

NOTES

FKS1 Mutations Responsible for Selective Resistance of *Saccharomyces cerevisiae* to the Novel 1,3- β -Glucan Synthase Inhibitor Arborcandin C

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Arborcandin C is a novel antibiotic with potent antifungal activity that inhibits 1,3- β -glucan synthase in fungi. We examined spontaneous *Saccharomyces cerevisiae* mutants which are selectively resistant to arborcandin C and revealed that a single amino acid replacement in Fks1p of Asn⁴⁷⁰ with Lys or of Leu⁶⁴² with Ser confers selective resistance on Fks1p mutants.

The 1,3- β -glucan polymer is a major component of the cell wall in yeast and filamentous fungi (11) and is synthesized by 1,3- β -glucan synthase (GS). In *Saccharomyces cerevisiae*, GS consists of at least two subunits: a putative catalytic subunit encoded by related genes *FKS1* and *FKS2* (7, 10) and a regulatory subunit, a GTP-bound protein encoded by *RHO1* (16). Several types of GS inhibitors, such as echinocandins (5, 13), papulacandins (20), and FR901469 (8), have been identified. GS inhibitors are a new class of antifungal antibiotics with clinical significance because of their strong fungicidal activity and low toxicity (1, 3).

Arborcandins are recently isolated cyclic lipopeptides which inhibit GS from *Candida albicans* and *Aspergillus fumigatus* (14) and which also have growth-inhibitory activity (14). Although we reported that arborcandin C noncompetitively inhibited GS (14), the molecular target of the inhibition still remains to be elucidated. In this study, we isolated selective arborcandin C-resistant *S. cerevisiae* mutants with antibiotic-resistant-GS activity and revealed that two single-amino-acid mutations in Fks1p conferred selective resistance to arborcandin C.

Standard procedures were used for DNA manipulations (17). Genetic manipulations for yeast were carried out as described previously (9). Yeast strains and plasmids used in this study are listed in Table 1. All yeast strains were derivatives of *S. cerevisiae* YPH250 (18). Yeast cells were grown either in YPDAU medium (2% peptone, 1% yeast extract, 20 μ g of adenine sulfate/ml, 20 μ g of uracil/ml, 2% glucose) or SD medium (9) supplemented appropriately. *FKS1* was cloned by screening the genomic DNA library of the wild-type strain (YPH250) for complementing *fks1 Δ ::LEU2* on SD medium containing 1 μ g of FK506/ml. An arborcandin C resistance

gene was cloned by screening the genomic DNA libraries of the arborcandin C-resistant mutants ACR79-5 and ACR1A3 for genes that conferred growth in SD medium containing 5 μ g of arborcandin C/ml on YPH250. The DNA sequence was determined by automated DNA sequencer model ABI3700 with a dye terminator cycle sequencing kit (Applied Biosystems).

Growth inhibition was determined by the broth microdilution method in a 96-well microplate using YPDAU medium. Briefly, 10⁴ yeast cells were inoculated into 150 μ l of medium in each well and incubated at 30°C for 16 to 24 h in the presence or absence of GS inhibitors. The optical density at 595 nm of the exponentially growing culture was measured by microplate reader (ARVOsx; Perkin-Elmer).

Preparation and measurement of activity of GS from wild-type and mutant *S. cerevisiae* strains were performed according to the procedure described by Inoue et al. (10) and Cabib and Kang (4), respectively. The solubilized membrane fraction (10) was used as GS.

Isolation of selective arborcandin C-resistant mutants. Arborcandin C is structurally different from echinocandins and papulacandins (14). To examine whether arborcandin C has a molecular target different from those of known GS inhibitors, we took a genetic approach. Spontaneously resistant *S. cerevisiae* mutants were isolated at a concentration of 1 μ g of arborcandin C/ml on a YPDAU medium plate. We selected two mutants, ACR79-5 and ACR1A3, which are resistant only to arborcandin C, not to any other type of GS inhibitors, for further study. As the echinocandin-type and papulacandin-type inhibitors, we used pneumocandin A₀ (2) and F-10748 C₁ (15), respectively. As shown in Table 2, both mutants were 100-fold more resistant to arborcandin C than the parental wild-type strain and there was a modest change in the susceptibility to pneumocandin A₀ and F-10748 C₁. We did not observe significant differences between the wild-type strain and the mutants in their phenotypes, such as their cell morphology and cal-

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TABLE 1. Yeast strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Plasmids		
pAUR112	Centromere-type shuttle vector	Takara Bio, Inc.
pCE112	Centromere-type shuttle vector derived from pAUR112	This study
pCE- <i>FKS1</i>	pCE112 containing cloned <i>FKS1</i> from YHP250	This study
pCE- <i>FKS1</i> ^{N470K}	pCE- <i>FKS1</i> replaced with ACR75-5 mutated region	This study
pCE- <i>FKS1</i> ^{L642S}	pCE- <i>FKS1</i> replaced with ACR1A3 mutated region	This study
Strains		
YHP250	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-c200 leu2-Δ1</i>	18
ACR75-5	Arborcandin C-resistant mutant derived from YHP250	This study
ACR1A3	Arborcandin C-resistant mutant derived from YHP250	This study
<i>fks1Δ</i>	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-c200 leu2-Δ1 fks1Δ::LEU2</i>	This study
<i>fks1ΔP_{GAL10}-FKS2</i>	<i>MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-c200 leu2-Δ1 fks1Δ::LEU2 P_{GAL10}-FKS2::TRP1</i>	This study
CE- <i>FKS1</i>	<i>fks1ΔP_{GAL10}-FKS2</i> harboring plasmid pCE- <i>FKS1</i>	This study
CE- <i>FKS1</i> ^{N470K}	<i>fks1ΔP_{GAL10}-FKS2</i> harboring plasmid pCE- <i>FKS1</i> ^{N470K}	This study
CE- <i>FKS1</i> ^{L642S}	<i>fks1ΔP_{GAL10}-FKS2</i> harboring plasmid pCE- <i>FKS1</i> ^{L642S}	This study

cofluor white sensitivity phenotypes, which reflect structural changes in the cell wall (data not shown).

To determine whether the selective resistance of the mutants to arborcandin C was due to a change of sensitivity of GS, the sensitivity of GS from the mutants was tested. As shown in Table 3, GS from either of the resistant mutants was highly resistant to arborcandin C. The 50% inhibitory concentration of arborcandin C against the mutant's GS was more than 100-fold higher than that of arborcandin C against the wild-type enzyme. On the other hand, the sensitivity of both mutant enzymes to the other types of GS inhibitors tested was not significantly changed. These results suggested that the selective resistance of the mutants was due to the selective resistance of GS.

Cloning of mutated genes responsible for selective arborcandin C resistance. To identify the mutated genes in ACR79-5 and ACR1A3, we screened the genomic DNA library of each mutant using the centromere-type vector (pCE112) for plasmids conferring arborcandin C resistance on the wild-type strain. Four and three arborcandin C-resistant colonies were isolated by the transformation with the libraries of ACR79-5 and ACR1A3, respectively. All plasmid DNAs recovered from the resistant transformants conferred arbor-

candin C resistance (data not shown). Partial sequence analysis of the plasmids revealed that the minimum region conferring arborcandin C resistance from both the mutants contained an *FKS1* locus as a complete open reading frame (data not shown). Therefore, it was strongly suggested that the gene mutated in both the resistant mutants was *FKS1*.

Mutation of Asn⁴⁷⁰ to Lys or Leu⁶⁴² to Ser in Fks1p confers selective resistance to arborcandin C. By DNA sequence analysis of the *FKS1* open reading frame, the mutations in ACR79-5 and ACR1A3 were determined to be the replacement of Asn⁴⁷⁰ with Lys and of Leu⁶⁴² with Ser in Fks1p, respectively (Fig. 1). We subcloned each of the mutated regions to wild-type *FKS1* and examined the effect of each mutation in the *fks1Δ P_{GAL10}-FKS2* cells. In the *fks1Δ P_{GAL10}-FKS2* strain, *FKS2* gene expression was controlled by the *GAL10* promoter and suppressed in the presence of glucose. Therefore, the effect of the substitution in Fks1p can be analyzed under the depletion of endogenous Fks1p and Fks2p in the *fks1Δ P_{GAL10}-FKS2* cells in glucose medium. The introduction of mutated *FKS1* (*FKS1*^{N470K} or *FKS1*^{L642S}) to the *fks1Δ P_{GAL10}-FKS2* strain conferred selective resistance to arborcandin C, as shown by arborcandin C-resistant mutants ACR79-5 and ACR1A3 (Table 2). Moreover, the GSs from cells with *FKS1*^{N470K} or *FKS1*^{L642S} conferred selective resistance to arborcandin C (Table 3). These results show that Asn⁴⁷⁰ and

TABLE 2. Susceptibilities of selective arborcandin C-resistant mutants to GS inhibitors

Strain	IC ₅₀ ^a (μg/ml)			MIC ^b (μg/ml)		
	Arborcandin C	Pneumocandin A ₀	F-10748 C ₁	Arborcandin C	Pneumocandin A ₀	F-10748 C ₁
YHP250 (wild type)	0.083	1.3	0.83	0.5	8	8
ACR79-5	50	0.70	0.75	>64	4	8
ACR1A3	9.2	0.75	3.0	>64	4	16
CE- <i>FKS1</i>	0.086	1.3	0.90	0.5	8	8
CE- <i>FKS1</i> ^{N470K}	59	0.88	1.1	>64	4	8
CE- <i>FKS1</i> ^{L642S}	14	0.83	4.0	>64	4	16

^a IC₅₀, concentration of compound that gives 50% inhibition of cell growth compared with the control.

^b MIC, minimum concentration of compound that gives 100% inhibition of cell growth compared with the control.

TABLE 3. Sensitivity of GS from selective arborcandin C-resistant mutants to GS inhibitors

Strain	IC ₅₀ ^a (μg/ml)			GS sp act (nmol/min/mg of protein)
	Arborcandin C	Pneumocandin A ₀	F-10748 C ₁	
YHP250 (wild type)	0.67	0.86	8.7	6.6
ACR79-5	>100	4.8	6.9	3.9
ACR1A3	>100	0.63	1.6	3.3
CE- <i>FKS1</i>	0.26	1.0	5.5	5.9
CE- <i>FKS1</i> ^{N470K}	>100	5.3	3.5	3.8
CE- <i>FKS1</i> ^{L642S}	>100	0.80	1.2	3.9

^a IC₅₀, concentration of a compound that gives 50% inhibition of GS activity compared with the control.

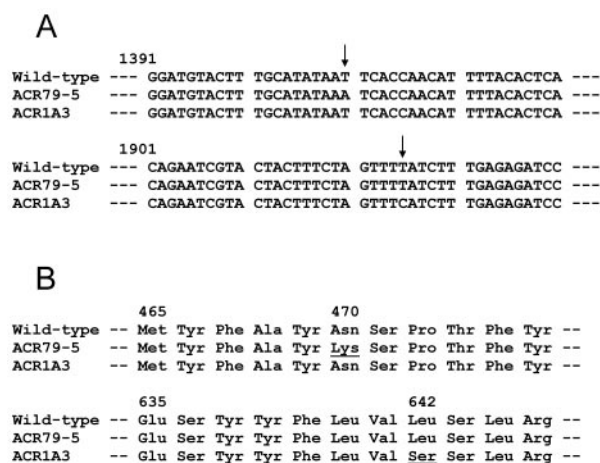


FIG. 1. Nucleotide sequence analysis of *FKS1* open reading frames of ACR79-5 and ACR1A3. (A) Partial nucleotide sequences of *FKS1* from wild-type and arborcandin C-resistant mutants. Arrows, points of nucleotide mutations found in the resistant mutants. (B) Predicted amino acid sequences of Fks1p from the resistant mutants. The substituted residues in the resistant mutants are underlined.

Leu⁶⁴² of Fks1p are critical amino acids in the GS-inhibitory activity of arborcandin C and strongly suggest that the target molecule of arborcandin C is Fks1p, the putative catalytic subunit of GS.

The results we obtained also suggested that Asn⁴⁷⁰ and Leu⁶⁴² in Fks1p are topologically proximate. The model of membrane topology of Fks1p was predicted by hydropathy plot analysis (6). In the model, Asn⁴⁷⁰ and Leu⁶⁴² exist in the transmembrane domain and cytoplasmic loop domain, respectively, and Asn⁴⁷⁰ is topologically distant from Leu⁶⁴² (Fig. 2A). On the other hand, in the model predicted by the

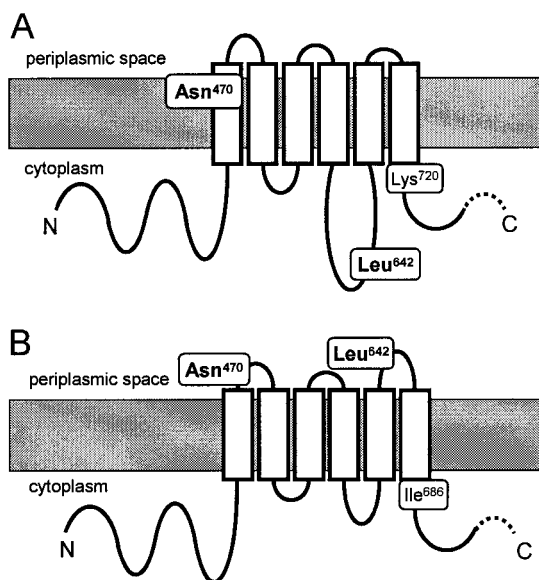


FIG. 2. Model of the predicted membrane topology of the N-terminal region of Fks1p. Solid line, polypeptide chain; boxes, putative transmembrane helices. (A) Model predicted by hydropathy plot analysis. (B) Model predicted by the PHDhtm server.

<i>S. cerevisiae</i>	459	HISIFWMYFAYNSPTFYTHNYQQLV
<i>C. albicans</i>	461	HGTIYWMYTAYNSPTLYTKHYVQTI
<i>A. fumigatus</i>	493	HLGAFWFFTAENAQSLYTDNYQQQV
<i>Cr. neoformans</i>	360	HISVFWFFTAYNAPSIIYAPSGSTTA

<i>S. cerevisiae</i>	632	KYSESYFFLVLSLRDPIRILSTTAM
<i>C. albicans</i>	634	KLVESYFFSTLSLRDPIRNLSTMTM
<i>A. fumigatus</i>	667	KLAESYFFLTLSFKDPIRILSPMQI
<i>Cr. neoformans</i>	526	KFTESYFFLTLSFRDPMKVMNGMKV

FIG. 3. Amino acid sequence alignment of Fks proteins from various fungi. The amino acid sequences of *S. cerevisiae* Fks1p, residues 459 to 483 and 632 to 656, are aligned to the relevant sequences of Fks proteins from *C. albicans*, *A. fumigatus*, and *C. neoformans* (GenBank accession numbers D88815, U79728, and AF102882, respectively). Gray boxes and filled boxes, conserved residues among Fks proteins and mutated residues in arborcandin C-resistant mutants, respectively.

PHDhtm server (<http://cubic.bioc.columbia.edu/predictprotein/>), Asn⁴⁷⁰ and Leu⁶⁴² exist at the first and third extracellular regions, respectively, and it is possible that these amino acid residues are close to each other (Fig. 2B). Our results support the model of Fks1p by the PHDhtm server and contribute to the understanding of the exact structure of Fks1p.

Amino acid residues corresponding to Asn⁴⁷⁰ and Leu⁶⁴² and the primary structure of the regions around those amino acid residues are well conserved among arborcandin-sensitive fungi (Fig. 3). However, these amino acid residues are also conserved in *Cryptococcus neoformans*, which is resistant to arborcandins (14). Although *C. neoformans* has GS and although the unique *FKS1* gene of this organism is essential for its growth (19), *C. neoformans* is resistant to all the known GS inhibitors (8, 12, 20). A common mechanism of resistance to these GS inhibitors, including arborcandins, may exist in *C. neoformans*.

Isolation of the selective arborcandin C-resistant mutants and the identification of amino acids responsible for the resistance provide new insights into the interaction between Fks1p and GS inhibitors. Based on the results of this study, future studies will monitor the direct interaction between arborcandin C and the N-terminal putative extracellular region. Structural analysis of the N-terminal region of Fks1p would also help to improve the antifungal activity of arborcandins.

Nucleotide sequence accession numbers. Whole nucleotide sequences of *FKS1* open reading frames of the wild type, ACR79-5, and ACR1A3 are available in the GenBank database under the accession numbers AY395693, AY395694, and AY395695, respectively.

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