptide sequence predicted a folding structure like that of SMKT.

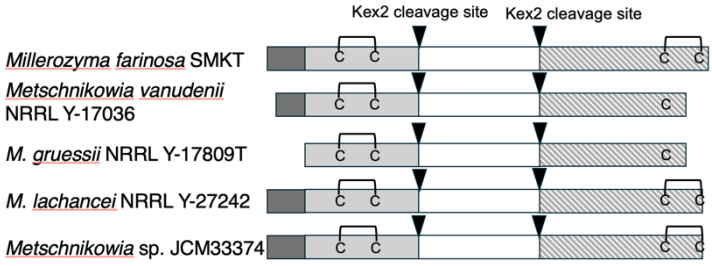

Fig. 1 The preprostructure of SMKT-like proteins of *Metschnikowia* sp.

## Conclusion

The fact that the *spf1gpi10* double mutant strain becomes susceptible to SMKT suggests that the MP of the *Δspf1* strain contributes to killer resistance. The MP layer of the *Δspf1* strain is significantly thinner than that of the wild-type strain, and the *Δspf1Δpmr1* mutation affects not only the MP layer but also the cell wall thickness, suggesting that the regulation of Spf1p membrane protein orientation plays some role in the MP layer and cell wall formation. The presence of SMKT-like proteins in yeasts of the genus *Metschnikowia* is of interest in the evolution and propagation of KT.

## References


1) Suzuki C* (2004) Acidophilic structure and killing mechanism of the *Pichia farinosa* killer toxin SMKT. pp 189-214. Schmitt, M. J. and Schaffrath, R. Eds. In Microbial Protein Toxins, Topics in Current Genetics, pp.1-233, Sprinter
2) McKenna et al. (2020) The endoplasmic reticulum P5A-ATPase is a transmembrane helix dislocase. Science 369(6511): eabc5809