



RESEARCH ARTICLE

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Effects of efflux pumps on antifungal activity of chitosan against *Candida albicans*

Sureeporn Muangsawat^{a,b}, Patcharaporn Chaiyosang^c, Patrawee Sinkanarak^c, Juthamas Sukted^{d,e}, Panida Thanyasrisung ^a and Oranart Matangkasombut ^{a,e}

^aDepartment of Microbiology and Center of Excellence on Oral Microbiology and Immunology, Faculty of Dentistry, Chulalongkorn University, Pathumwan, Bangkok, Thailand; ^bInterdisciplinary Program on Medical Microbiology, Graduate School, Chulalongkorn University, Bangkok, Thailand; ^cDDS Program, Faculty of Dentistry, Chulalongkorn University, Pathumwan, Bangkok, Thailand; ^dGraduate Program in Applied Biological Sciences, Chulabhorn Graduate Institute, Bangkok, Thailand; ^eResearch Laboratory of Biotechnology, Chulabhorn Research Institute, Bangkok, Thailand

ABSTRACT

Background: Antifungal resistance is a major problem, commonly caused by drug-efflux pump overexpression. To evaluate if chitosan could be effective in drug-resistant *Candida* infections, we investigated the effects of efflux pumps on antifungal activity of chitosan.

Materials and Methods: The minimal fungicidal concentration (MFC) of oligomer (7–9 kD) and polymer (900–1,000 kD) chitosan against *Saccharomyces cerevisiae* and *Candida albicans* were evaluated by broth and agar dilution methods. The MFCs of *S. cerevisiae* with single deletion of efflux pump genes, with deletion of seven efflux pumps (*ADΔ*), and *ADΔ* overexpressing *C. albicans* efflux pump genes (*CDR1*, *CDR2* and *MDR1*) were determined. *C. albicans* with homozygous deletions of *CDR1* and of *CDR2* were generated using CRISPR-Cas9 system and tested for chitosan susceptibility.

Results: While deleting any individual efflux pump genes had no effect on chitosan susceptibility, simultaneous deletion of multiple pumps (in *ADΔ*) increased sensitivity to both types of chitosan. Interestingly, the overexpression of *CDR1*, *CDR2* or *MDR1* in *ADΔ* barely affected its sensitivity. Moreover, *C. albicans* with homozygous deletions of *CDR1* and/or *CDR2* showed similar sensitivity to wildtype.

Conclusion: Thus, *C. albicans* susceptibility to chitosan was not affected by drug-efflux pumps. Chitosan may be a promising antifungal agent against pump-overexpressing azole-resistant *C. albicans*.

KEY MESSAGES

1. Neither deletion of efflux pump genes, nor overexpression of major *C. albicans* efflux pumps in pump-deficient *S. cerevisiae*, nor deletion of major efflux pumps in *C. albicans* affects yeast susceptibility to chitosan.
2. Chitosan may be an effective antifungal agent against drug-resistant *C. albicans*.

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efflux pump; Antifungal agent; Chitosan; *Saccharomyces cerevisiae*; *Candida albicans*; drug resistance



Introduction

Candida can cause superficial and invasive infections that affect millions of people worldwide, especially in immunocompromised persons [1]. *Candida albicans* is the most prevalent species, and invasive infections have up to 40% mortality rate [2]. It is listed as one of the four highest priority fungal pathogens by the World Health Organization (WHO) [3]. The limited repertoire of antifungal drugs coupled with increasing antifungal drug resistance has made treatments even more difficult [4].

Azoles, especially fluconazole, are the most commonly used antifungal drugs for *Candida* infections, but drug resistance is increasingly prevalent, mainly through overexpression of drug efflux pumps [5]. ATP binding cassette (ABC) and Major facilitator superfamily (MFS) transporter proteins are two

main classes of efflux pumps in yeasts [6]. Upregulation of *CDR1* and *CDR2* of the pleiotropic drug resistance (PDR) subfamily of ABC transporters, and *MDR1*, an MFS transporter, results in reduced intracellular drug accumulation and antifungal drug resistance [5].

The rise in antifungal drug resistance necessitates the development of novel antifungal agents with low toxicity. Chitosan, a natural polymer from the deacetylation of chitin in crustacean shells, has high biocompatibility and antibacterial and antifungal activities [7]. Many chitosan derivatives exist with different molecular weights (MW), degrees of deacetylation and modifications, which could affect their antimicrobial activity [8]. Several derivatives with various MW were shown to be effective against planktonic cells and biofilm of *Candida* species [9–11]. Mechanistically,

CONTACT Oranart Matangkasombut  oranart.m@chula.ac.th  Department of Microbiology and Center of Excellence on Oral Microbiology and Immunology, Faculty of Dentistry, Chulalongkorn University, Pathumwan, 34 Henri Dunant Rd, Bangkok 10330, Thailand

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high MW chitosan could disrupt cell membrane functions, while low MW chitosan could enter the cells and interfere with DNA and RNA synthesis [8].

Importantly, it is unknown if chitosan activity is affected by the common antifungal resistance mechanisms, especially drug efflux pumps. Taking advantage of the gene deletion library and multiple efflux pump deletion strains in *Saccharomyces cerevisiae*, we first employed this model to test the effects of individual vs. multiple efflux pump gene deletion on susceptibility to chitosan. Then, we examined the effects of *Candida* efflux pump gene overexpression in *S. cerevisiae* and gene deletions in *C. albicans*. We hypothesized that high MW chitosan that likely acts on the outer cell membrane would not be affected by efflux pumps, while low MW chitosan may be a pump substrate. If chitosan is not affected by efflux pumps, it may be useful against efflux pump-overexpressing azole-resistant *C. albicans*.

Materials and methods

Yeast strains and growth condition

Yeast were cultured in Yeast-extract-Peptone-Dextrose (YPD) broth and incubated overnight at 30°C with shaking (180 rpm). The culture was adjusted to an optical density at 600 nm (OD₆₀₀) of 0.1 and incubated until log phase (OD₆₀₀ = 0.4–0.6).

S. cerevisiae BY4741 and *C. albicans* SC5314 were wild-type controls. *S. cerevisiae* with single deletion of efflux pump strains were from Mat-a haploid yeast deletion library (Invitrogen, USA). Strains used in this study included *S. cerevisiae* with double deletion of *PDR1* and *PDR3*, major transcription factors of several pump genes (gift from Prof. L. Jensen) [12], *ADA* strain, with simultaneous deletions of seven pump genes [13] and *ADA* overexpressing *C. albicans* efflux pump genes (*ADA/CaCDR1A-HIS*, *ADA/CaCDR1B-HIS*, *AD/CaCDR2A-HIS* and *AD/CaMDR1A*) [13,14] (gifts from Prof. R. Cannon).

C. albicans with homozygous deletions of *CDR1* and *CDR2* genes were created using the transient CRISPR-Cas9 system [15,16]. The sequence of sgRNA (GTTTTGGGGAGACCCGGTGC) was chosen from a previously proposed guide RNA sequence, amplified and cloned into pV1093 (gift from Prof. G. Fink) [15]. *C. albicans* were electroporated with *CaCas9*, sgRNA and *CaSAT1* flipper cassette carrying nourseothricin-resistant marker (amplified from pSFS2AS, gift from Prof. J. Shieh, using primers: CDR1S1_F:CTCTTCTTCAGAAA ACCATTCCATTAATGAATACCACGGGTTTGATGCCCATACAAGTGAAACATTGAGAATTTAGCCAGGAAGCTTCGTACGCTGCAGGTC, CDR1S2_R: CAACAACAATAGTCTAAAAACGTCTATTATATT-

TTAGACGTTTGGAGATACCACCATGTCAAAAAA-CAAACGTGTTTAATTCTGATATCATCGATGAATTCGAG, CDR2S1_F:CTTGAAAAAATTATTTGAATCTGATTCTGATTATTATAAGCCATCGAAATTAGGGTTGCCTATAGAAATTTAAGAGCTTACGGGAGCTTCGTACGCTGCAGGTC, CDR2S2_R:CAAAACAAACAAATAACGTATAATAATAATAAGAAAAAATATGAATACTAATTGTAATAAGACCCCATCCTCTGATATCATCGATGAATTCGAG) [17]. Transformants were selected on YPD containing 400 µg/ml nourseothricin (ClonNAT, Werner bioagents GmbH, Germany) and verified by PCR. Cells were grown on YEP + 2% maltose to induce flippase for *CaSAT1*-marker removal, and re-transformed with CRISPR-Cas9 fragments to generate homozygous deletion.

Chitosan and antifungal activity measurement

Shrimp oligomer (7–9 kDa) and polymer (900–1000 kDa) chitosan (Taming Enterprises, Samut-Sakhon, Thailand) with >85% degree of deacetylation were dissolved in 1% acetic acid at 30 mg/ml (w/v) and autoclaved.

Minimum Fungicidal Concentration (MFC) of chitosan was determined by broth and agar dilution methods. For broth dilution, log-phase cultures ($\approx 10^6$ cells/ml) was incubated with two-fold serial dilutions of chitosan in YPD at 30°C with shaking for 18 h, and 100 µl aliquot was plated on YPD agar and incubated for 48 h. For agar dilution, 5 µl of log-phase cultures ($\approx 10^6$ cells/ml) were spotted on YPD agar containing various concentrations of chitosan and incubated for 48 h. The lowest concentration of chitosan that showed no growth was recorded as MFC.

Results

MFC of chitosan in wild-type *S. cerevisiae* and *C. albicans*

The MFC of two types of chitosan against wild type *S. cerevisiae* and *C. albicans* were examined. Since chitosan stock (30 mg/ml) was dissolved in 1% acetic acid, we first determined the highest concentration of acetic acid that does not affect fungal viability to exclude antifungal effects of the acid. Figure 1(a) shows that 0.2% acetic acid was the highest concentration tolerated by both *S. cerevisiae* and *C. albicans*. Thus, the maximum concentration of chitosan tested in this study was 6 mg/ml, which has 0.2% acetic acid. Yeast growth was partially inhibited by oligomer and polymer chitosan at 6 mg/ml (Figures 1(b,c)). Using agar dilution, *S. cerevisiae* was inhibited at 3 mg/ml oligomer and 6 mg/ml polymer, while *C. albicans* was inhibited at 3 mg/ml of both types of chitosan (Figures 1d,e).

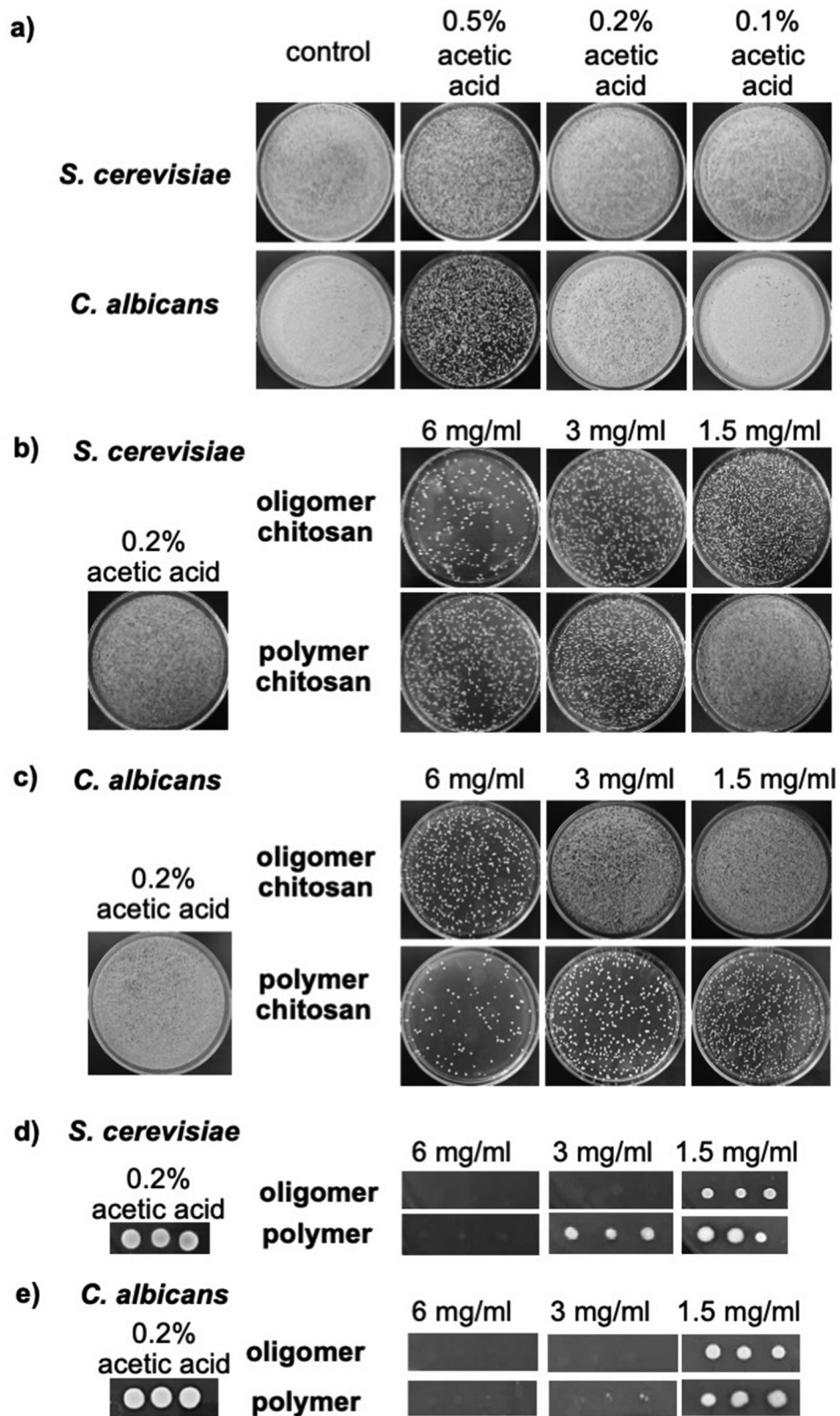


Figure 1. Minimum Fungicidal Concentration (MFC) of chitosan derivatives. (a) Effect of acetic acid on *S. cerevisiae* and *C. albicans* was determined by broth dilution method. MFC of oligomer and polymer chitosan against *S. cerevisiae* (b, d) and *C. albicans* (c, e) was determined by broth and agar dilution assays, respectively.

Effects of efflux pump gene deletions and *C. albicans* efflux pump overexpression

To examine the effects of efflux pumps, we tested chitosan susceptibility of *S. cerevisiae* with pump gene deletions using agar dilution. At 1.5 mg/ml

oligomer, individual efflux pump deletion strains and the double deletion of major transcription factors that regulate the expression of several efflux pumps, *pdr1Δpdr3Δ* strain, showed similar growth as wild type. In contrast, *ADAΔ* strain, which lacks seven efflux

pump genes, showed hypersensitivity to chitosan (Figure 2a). *ADA* strain was also extremely sensitive to 6 mg/ml polymer, while single pump deletions and *pdr1Δpdr3Δ* strains were inhibited similarly to the wild type (Figure 2b).

The effect of *C. albicans* efflux pump overexpression on chitosan susceptibility was examined in *ADA* that overexpressed *CDR1*, *CDR2* or *MDR1*. Comparing to *ADA* strain (MFC = 0.2 mg/ml oligomer), overexpression of *MDR1*, and to a lesser extent *CDR1*, in *ADA* strain showed a small number of colonies at 0.2 mg/ml, but no growth at 0.3 mg/ml oligomer (Figure 3a). Likewise, at the MFC of *ADA* strain (0.5 mg/ml polymer), overexpression of *CDR1* or *MDR1* allowed only minimal growth (Figure 3b).

Effect of efflux pump deletions in *Candida albicans*

We next analysed the roles of efflux pumps directly in *C. albicans* by generating homozygous deletions of single or double *CDR1* and *CDR2* genes. Using broth dilution, the MFC of *C. albicans* with single (*cdr1^{-/-}* or *cdr2^{-/-}*) or double (*cdr1^{-/-}cdr2^{-/-}*) homozygous deletion strains was similar to that of wild-type for both types of chitosan (Figure 4a,b). Similar results were observed using agar dilution (Figure 4c).

Discussion

Here, we showed that while simultaneous deletion of multiple efflux pumps in *S. cerevisiae* led to increased sensitivity to both oligomer and polymer chitosan, overexpression of *Candida* efflux pumps, Cdr1, Cdr2 or Mdr1 had minimal effects on increasing chitosan resistance. Furthermore, the deletion of the *CDR1* and/or *CDR2* in *C. albicans* did not affect the susceptibility to both types of chitosan. Thus, our results

suggest that chitosan could serve as an alternative or adjunctive antifungal agent against azole-resistant *C. albicans* that overexpress efflux pumps.

Numerous chitosan derivatives exist with various MW, which is an important determinant of their antimicrobial mechanism. It has been proposed that high MW chitosan binds to the cell membrane, thereby blocking nutrient transports and causing leakage, whereas low MW chitosan could enter the cells and disrupt DNA, RNA and protein metabolism [8]. MW could also determine if chitosan would be substrates of efflux pumps. Thus, we examined the roles of efflux pumps on two types of chitosan with different MW, oligomer and polymer. We first employed *S. cerevisiae* since its efflux pump genes were similar to *C. albicans* and pump-deletion strains were available [13]. We hypothesized that if any efflux pump contributed to chitosan resistance, the pump-deletion strain would be hypersensitive to chitosan. Our result showed that all single pump deletions showed similar sensitivity to wild-type, suggesting that no single pump plays a predominant role in transporting chitosan. Chitosan susceptibility was unaltered even in the *pdr1Δpdr3Δ* strain, which has downregulation of multiple efflux pumps. Meanwhile, *ADA* strain with multiple pump deletion was hypersensitive to both types of chitosan. This implies that multiple pumps may play redundant roles in chitosan susceptibility, but it is also possible that simultaneous deletions of multiple pumps may disrupt membrane functions and cause cellular stress that enhances the effect of chitosan. Because ABC pumps play important roles in sterol transport and regulation of asymmetric lipid distribution across the plasma membrane lipid bilayer, multiple pump deletion may interfere with regulation of membrane traffic and cell division [18]. In addition, *ADA* strain was shown to be hypersensitive to a wide range of

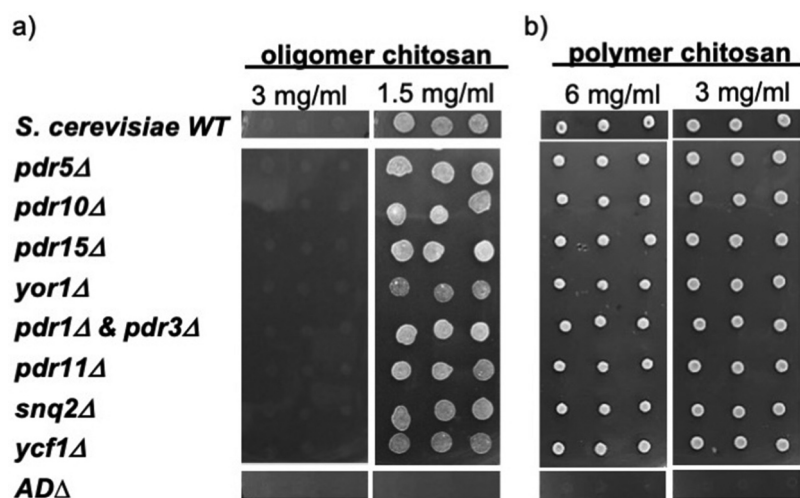


Figure 2. Susceptibility of *S. cerevisiae* strains with indicated efflux pump gene deletion to chitosan, (a) oligomer and (b) polymer, by agar dilution assay.

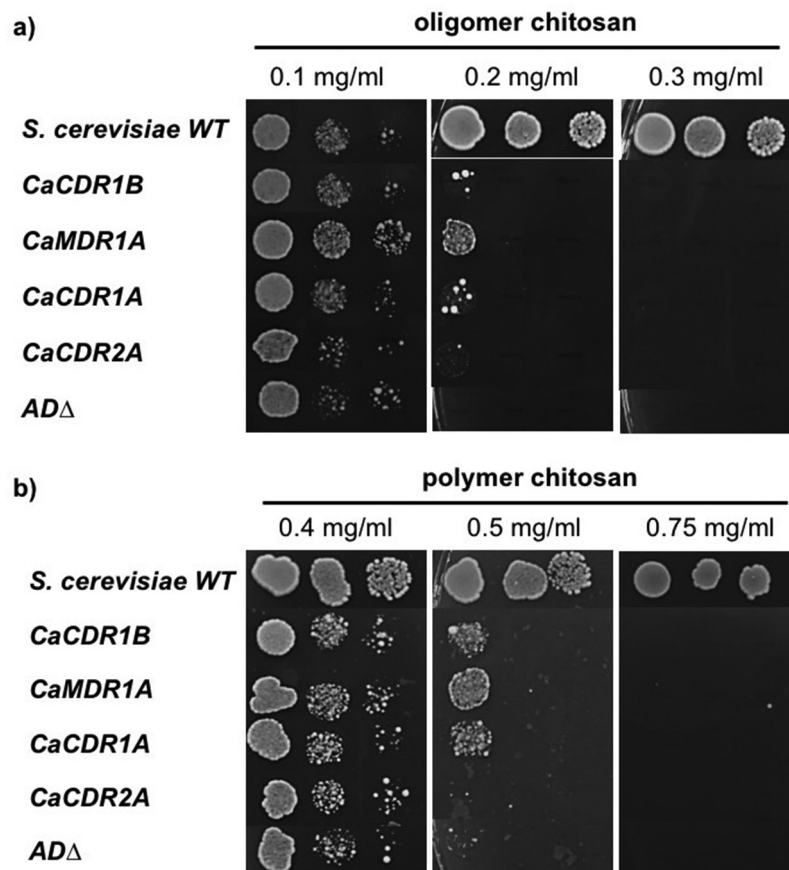


Figure 3. Effects of overexpression of *C. albicans* efflux pump genes in *AD*Δ strains on susceptibility to chitosan, (a) oligomer and (b) polymer, by agar dilution assay.

xenobiotics, which may be direct effects of the pumps or secondary consequences of membrane disturbance [19].

Interestingly, overexpression of *C. albicans* efflux pump genes important in clinical drug resistance (*CDR1*, *CDR2* or *MDR1*) only minimally increased the resistance to chitosan in *AD*Δ strains. This is in contrast to the case of fluconazole susceptibility, where the MIC was increased by 400–600 folds, from 0.5 μg/ml in *AD*Δ to 200–300 μg/ml or to 75–150 μg/ml, when *CDR1* or *CDR2* was overexpressed, respectively [13]. Although CDRs show substrate promiscuity and their overexpression in pump-deficient yeasts increased resistance to various structurally unrelated compounds [6], our results showed that their overexpression affects neither oligomer nor polymer chitosan antifungal activity.

Among multiple *C. albicans* CDR genes, *CDR1* plays predominant roles in drug resistance, while *CDR2* have less effects [6]. The deletion of *CDR1* and *CDR2* in *C. albicans* drastically increased azole sensitivity in both laboratory strains and clinically drug-resistant isolates [15]. However, single or double *CDR1* and *CDR2* deletions had no effect on *C. albicans* sensitivity to chitosan. This supports the hypothesis that these major efflux pumps do not affect chitosan's activity and that neither oligomer nor polymer is substrates of these pumps.

Chitosan has broad applications in medical and cosmetics industries because of its high biocompatibility, biodegradability and antibacterial and antifungal activities [7]. We previously reported chitosan derivatives that are effective against common oral *Candida* species, including clinical isolates and biofilms [9,10,20]. Importantly, oligomer and polymer chitosan were effective against most fluconazole-resistant isolates from HIV-infected individuals, emphasizing their potential as a promising antifungal agent against azole-resistant *Candida* [10]. Furthermore, chitosan can serve as an active carrier to deliver and enhance the activity of antimicrobial drugs, and its mucoadhesive property can provide retention on the mucosa or under denture to prevent or treat denture stomatitis [7,9]. Therefore, future research into chitosan applications as alternative and/or adjunctive antifungal agent is warranted.

Conclusion

In conclusion, our results showed that *S. cerevisiae* and *C. albicans* were susceptible to both oligomer and polymer chitosan with minimal effects from drug efflux pumps. Thus, chitosan may be an effective alternative or adjunctive antifungal agent against drug-resistant *C. albicans* that overexpress drug efflux pumps.

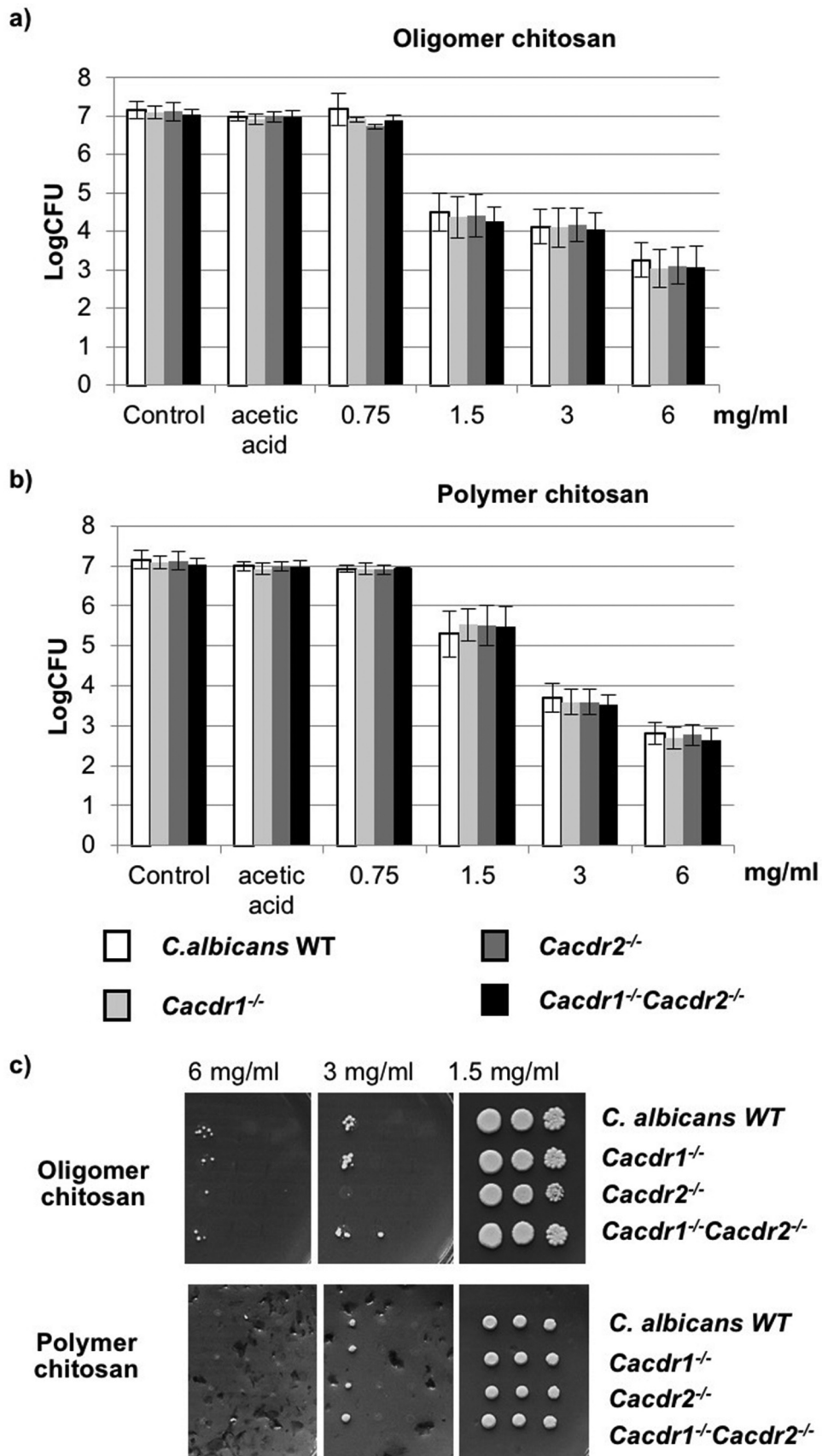


Figure 4. Effects of efflux pump gene deletion on *C. albicans* susceptibility to chitosan, (a) oligomer and (b) polymer by broth dilution, and by agar dilution assay (c).

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions statement

S.M. participated in study design, conducted experiments, data analysis and interpretation and drafted the initial manuscript; P.C., P.S. and J.S. participated in study design, conducted experiments, data analysis and critically revised the manuscript; P.T. participated in study design, data interpretation and critically revised the manuscript; O. M. conceived the idea, designed the study, supervised data collection and analysis, interpreted the data, drafted and critically revised the manuscript. All the authors have read and approved the final manuscript.

Data availability statement

The data generated in the current study are available from the corresponding author on reasonable request.

ORCID

Panida Thanyasrisung  <http://orcid.org/0000-0002-1378-695X>

Oranart Matangkasombut  <http://orcid.org/0000-0003-4029-6830>

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