

## Protein O-Mannosyltransferase Isoforms Regulate Biofilm Formation in *Candida albicans*

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**Five isoforms of protein mannosyltransferase (Pmt) O-mannosylate secretory proteins in *Candida albicans*. *pmt* mutants were differentially defective for biofilm formation on plastic in static and flow-through systems, and a Pmt inhibitor blocked early stages of biofilm formation. Conceptually, Pmt inhibition may prevent surface anchoring and biofilm-dependent resistance of fungal pathogens.**

The human fungal pathogen *Candida albicans* is able to form biofilms on a variety of inert surfaces, including materials used for medical implants, e.g., central venous catheters (7, 8, 17), which consist of a bottom layer of mostly yeast cells, an upper layer containing mostly hyphal cells, and yet-undefined extracellular material (2, 4, 5). *C. albicans* biofilms are highly resistant to most antifungals, but the mechanisms of resistance are not clear (1, 3, 5, 6, 12, 13).

Some compounds blocking hyphal development and consequently biofilm formation and biofilm-mediated resistance have been described (11, 16). We speculated that surface mannoproteins could also represent targets for biofilm inhibitors. Surface mannoproteins in fungi are typically O-mannosylated at serine or threonine residues, and protein O-mannosyltransferases (Pmt proteins) initiate this modification in the endoplasmic reticulum (9). We previously characterized the *PMT* gene family of *C. albicans*, which encodes five Pmt isoforms (15, 19, 20). One isoform, Pmt2p, was required for growth, while the Pmt1p and Pmt4p isoforms contributed to high levels of basal resistance towards a range of antifungals. Most *pmt* mutants (except *pmt5*) were defective in hyphal formation in some conditions, and all five Pmt isoforms contributed to virulence (15, 18).

We compared the biofilm-forming ability of the control strain CAF2-1, isogenic homozygous *pmt* mutants (*pmt1*, *pmt4*, and *pmt6*), and the heterozygous *PMT2/pmt2* strain (15) in a batch system. Strains were pregrown for 24 h at 37°C in SD medium (0.67% yeast nitrogen base, 2% glucose) (yeast form only) and resuspended in 5 ml phosphate-buffered saline. Cells were separated in a bath sonifier and resuspended in RPMI 1640 medium containing 2% glucose (0.165 M morpholinepropanesulfonic acid, pH 7) at 10<sup>6</sup> cells per ml. Each strain was used for inoculation of a separate 24-well-culture polystyrene dish (Falcon), adding 500 µl of the cell suspension to each well. Plates were incubated for 48 h at 37°C in a wet chamber. The medium was discarded, each well was washed with 500 µl of

phosphate-buffered saline, and plates were dried for 24 h at 37°C to determine dry weights. Results were evaluated by an unpaired *t* test, calculating two-tail *P* values (GraphPad Prism 4). Clear quantitative differences were observed between biofilms of *pmt* mutants and the control strain (Fig. 1A). Biofilm formation of the *pmt1* mutant and the heterozygous *PMT2/pmt2* strain was significantly reduced, while *pmt4* and *pmt6* mutations caused moderate defects; in contrast, biofilm formation of the *pmt5* mutant was not affected. A *pmt1 pmt6* double mutant (20) revealed no additional contribution of the *pmt6* mutation to the *pmt1* biofilm phenotype, while the *pmt4 pmt6* double mutant (14), unexpectedly, had the wild-type phenotype. To confirm mutant phenotypes, we compared biofilm formation of *pmt* mutants containing chromosomally integrated vectors carrying the corresponding *PMT* gene to that of strains carrying an empty vector (pRC18) (18). This experiment confirmed strong and moderate requirements for *PMT1* and *PMT4*, respectively, in biofilm formation (Fig. 1B). Importantly, biofilm defects were not due to defects in growth or morphogenesis, because growth rates and hyphal formation abilities of *pmt* mutants were equivalent to those of the control strain during planktonic growth under the conditions used for biofilm formation (data not shown). Furthermore, electron microscopy (Philips ESEM XL30 FEG microscope) showed similar appearances of biofilms of the control, *pmt4*, *pmt5*, *pmt6*, and *PMT2/pmt2* strains, consisting of a dense mat of intertwined hyphal filaments mixed with yeast cells. In contrast, the *pmt1* mutant formed very few microcolonies on the polystyrene surface, which nevertheless consisted of both yeast and hyphal cells (Fig. 2).

Certain rhodanine compounds inhibit the enzymatic activity of Pmt1p, leading to *pmt1* mutant phenotypes, including aminoglycoside supersensitivity, defective hypha formation under some conditions, and increased aggregation, while not affecting growth rates (13; data not shown). Addition of compound OGT2599 inhibited biofilm formation in polystyrene wells at low concentrations in a dose-dependent manner (Fig. 3). To clarify if this inhibition was caused by interference with adherence of cells or by inhibition of later stages of biofilm formation, we either added the inhibitor simultaneously with cells or

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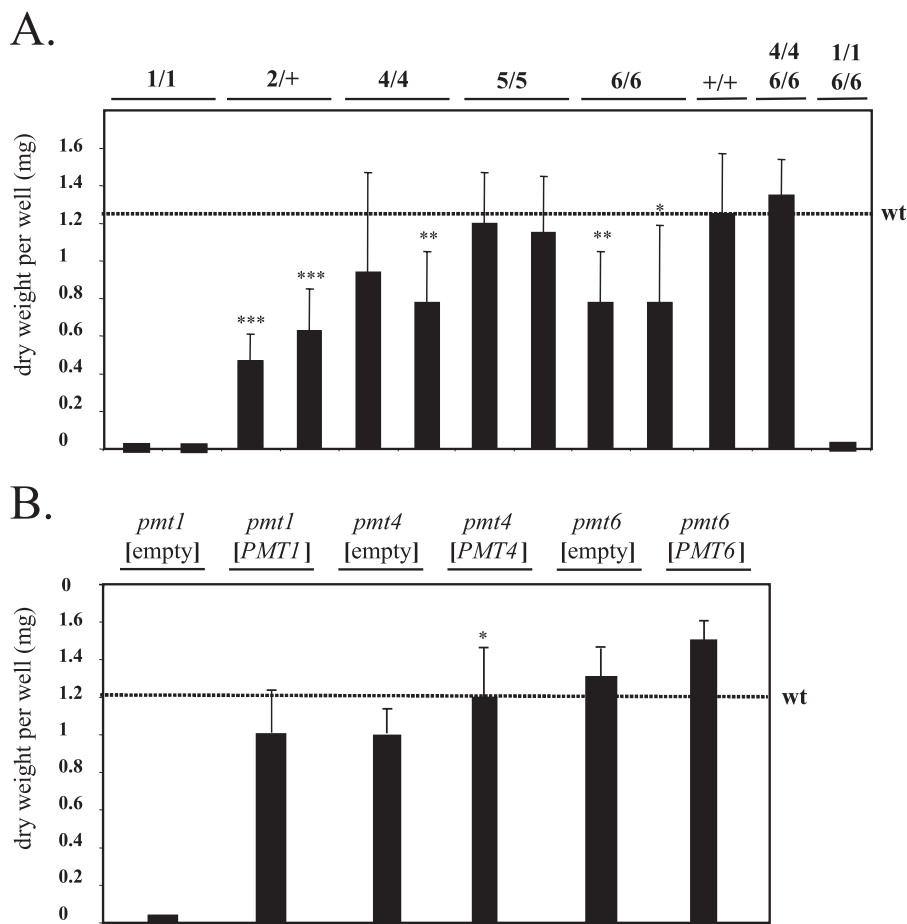


FIG. 1. Biofilm formation in polystyrene cell culture wells. (A) Wells were inoculated with  $5 \times 10^5$  *C. albicans* cells, and biofilm formation was allowed to proceed in RPMI medium for 48 h at 37°C. Strains included the control strain CAF2-1 (+/+) and two independently constructed homozygous mutant strains: *pmt1* mutants SPCa2 and SPCa3 (1/1), *pmt2* heterozygotes SPCa4 and SPCa5 (2/+), *pmt4* mutants SPCa6 and SPCa7 (4/4), *pmt5* mutants SPCa10 and SPCa11 (5/5), *pmt6* mutant SPCa8 and SPCa9 (6/6), *pmt4 pmt6* mutant PP46-428 (4/4 6/6), and *pmt1 pmt6* mutant CPP117 (1/1 6/6) (15). Biofilms were quantitated by dry weights of wells. Mean values and standard deviations were determined from eight independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.0001$  (*pmt* mutant versus control strain). The mass of the control strain is indicated as a dashed line (wt). (B) Biofilm formation of *PMT*-reconstituted strains. *pmt* mutants containing an empty plasmid (pRC18) or a *PMT*-carrying plasmid, integrated in the *LEU2* locus, were allowed to form biofilms in polystyrene cell culture wells. Strains included CAP1-3121(pRC18) (*pmt1* [empty]), CAP1-3121(pCT30) (*pmt1* [PMT1]), CAP4-2164(pRC18) (*pmt4* [empty]), CAP4-2164(pSP1) (*pmt4* [PMT4]), CAP2-2391(pRC18) (*pmt6* [empty]), and CAP2-2391(pCT34) (*pmt6* [PMT6]) (18). Dry weights of biofilms were determined, and mean values and standard deviations were calculated from at least five independent experiments. \*,  $P < 0.05$  (mutant with empty vector versus mutant carrying *PMT* gene).

after 24 h, when biofilm formation was incomplete. The results indicate that simultaneous addition of the inhibitor completely blocked biofilm formation, while after 24 h the inhibitor was ineffective (Fig. 3). Because mannoproteins synthesized during pregrowth appeared insufficient for biofilm formation, we conclude that the inhibitor blocks mannosylation of newly synthesized, biofilm-relevant proteins.

The *pmt* mutant strains were evaluated for their ability to form a biofilm in a continuous-flow microfermenter model. Biofilms were produced as described previously (10) except that SD medium contained 0.4% glucose, arginine (0.1 g/liter), histidine (0.1 g/liter), methionine (0.2 g/liter), and uridine (0.01 g/liter). Stationary-phase yeast cells were placed for 30 min on a slide of Thermanox plastic (a polyolefin polyester) and gently washed by dipping into SD medium (five times), and adherent cells were allowed to form a biofilm under a continuous flow of minimal medium and air. We first evaluated adherence abili-

ties of *pmt* mutants in this model. The *pmt1* mutant was severely impaired for adherence to Thermanox, while the *pmt4*, *pmt5*, and *pmt6* mutants adhered as did the control strain (Fig. 4 A). Assessment of the heterozygous *PMT2/pmt2* mutant was prevented by its occasional flocculation during planktonic growth (data not shown). The results indicate that the *pmt5* and *pmt6* homozygous mutants formed biofilms as efficiently as the control strain, while a twofold reduction in biofilm biomass was observed for the *pmt4* mutant (Fig. 4B). Thus, in the continuous system, Pmt1p is required for early phases of biofilm formation, while Pmt4p is necessary for subsequent phases.

Defective biofilm formation by *pmt* mutants and after Pmt inhibition may be the consequence of altered cell wall composition and overall hydrophobicity in cells defective in O mannosylation (15). Novel strategies to combat *C. albicans* as a commensal or infectious agent could include Pmt inhibition to prevent anchoring and biofilm formation on biological surfaces

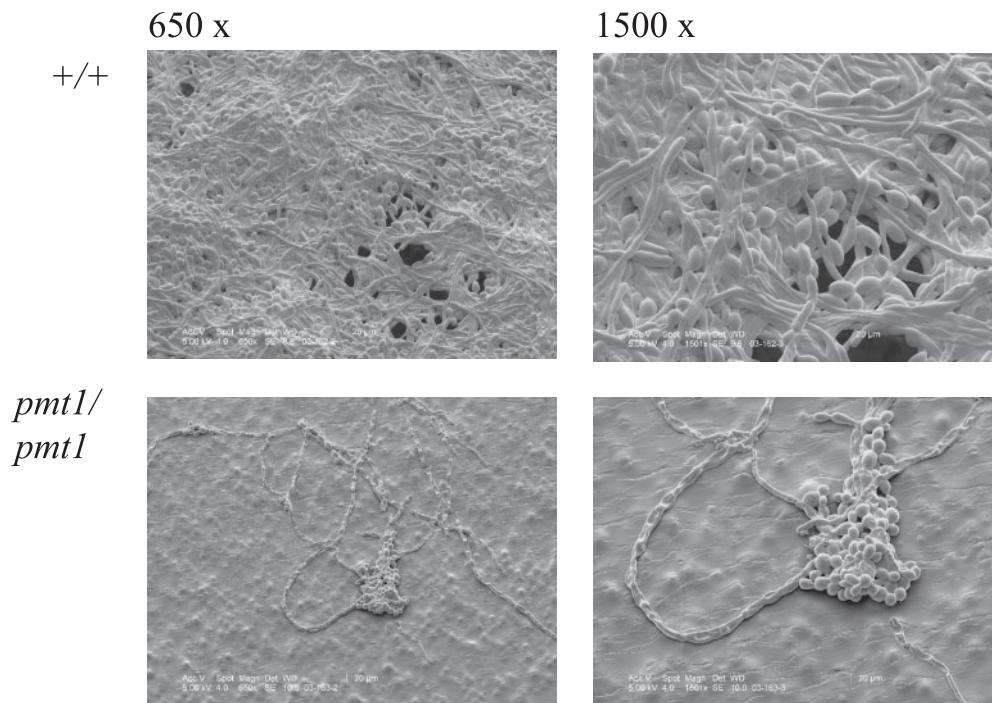


FIG. 2. Scanning electron microscopy of *C. albicans* biofilms on polystyrene. Dense biofilms of the control strain CAF2-1 (+/+) are compared to rare attached cells and microcolonies of the *pmt1* mutant SPCa2.

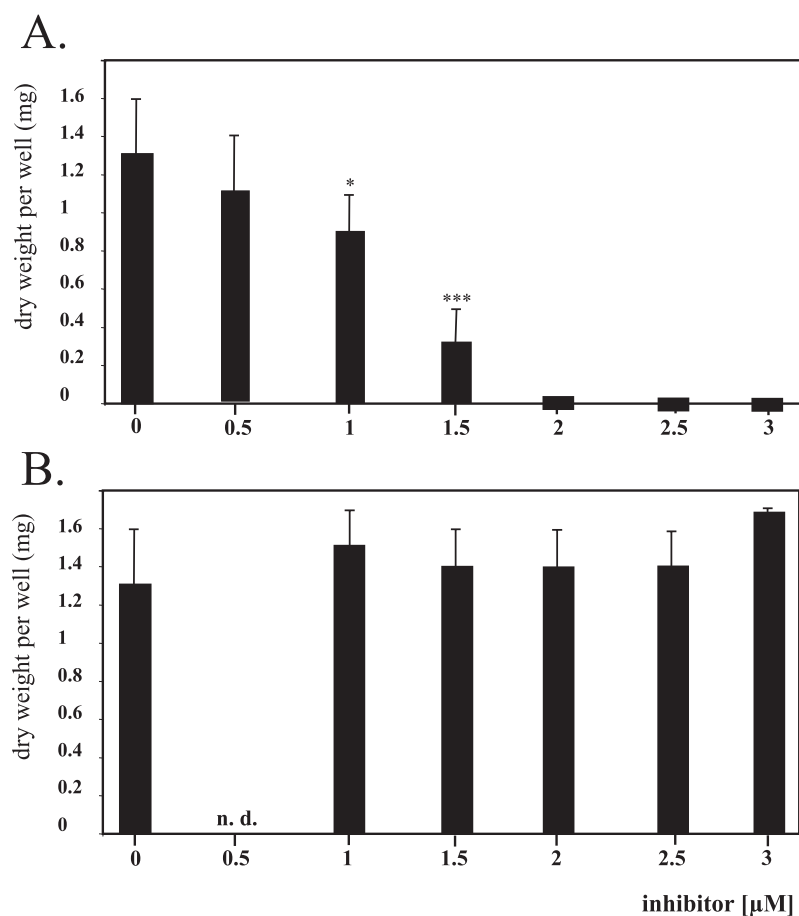


FIG. 3. Inhibition of biofilm formation by O glycosylation inhibitor OGT2599. (A) Increasing concentrations of OGT2599 were added during addition of *C. albicans* CAF2-1 to polystyrene cell culture wells. Biofilm formation was allowed to proceed and was quantitated as for Fig. 1. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.0001$  (culture without inhibitor versus culture with added inhibitor). (B) The inhibitor OGT2599 was added at the indicated concentrations 24 h after addition of *C. albicans* cells. Values represent the mean  $\pm$  standard deviation for at least five independent measurements. n.d., not done.

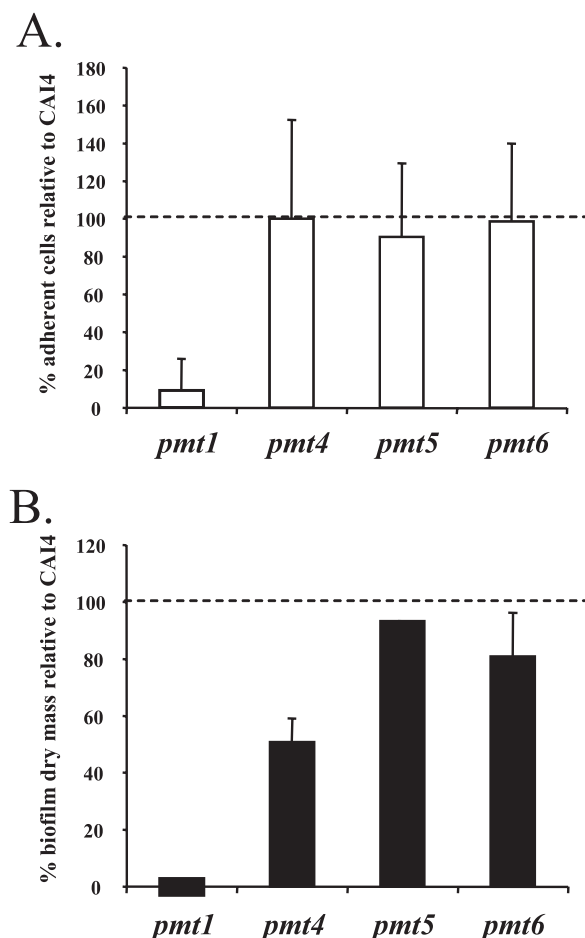


FIG. 4. Defective adherence and biofilm formation of *pmt* mutants in the continuous-flow microfermenter model. A. Stationary-phase cells of the wild-type (CAI4) and mutant (*pmt1* $\Delta$ , SPCa2; *pmt4* $\Delta$ , SPCa6; *pmt5* $\Delta$ , SPCa10; *pmt6* $\Delta$ , SPCa8) strains were put into contact with a Thermanox slide for 30 min. Following removal of nonadherent cells, at least 10 microscope fields were counted for adherent cells. Data obtained from two independent experiments are shown as the ratio between adherent mutant and wild-type cells along with the standard deviation. B. Biofilm formation was monitored in duplicate following adhesion of yeast cells to Thermanox and growth for 41 h in the microfermenter model. Dry biomasses of the biofilms were quantified and expressed as a ratio of mutant versus wild type. This experiment is representative of the data obtained in three separate experiments.

(extracellular materials and cell surfaces) and on nonbiological surfaces including medical implants. Conceivably, surface-bound or -incorporated inhibitors could protect medical devices from colonization by *C. albicans*. Blockage of biofilm formation by Pmt inhibition would also abolish an important mechanism of antifungal resistance (4, 5, 9, 12, 13).

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