JOURNAL OF BACTERIOLOGY, Oct. 1995, p. 5732–5739 Vol. 177, No. 20
0021-9193/95/$04.00+0

Papulacandin B Resistance in Budding and Fission Yeasts: Isolation and Characterization of a Gene Involved in (1,3)β-D-Glucan Synthesis in *Saccharomyces cerevisiae*

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Received 27 February 1995/Accepted 3 August 1995

Papulacandin B, an antifungal agent that interferes with the synthesis of yeast cell wall (1,3)β-D-glucan, was used to isolate resistant mutants in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. The resistance to papulacandin B always segregated as a recessive character that defines a single complementation group in both yeasts (*pbr1*$^+$ and *PBR1*, respectively). Determination of several kinetic parameters of (1,3)β-D-glucan synthase activity revealed no differences between *S. pombe* wild-type and *pbr1* mutant strains except in the 50% inhibitory concentration for papulacandin B of the synthases (about a 50-fold increase in mutant activity). Inactivation of the synthase activity of both yeasts after in vivo treatment with the antifungal agent showed that mutant synthases were more resistant than the corresponding wild-type ones. Detergent dissociation of the *S. pombe* synthase into soluble and particulate fractions and subsequent reconstitution indicated that the resistance character of *pbr1* mutants resides in the particulate fraction of the enzyme. Cloning and sequencing of *PBR1* from *S. cerevisiae* revealed a gene identical to others recently reported (*FKS1*, *ETG1*, *CWH53*, and *CND1*). Its disruption leads to reduced levels of both (1,3)β-D-glucan synthase activity and the alkali-insoluble cell wall fraction. Transformants containing the *PBR1* gene reverse the defect in (1,3)β-D-glucan synthase. It is concluded that Pbr1p is probably part of the (1,3)β-D-glucan synthase complex.

The major structural polymer of the yeast cell wall, β-glucan, is a glucose homopolymer linked through either (1,3)β- or (1,6)β-glycosidic bonds. Biosynthesis of (1,3)β-D-glucan is mainly attributed to (1,3)β-D-glucan synthase activity, first described for *Saccharomyces cerevisiae* (60), which uses UDP-glucose as a substrate, is localized on the plasma membrane, and is stimulated in vitro by nucleoside triphosphates, mainly guanosine derivatives (45, 60, 61). This property appears to be a general feature displayed by other yeast and fungal (1,3)β-D-glucan synthases (48, 65). Dissociation of the enzymatic activity by treatment with detergents (32) has provided evidence for the existence of a membrane-bound fraction, which seems to contain the catalytic center of the enzyme, and a solubilized GTP-binding fraction. It has been proposed that this second component, recently purified (43), may be involved in the regulation of cell wall (1,3)β-D-glucan synthesis (32). Additionally, a substrate-binding protein from the (1,3)β-D-glucan synthase of *Neurospora crassa* has been identified recently (2).

To gain further insight into the physiologically relevant mechanisms of yeast cell wall synthesis, several genetic approaches have been developed. For instance, taking advantage of the interference caused by calcofluor white in cell wall assembly, a recent report (51) has described the screening of hypersensitivity to calcofluor white as a general procedure for isolating mutants defective in yeast cell wall synthesis. In the case of *S. cerevisiae* chitin synthesis, the isolation of mutants either resistant to the fluorochrome calcofluor white (55, 67) or defective in chitin synthesis in vivo (10) has led to the identification of a third chitin synthase (10, 59, 67). Other types of screenings directed more specifically towards the detection of β-glucan-defective mutants have been undertaken. In some cases, the aim has been to isolate osmotically fragile mutants defective in β-glucan synthesis in *S. cerevisiae* (4, 36) and in *Schizosaccharomyces pombe* (52). In this way, *S. pombe* mutants affected either directly or indirectly in the particulate or in the detergent-solubilized component of (1,3)β-D-glucan synthase have been isolated (14, 52). In other cases, mutants resistant to killer toxins whose primary receptors are cell wall components, i.e., *S. cerevisiae* K1 killer toxin (30) and *Hansenula mrakii* K9 killer toxin (71), have been selected. By analyzing *S. cerevisiae* K1 killer toxin resistance, several genes (*KRE* and related) involved in (1,6)β-D-glucan synthesis have been isolated and characterized (6, 8, 9, 39, 53, 54). (1,6)β-D-glucan synthesis appears to operate along a secretory pathway (54), although the precise function of most of these genes in the biochemistry of the (1,6)β bond remains unknown. Analysis of resistance to *H. mrakii* K9 killer toxin has to date allowed the characterization of a gene, *KNR4*/*SMI1*, whose disruption is not lethal but which promotes a considerable reduction in both (1,3)β-D-glucan synthase activity and (1,3)β-D-glucan contents in the cell wall (29). Cloning of the *gs-1* gene from *N. crassa* (20) by functional complementation of the cell wall-less defect of a (1,3)β-D-glucan synthase-deficient mutant has been reported recently, and the predicted encoded protein is partially homologous to the Knr4p/Smi1p mentioned above. Overriding the effect of *H. mrakii* K9 killer toxin by overexpression of wild-type genes has allowed the isolation and characterization of *HKR1* (33), an essential gene that appears to regulate β-glucan synthesis in vivo.

Another approach involves the isolation of mutants resistant to antifungal agents that act on β-glucan synthesis, such as aculeacin A (25) or echinocandin (16, 18). Papulacandin B is

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TABLE 1. Yeast strains used in this study

Strain[a]	Genotype	Source[b]
S. pombe		
972	h$^-$	P. Munz
JCR1 to JCR8	h$^-$ *pbr1*	This work
975	h$^+$	P. Munz
JCR11	h$^-$ *pbr1-1*	This work
JCR16	h$^-$ *pbr1-6*	This work
JCR18	h$^-$ *pbr1-8*	This work
2178	h$^-$ *ura1-61 leu3-155 ade6-704*	NCYC
JCR20	*mat2P-102 pbr1-8 lys1-131*	This work
1900	h$^-$ *his3-237*	NCYC
JCR21	*mat2P-102 pbr1-8 ura4-94 arg1-230 ade5-36*	This work
1685	h$^-$ *cdc11-136*	NCYC
JCR22	h$^+$ *pbr1-8 ade5-36*	This work
S. cerevisiae		
X2180-1A	*MAT***a**	YGSC
HV1 to HV5	*MAT***a** *pbr1*	This work
α131-20	*MAT*α *ade2 ura3 leu1*	J. E. Haber
CC1	*MAT***a** *pbr1-1 leu1*	This work
CC10B	*MAT***a** *pbr1-1 leu1 ura3-373-251-328*	This work
YNN295	*MAT*α *ura3 lys2 his7 trp1 ade*$^-$	YGSC
CVX12-3A	*MAT*α *ura3-373-251-328 his4-34 leu2-3-112*	C. R. Vazquez
TD28	*MAT***a** *ura3-52 ino1 can1*	G. R. Fink
CC15	*MAT*α *ura3-373-251-328 his4-34 leu2-3-112 pbr1*Δ*::URA3*	This work

[a] Strains JCR1 to JCR8 are the original *pbr1* isolates of strain 972 with different alleles at the *pbr1*$^+$ locus (*pbr1-1* to *pbr1-8*). Strains JCR11, JCR16, JCR18 are segregants from crosses JCR1/975, JCR6/975, and JCR8/975, respectively. JCR20, JCR21, and JCR22 are *pbr1-8* derivatives of strain JCR18. Strains HV1 to HV5 are the original *pbr1* isolates of strain X2180-1A with different alleles at the *PBR1* locus (*pbr1-1* to *pbr1-5*). HV1 and α131-20 are the parents of CC1. CC1 and CVX12-3A are the parents of CC10B.

[b] NCYC, National Collection of Yeast Cultures; YGSC, Yeast Genetic Stock Center.

one of the first antibiotics reported to inhibit yeast growth (66) by specifically interfering both in vivo and in vitro with (1,3)β-D-glucan synthesis in yeasts and filamentous fungi (3, 47, 48, 68). On the basis of these results, we have undertaken a search for papulacandin B-resistant mutants in the budding yeast *S. cerevisiae* and also in the fission yeast *S. pombe*. In this paper, we report on the characterization of these mutants as well as on the partial characterization of a gene that complements papulacandin B resistance in *S. cerevisiae*. This gene has been reported recently by several groups as complementing FK506 and cyclosporin A hypersensitivity (*FKS1*) (21), hypersensitivity to calcofluor white (*CWH53*) (51), or resistance to echinocandin (*ETG1*) (15) or as promoting synthetic lethality with calcineurin mutants (*CND1*) (cited in reference 51), and it may be a structural component of the (1,3)β-D-glucan synthase complex. In a very recent report (19), a new *S. cerevisiae* gene, *GNS1*, has been cloned by complementing an echinocandin resistance phenotype that is also characterized by a great decrease in (1,3)β-D-glucan synthase activity in vitro.

(A preliminary report of parts of this work was presented at the 16th International Conference on Yeast Genetics and Molecular Biology, Vienna, Austria, and at the Biotec-92 Congress, Santiago de Compostela, Spain, 1992.)

MATERIALS AND METHODS

Strains. *Escherichia coli* DH5α and MV1190 were used as hosts for transformations and plasmid propagation. Yeast strains used in this work are listed in Table 1. The genotypes of the other strains employed will be referred to in the text. The general methods for culture and genetic manipulation of the yeast cells were carried out as described previously (44, 62).

Isolation of papulacandin B-resistant mutants. *S. pombe* cells (10^8) were mutagenized in liquid YED medium (2% glucose, 1% yeast extract) with ethylmethane sulfonate (25 μl/ml) for 45 min at 30°C. Mutagenized cells (about 15% survivors) were washed twice, resuspended in YED medium supplemented with papulacandin B (20 μg/ml), distributed in 1-ml aliquots, and incubated for 3 days at 30°C. Aliquots from tubes in which growth was observed were plated on solid YED medium containing 20 μg of papulacandin B per ml from which, after 3 days at 30°C, one resistant clone per tube was recovered. A similar procedure was used for the isolation of *S. cerevisiae* papulacandin B-resistant mutants under conditions in which about 30% of the cells remained viable.

Antifungal compounds and susceptibility assays. Papulacandin B, papulacandin D, and aculeacin A were generous gifts from K. Scheibli and P. Traxler (Ciba-Geigy, Basel, Switzerland) and K. Mizuno (Tokyo Jozo Co. Ltd., Tagata-gun, Shizuoka-ken, Japan), respectively. These antibiotics were assayed at the concentrations indicated for each experiment in stock solutions (10 mg/ml in methanol, kept at −20°C).

For parallel tests of a large number of strains, late-exponential-phase cultures in YED medium were diluted at a cell density of 5×10^6 cells per ml in test tubes containing 5 ml of the same medium, and the antibiotic (20 μg/ml) or an equivalent volume of the solvent was added. Incubation was at 30°C, and the optical density at 600 nm was determined at 24 h. The same procedure was used for MIC determinations, with 1:2 serial dilutions of the antibiotic. Qualitative analyses of susceptibility to papulacandin B were also carried out on solid YEPD medium (2% glucose, 2% peptone, 1% yeast extract). Under these conditions, resistance to the antibiotic, mainly in *S. cerevisiae*, was inoculum dependent, and conventional replica plating or plating by patching tended to produce false resistance. Therefore, sensitivity or resistance to the antibiotic was scored by diluting each clone ($<10^7$ cells per ml) in microtiter plate wells and replica plating them with a multireplicator device that deposits drops (about 2 μl) on plates supplemented with papulacandin B at a final concentration of 20 μg/ml. Scoring of resistance was improved by the use of plates with 1% agarose (FMC Bioproducts, Rockland, Maine).

Labeling and fractionation of cell wall polysaccharides. Exponentially growing cultures of *S. cerevisiae* wild-type or mutant strains in YEPD medium (with 1% glucose) were supplemented with D-[U-^{14}C]glucose (7.5 μCi/ml). After three doubling times, cells were harvested (6×10^7/ml), supplemented with unlabeled cells as the carrier, and subjected to mechanical breakage by vortexing in the presence of glass beads. Cell walls were purified by repeated washings and differential centrifugation (three times with 5% NaCl and three times with 1 mM EDTA) and then extracted twice with 6% NaOH for 90 min at 80°C. The resulting alkali extract was divided into two aliquots. In one of them, precipitation of mannan with the Fehling reagent was performed as described previously (1). In the other, alkali-soluble β-glucan and mannan were coprecipitated by the addition of 2 volumes of absolute ethanol. The difference between the counts obtained in the ethanol-precipitated pellet and that produced by the Fehling reagent was taken to represent the alkali-soluble β-glucan fraction. The residue remaining after NaOH extraction was washed by centrifugation until neutral pH was reached and corresponded to the alkali-insoluble β-glucan plus chitin. All determinations were carried out in duplicate.

Enzyme preparation and assays. Early-logarithmic-phase cells resuspended in 1 mM EDTA (pH 8) were subjected to mechanical breakage with a Braun homogenizer and glass beads. Cell debris and unbroken cells were removed by low-speed centrifugation ($5,000 \times g$ for 5 min at 4°C). The resulting supernatant was centrifuged at $48,000 \times g$ for 30 min, and the pellets were washed once with 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 1 mM β-mercaptoethanol. The final pellets were resuspended in the same buffered solution containing 33% glycerol and stored at −20°C.

(1,3)β-D-Glucan synthase activity was determined as outlined by Shematek et al. (60), with minor modifications. The standard incubation mixture contained 5.3 mM UDP–D-[^{14}C]glucose (4×10^4 cpm/mmol), 80 μM GTP-γ-S, 0.8% bovine serum albumin, 2.2 mM EDTA, 75 mM Tris-HCl (pH 8), 8.25% (vol/vol) glycerol, and enzyme (usually between 100 and 200 μg of protein), in a total volume of 40 μl. The reaction was carried out at 30°C for 30 min and stopped by adding 1 ml of 10% trichloroacetic acid. The reaction mixture was transferred onto a fiberglass filter mat (Whatman GF/C), washed three times with 1 ml of 10% trichloroacetic acid and twice with 1 ml of 95% ethanol, and then counted in a liquid scintillation counter. Assays of chitin synthase I activity were performed as described previously (17). One unit of enzyme was defined as the amount that catalyzes the incorporation of 1 μmol of substrate, i.e., glucose or *N*-acetylglucosamine, into glucan or chitin, respectively, per min at 30°C. Specific activity was expressed as milliunits per milligram of protein. Protein was quantitated by the method of Peterson (50), with bovine serum albumin as the standard.

Recombinant DNA manipulations. Transformation of *E. coli*, plasmid preparation, restriction mapping, DNA ligations, colony hybridization, Southern blotting, and other DNA manipulations were done by standard techniques (57). Transformation of yeast cells was carried out by the lithium acetate procedure (31). Yeast genomic DNA for Southern blots was prepared as described by Struhl et al. (64). DNA fragments to be used as probes were labeled by the

random priming procedure (23) with Klenow enzyme and hexamers from Amersham and subsequently purified on Sepharose CL-6B columns.

Nucleotide sequences were determined by the enzymatic dideoxy chain termination method (58). Unidirectional deletions of DNA fragments, subcloned into the Bluescript KS^+/SK^+ vectors, were generated with exonuclease III and S1 nuclease (28), and single-stranded DNA sequencing was carried out with the Sequenase version 2.0 kit, as described by the manufacturer (U.S. Biochemical Corporation). Occasionally, specific oligonucleotides synthesized on the basis of the complementary strand were used as primers. The DNA sequence reported here has been deposited in the EMBL database under accession number Z46262.

Construction of a *S. cerevisiae pbr1* null mutant was carried out as follows. A 1.1-kb *Hin*dIII fragment containing the *URA3* gene was treated with Klenow fragment and then blunt ligated into the *Eco*RV site at the polylinker of the pBluescript KS^+ vector. From this plasmid, the *URA3* gene was recovered by digesting at the *Hin*dIII and *Xba*I flanking sites and then used to replace the 2.6-kb *Hin*dIII-*Xba*I internal region in a *PBR1*-bearing plasmid to create *pbr1*Δ::*URA3*. A linear 2.0-kb *Kpn*I fragment containing the *URA3* gene, flanked on either side by *PBR1* sequences, was excised from this construct and used to transform two *ura3*, papulacandin B-sensitive hosts, one haploid (CVX12-3A) and one diploid (CVX12-3A/TD28). Correct integrations were confirmed by genomic Southern blots.

RNA preparation, Northern (RNA) analysis, and transcript mapping. Total RNA was prepared from exponentially growing cells by a method similar to that described by Percival-Smith and Segall (46). Poly(A)$^+$ RNA was isolated by affinity chromatography on oligo(dT)-cellulose. RNA samples were prepared for electrophoresis by glyoxalation by the procedure of Carmichael and McMaster (12). Northern blot analyses were performed essentially as described by Vrati et al. (69). Primer extension analysis was carried out essentially as described by Sambrook et al. (57) with a synthetic oligonucleotide (5′-TCCCTGGGTATA GTCCGTTTG-3′) complementary to nucleotide positions +51 and +31 of the *PBR1* coding sequence.

Other methods. Separation of *S. cerevisiae* chromosomes was achieved by alternating-field gel electrophoresis (13) with a Bio-Rad CHEF DR-II system at 200 V with a switching interval of 60 s for 15 h, followed by a 90-s switch for 8 h. *PBR1* was located to chromosome XII with a 1.3-kb *Sal*I-*Cla*I *PBR1*-specific random-primed probe to hybridize a yeast chromosomal blot from *S. cerevisiae* YNN295. Precise mapping of *PBR1* was achieved by use of the *PBR1* probe against a collection of overlapping recombinant clones containing inserts from the yeast genome prepared by L. Riles and M. V. Olson (gene mapping kit ATCC 77284). *PBR1* hybridized to lambda clones 6165, 3925, and 2019, which contain overlapping inserts from the right arm of chromosome XII between the reference loci *CDC3* and *ILV5*. Extraction and determination of phospholipids and ergosterol were performed as described previously (5, 27, 34).

RESULTS AND DISCUSSION

Isolation and genetic characterization of papulacandin B-resistant mutants. *S. pombe* and *S. cerevisiae* cells were mutagenized with ethyl methanesulfonate. Surviving cells were selected for papulacandin B resistance by growing them in the presence of 20 μg of papulacandin B per ml (MICs of papulacandin B and aculeacin A for *S. pombe* and *S. cerevisiae* were 5 μg/ml for both antifungal agents). A total of eight *S. pombe* and five *S. cerevisiae* independent clones resistant to papulacandin B were selected and further analyzed. In each case, the heterozygous diploids did not grow on cultures supplemented with 20 μg of papulacandin B per ml. More than 40 complete tetrads from each cross were analyzed, and in all cases, the sensitivity to papulacandin B segregated in a 2+:2− fashion, indicating that resistance phenotypes were monogenic and nuclear. All papulacandin B-resistant mutants analyzed were also aculeacin A resistant except in the case of the *S. pombe pbr1-6* allele, which was resistant to papulacandin B but sensitive to aculeacin A. The resistance to papulacandin B and aculeacin A in mutants from both yeasts cosegregated in genetic crosses. Complementation tests between *S. pombe* or *S. cerevisiae* mutants indicated the existence of a single complementation group for each yeast, a clear indication that either the mutant search is saturated or that the method has a bias for selecting a special kind of mutant. The mutants were designated *pbr1* (papulacandin B resistance).

Assignment of the *S. pombe pbr1*$^+$ locus to a specific linkage group was carried out following the fluorophenylalanine-induced haploidization method, as described by Kohli et al. (35). A diploid strain that carried the *pbr1* marker in addition to auxotrophic markers on chromosomes I (*lys1 ura1*), II (*leu3*), and III (*ade6*) was constructed. The uncovering of recessive markers by chromosome loss in this (diploid 2178/JCR20: *h*$^-$ *ura1-61 leu3-155 ade6-704*/*mat2P-102 pbr1-8 lys1-131*), achieved by fluorophenylalanine treatment, showed that although it was possible to find clones coordinately expressing *pbr1-lys1*, *pbr1-leu3*, and *pbr1-ura1*, all of the 303 papulacandin B-resistant colonies analyzed were able to grow in the absence of adenine. Therefore, *pbr1*$^+$ was assigned to chromosome III. UV-induced mitotic recombination analysis (24) performed on diploid 1900/JCR21 (*h*$^-$ *his3-237*/*mat2P-102 pbr1-8 ura4-94 arg1-230 ade5-36*) localized *pbr1*$^+$ on the right arm of this chromosome, between *arg1*$^+$ and *ade5*$^+$ (data not shown). A more precise location was determined by tetrad analysis in a three-point cross with the right arm markers *cdc11* and *ade5* (1685/JCR22: *h*$^-$ *cdc11-136*/*h*$^+$ *pbr1-8 ade5-36*). Analysis of 154 tetrads yielded 103 parental ditypes (PD), 1 nonparental ditype (NPD), and 50 tetratypes (T) for the interval between *pbr1*$^+$ and *ade5*$^+$, whereas 63 PD, 9 NPD, and 82 T were found for the *pbr1*$^+$-*cdc11*$^+$ interval, and 53 PD, 17 NPD, and 84 T were found for the *cdc11*$^+$-*ade5*$^+$ interval. The genetic distances calculated from the segregation ratios (49) allow these loci to be arranged, relative to each other, in the map order *cdc11*$^+$-*pbr1*$^+$-*ade5*$^+$, separated at approximately 44.1 and 18.1 cM, respectively. Mapping of the *S. cerevisiae PBR1* locus was described in Materials and Methods.

Physiological characterization of papulacandin B-resistant mutants. The growth rates of *pbr1* mutants from the budding and fission yeasts were identical to the corresponding wild-type strains except in the case of *S. pombe pbr1-6* allele, in which the rate of growth was slightly lower (results not shown). The MIC of papulacandin B for *S. pombe pbr1-8* strain was 50-fold higher than that for the wild type; at concentrations above this, mutant cells lysed. For aculeacin A, the MIC only increased up to 20-fold; higher concentrations affected the morphology of the cells, which became rounder at the poles and eventually lysed. The MIC of papulacandin B for *S. cerevisiae* mutants was at least 20-fold higher than that of the wild type. The *S. pombe* wild-type strain was affected by papulacandin D (a papulacandin B derivative in which the galactosyl residue and the short fatty acid have been removed), and all of the *S. pombe* mutant strains were also resistant to this drug. However, the wild-type *S. cerevisiae* strain was not affected by 100 μg of papulacandin D per ml (data not shown). The reason for this different behavior is unknown.

It has been reported (25) that aculeacin A-resistant mutants undergo changes in cell surface hydrophobicity that may reflect changes in membrane lipid composition. However, the susceptibility of *S. pombe* or *S. cerevisiae* mutants to other antifungal agents such as polyenes (2 μg of nystatin per ml) or imidazoles (20 μg of miconazole per ml) was not altered (data not shown). Furthermore, the phospholipid (phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, and phosphatidic acid) and ergosterol levels of the mutant strains were almost identical to those of the corresponding wild-type strains (data not shown).

In vitro (1,3)β-D-glucan synthase activity of *pbr1* mutants. Papulacandin B and aculeacin A were the first antibiotics described to exert their antifungal activity by interfering specifically with cell wall (1,3)β-D-glucan synthesis, hence lysing the cells by weakening their walls (3, 40–42). In vitro-specific inhibition of fungal and fission yeast (1,3)β-D-glucan synthases has also been described (48, 68), although the precise mechanism of action of these drugs is still unknown. Therefore, we tested whether resistance to papulacandin B was somehow

TABLE 2. Inhibition of *S. pombe* and *S. cerevisiae* (1,3)β-D-glucan synthase by papulacandin B

Strain	(1,3)β-D-Glucan synthase activity (mU/mg of protein)[a]			
	− PB treatment in vivo		+ PB treatment in vivo	
	− PB treatment in vitro	+ PB treatment in vitro	− PB treatment in vitro	+ PB treatment in vitro
S. pombe wild type	8.5	2.9 (34)	4.3 (51)	1.6 (19)
S. pombe pbr1-8	7.6	6.6 (87)	5.9 (78)	4.9 (64)
S. cerevisiae wild type	18.6	ND[b]	0.56 (3)	ND
S. cerevisiae pbr1-1	20.8	ND	10.8 (52)	ND

[a] In vivo log-phase cultures were supplemented (+) or not (−) with papulacandin B (PB; 8 μg/ml), and after 60 min (growth was unaffected in this period), cells were harvested, cell extracts were prepared, and activity was measured. In vitro, papulacandin B (50 μg/ml) was added (+) or not (−) to the assay. In parentheses, the remaining activity is expressed as a percentage of control values (without any papulacandin B treatment). Strains used were *S. pombe* 972, *S. pombe* JCR18, *S. cerevisiae* X2180-1A, and *S. cerevisiae* CC1.

[b] ND, not determined.

affecting (1,3)β-D-glucan synthase activity. As shown in Table 2, specific activity levels in cell extracts from wild-type and mutant strains grown to the logarithmic phase of growth were 8.5 and 7.6 mU/mg of protein, respectively, for *S. pombe* and 18.6 and 20.8 mU/mg of protein, respectively, for *S. cerevisiae*. Cell extracts from wild-type and mutant strains were also assayed for some kinetic properties of such activity. Synthase from *S. pombe* wild-type and mutant strains was similarly stimulated (about sixfold) by GTP-γ-S. The enzyme from *S. pombe pbr1-8* had a K_m of 3.5 mM for UDP-glucose, whereas the wild-type synthase had a K_m of 3.2 mM. No differences in these parameters were observed between cell extracts from the *S. cerevisiae* wild type and a *pbr1-1* mutant strain.

The addition of papulacandin B (50 μg/ml) to the *S. pombe* (1,3)β-D-glucan synthase assay in extracts lowered the wild-type activity to 34%, but mutant activity remained at 87% (Table 2). Inhibition by papulacandin B increased up to a certain drug concentration (Fig. 1); the 50% inhibitory concentration for mutant enzymes was about 50-fold higher than that for the wild type. These results are similar to those reported (16) for a *S. cerevisiae* mutant resistant to an echinocandin analog that also inhibits yeast (1,3)β-D-glucan synthase. Therefore, the consideration of *S. pombe* mutants as typical permeability mutants can be excluded. Inhibition by aculeacin A was less than it was for papulacandin B, and the activity from the in vivo aculeacin A-resistant *S. pombe pbr1-8* allele was seen to be almost insensitive. The inhibition curve of the activity from the in vivo aculeacin A-sensitive *S. pombe pbr1-6* allele was intermediate between that of the wild type and that of the resistant one.

Similar titrations could not be done with *S. cerevisiae* since, as reported, papulacandin B and aculeacin A inhibit (1,3)β-D-glucan synthase in vitro only at very high drug concentrations (3, 11, 25, 70). However, we observed previously (48, 68) that although the addition of these antibiotics to *S. pombe* or *Geotrichum lactis* hardly affected growth, it led to a reduced specific activity of the synthase in extracts. The results from two such representative experiments on *S. pombe* and *S. cerevisiae* strains are also shown in Table 2. (1,3)β-D-Glucan synthase activity in extracts from mutant cells pretreated with papulacandin B was always higher than that observed in pretreated wild-type cells and particularly evident for *S. cerevisiae*, where residual activity for the wild type was almost zero. A

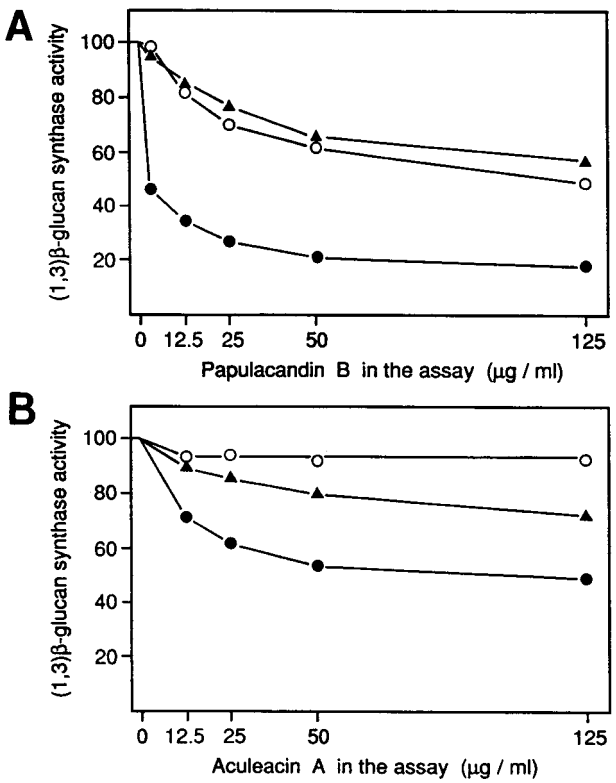

FIG. 1. Effect of papulacandin B (A) or aculeacin A (B) on (1,3)β-D-glucan synthase activity from the following *S. pombe* strains: 972 (●), JCR16 (△), and JCR18 (○). Results are expressed as percentages of the activity measured in the absence of the antifungal agent.

further decrease in *S. pombe* activity was achieved by the addition of papulacandin B to the assay (50 μg/ml), indicating that in vivo and in vitro effects were additive and perhaps reflect an inactivation process.

(1,3)β-D-Glucan synthase activity from *S. pombe* can be dissociated into two inactive fractions, one soluble (GTP binding) and another particulate (UDP-glucose binding), by extraction with NaCl and tergitol Nonidet P-40 (52); reconstitution of the activity occurs after remixing (52). Cell extracts from *S. pombe* wild-type and *pbr1-8* mutant strains were dissociated, and four reconstituted enzymes were prepared. The reconstituted enzymes were functional, and more than 80% of the activity was always recovered (Table 3). Reconstituted enzymes in which the particulate fraction came from the wild-type extract were

TABLE 3. Effect of papulacandin B or aculeacin A on fractionated and reconstituted (1,3)β-D-glucan synthase activity from *S. pombe* strains

Fractions[a]	Activity (mU/mg protein)		
	Control	Papulacandin B[b]	Aculeacin A[b]
$P_{wt} + S_{wt}$	7.4	0.8 (11)	3.3 (44)
$P_{pap1\text{-}8} + S_{pap1\text{-}8}$	6.3	4.0 (63)	4.8 (76)
$P_{wt} + S_{pap1\text{-}8}$	7.8	1.1 (14)	3.6 (46)
$P_{pap1\text{-}8} + S_{wt}$	6.8	4.1 (60)	5.2 (76)

[a] P, particulate (insoluble) fraction; S, soluble fraction; wt, wild type.

[b] Papulacandin B or aculeacin A (50 μg/ml) was added to the assay. In parentheses, the remaining activity is expressed as a percentage of control values. *S. pombe* strains used were 972 and JCR18.

clearly inhibited by papulacandin B and aculeacin A (11 to 14% and 44 to 46% residual activity, respectively). By contrast, reconstituted enzymes in which the particulate fraction came from *pbr1-8* extracts were more resistant to both drugs (60 to 63% and 76% residual activity, respectively). It may therefore be inferred that the particulate, UDP-glucose-binding fraction of the *S. pombe* mutant enzyme is responsible for the resistance to papulacandin B and aculeacin A. A similar result has been described for the (1,3)β-D-glucan synthase activity of an echinocandin-resistant *S. cerevisiae* mutant (16). The rates of incorporation of radioactivity from [^{14}C]glucose into the (1,3)β-D-glucan cell wall fraction and the cell wall susceptibility to digestion, either by zymolyase 100T or novozyme 234, for *S. pombe* wild-type and *pbr1-8* mutant strains are indistinguishable (results not shown). Since the catalytic function of mutant synthase is not apparently affected, either in vivo or in vitro, it can be concluded that the *S. pombe pbr1-8* mutation might specifically affect the interaction between drug and enzyme without interfering with UDP-glucose binding. Unfortunately, all efforts to clone the *S. pombe* wild-type allele by complementing the resistance character of the mutant strain have been, to date, totally unsuccessful.

Isolation of *S. cerevisiae PBR1*. The *S. cerevisiae* wild-type *PBR1* gene was isolated by functional complementation of the *pbr1-1* allele. Strain CC10B was transformed with a yeast DNA library prepared in the centromeric vector YCp50, which carries the *URA3* gene as a selectable marker (56). Of 14,000 transformants able to grow in the absence of uracil, only one was found to have a plasmid-dependent, papulacandin B-sensitive phenotype (see Materials and Methods). The plasmid contained an insert of about 13.3 kb. The smallest subclone capable of complementing *pbr1-1* was the 8.7-kb *Sph*I-*Pst*I fragment (Fig. 2). Aculeacin A resistance was also complemented in parallel by papulacandin B resistance. Southern blot analysis, using several probes encompassed within the 8.7-kb *Sph*I-*Pst*I fragment and several restriction enzymes, indicated that no rearrangement of the DNA structure had occurred during the cloning and that *PBR1* is present as a single-copy gene per haploid genome. However, hybridization under low-stringency conditions revealed an additional band, pointing to the existence of a *PBR1*-related sequence in the yeast genome (Fig. 2A).

Sequencing of the 8.7-kb *Sph*I-*Pst*I fragment revealed a large uninterrupted open reading frame of 5,628 nucleotides, predicted to code for an integral membrane protein (215 kDa) with 16 potential transmembrane domains. A Northern blot experiment (Fig. 2B) showed a single band of approximately 6 kb, sufficient for the predicted 1,876-amino-acid polypeptide. *PBR1* transcription start sites were determined by extending a ^{32}P-labeled synthetic primer with reverse transcriptase following hybridization to total RNA. A major cDNA product 309 nucleotides long was obtained (Fig. 2C), indicating that the initiation of transcription preferentially takes place at the cytosine residue 258 bases upstream from the predicted start of translation. This defines a presumptive 5′-nontranslated leader sequence that is considerably longer than that normally found in *S. cerevisiae* mRNAs (258 nucleotides as compared with the usual 20 to 60 nucleotides).

The *PBR1* gene sequence turned out to be identical to the sequences of the following other genes recently reported: *FKS1*/*ETG1* (15, 21), which complements hypersensitivity to calcineurin inhibitors FK506 and cyclosporin A (*FKS1*) and resistance to echinocandin (*ETG1*), respectively; *CWH53*, a gene that complements hypersensitivity to the fluorochrome calcofluor white (51); and *CND1* (EMBL accession number L35923), a gene whose mutation promotes synthetic lethality with calcineurin mutants (cited in reference 51).

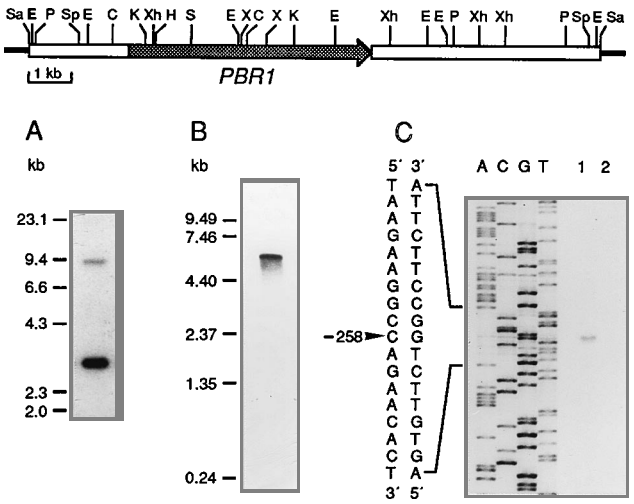

FIG. 2. Restriction map of the pPbr1 insert and characterization of the *PBR1* transcript. The position of the *PBR1* gene is shown as a stippled arrow indicating the direction of transcription. Restriction site abbreviations: C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sa, *Sau*3A; Sp, *Sph*I; X, *Xba*I; Xh, *Xho*I. (A) Southern blot of chromosomal DNA from *S. cerevisiae* CVX12-3A, digested with *Eco*RI and hybridized under low-stringency conditions to a 1.1-kb *Sal*I-*Eco*RI *PBR1*-containing probe. *Hin*dIII-digested lambda DNA was run in adjacent lanes as a size standard. (B) Northern analysis at the *PBR1* gene-containing region. Poly (A)-enriched RNA (5 μg) obtained from exponentially growing cells of strain X2180-1A was denatured with glyoxal, resolved by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized to a 1.3-kb *Sal*I-*Cla*I *PBR1*-containing probe. The positions and sizes of the RNA markers are indicated on the left. (C) Transcript mapping by primer extension. A synthetic oligonucleotide complementary to the sense strand of the *PBR1* gene between +51 and +31 was labeled at the 5′ end and annealed to 20 μg of total RNA from exponentially growing cells. The primer was then elongated with Rous-associated virus 2 reverse transcriptase, and the extended products (lane 1) were resolved on a sequencing gel next to a sequencing ladder of the noncoding strand (lanes A, C, G, and T) obtained with the same oligonucleotide as a primer. Total RNA was substituted by the same amount of yeast tRNA for the reaction shown in lane 2.

Disruption of *S. cerevisiae PBR1*. The *pbr1* mutations from *S. pombe* or *S. cerevisiae* were not apparently affecting the functionality of (1,3)β-D-glucan synthase even though a considerable increase in the 50% inhibitory concentration of papulacandin B or aculeacin A was observed in the case of *S. pombe*. Therefore, a null mutant was constructed (see Materials and Methods). Stable *pbr1*Δ*URA3* transformants were obtained in both haploid and diploid hosts, indicating that the disruption of the *PBR1* gene was not lethal for *S. cerevisiae*. Unexpectedly, the *pbr1*Δ disruptant was not resistant in vivo to either papulacandin B or aculeacin A. Genetic analysis in a *pbr1*Δ::*URA3*/*pbr1-1* diploid (CC15/CC10B) gave no cosegregation of *URA3*, and *pbr1* in 20 tetrads and analysis of 1,045 random spores from the diploid mentioned above gave the same result, suggesting that mutant and disrupted loci were the same.

Disruptant strains presented some additional characteristics. First, on YEPD medium, they grew in clumps and at a lower rate than the wild type or *pbr1-1* mutant cells; a significant proportion of cells were smaller than, and not as rounded as, the isogenic wild-type cells. The lower rate of growth in YEPD liquid medium as well as the altered morphology of the disruptant cells were corrected by supplementing the medium with 1 M sorbitol (results not shown). These results may be an indication that the disruptant has an osmotically fragile and partially affected cell wall. Second, and more interesting, the (1,3)β-D-glucan synthase-specific activity level from disruptant cell extracts was significantly lower than that from the isogenic wild-type strain, whereas the chitin synthase I level was not

TABLE 4. Phenotypes associated with *S. cerevisiae PBR1* locus

Allele at *PBR1* locus	Plasmid[a]	Papulacandin B or aculeacin A resistance[b]	(1,3)β-D-Glucan synthase (mU/mg protein)	Chitin synthase I (mU/mg protein)	[^{14}C]glucose incorporated[c]		
					Alkali-insoluble cell wall fraction	Alkali-soluble β-glucan	Mannan
Wild type	None	−	18.6	0.43	17.9	5.3	12.7
pbr1-1	None	+	20.8	0.48	13.8	3.8	17.9
*pbr1*Δ::*URA3*	None	−	8.2	0.60	8.5	3.0	26.2
*pbr1*Δ::*URA3*	pCC40	−	16.3	ND[d]	ND	ND	ND
*pbr1*Δ::*URA3*	pCC41	−	19.3	ND	ND	ND	ND

[a] pCC40 is a centromeric pRS315-derived plasmid (63) containing *PBR1*. pCC41 is a multicopy Yep13-derived plasmid (7) containing *PBR1*.
[b] Resistance or sensitivity to antifungal drugs is indicated by + or −, respectively.
[c] Results are expressed as percent incorporation of radioactivity from [^{14}C]glucose into the different cell wall polysaccharides or (counts per minute incorporated per fraction/total counts per minute incorporated) × 100. *S. cerevisiae* strains used were CVX12-3A, CC10B, and CC15.
[d] ND, not determined.

reduced (Table 4). Transformation of the *pbr1*Δ disruptant strain with a low-copy or multicopy plasmid carrying the *PBR1* gene restored the (1,3)β-D-glucan synthase wild-type level.

It was appropriate to determine whether in vivo β-glucan synthesis might be affected in the *pbr1*Δ disruptant strain. Therefore, wild-type, resistant mutant, and disruptant strains were grown in parallel on YEPD medium supplemented with 1 M sorbitol and radioactive glucose. Cells were harvested and fractionated as described previously, and the radioactivity incorporated in the alkali-insoluble residue as well as the alkali-soluble β-glucan and mannan cell wall fractions was determined (Table 4). Incorporation of label into the alkali-insoluble residue, mostly constituted by the alkali-insoluble β-glucan component and a small amount of chitin, was reduced considerably (more than 50%) in the disruptant strain. Incorporation into the corresponding *pbr1-1* mutant fraction was also reduced, but to a lesser extent (77%), in comparison with wild-type values. Incorporation into the alkali-soluble β-glucan fraction was also clearly reduced in the disruptant strain (57% of the control value). By contrast, incorporation into mannan was increased considerably in the disruptant as though a higher amount of this fraction tended to compensate for the defect in β-glucan synthesis.

In summary, the *PBR1* locus of *S. cerevisiae* is responsible for both papulacandin B and aculeacin A resistance as well as for a normal level of (1,3)β-D-glucan synthase activity. Furthermore, *S. cerevisiae* Pbr1p, as deduced from the hydropathy analysis, appears to be an integral membrane protein and therefore is located in a particulate fraction where the (1,3)β-D-glucan synthase catalytic subunit is expected to be if it were a structural component of a (1,3)β-D-glucan synthase. However, it should be mentioned that no UDP-glucose binding consensus sequence (RXGG) (22, 37) is found in the *S. cerevisiae PBR1*-encoded polypeptide, and this may be taken as an indication that it might not be the catalytic subunit but rather another component of a (1,3)β-D-glucan synthase complex.

There are peculiar differences between *cwh53-1* and *pbr1-1* alleles. *cwh53-1* has a low cell wall glucose/mannose ratio in comparison with that of the wild type (about four times lower), indicative of a low level of β-glucan (51), whereas the *pbr1-1* β-glucan defect is smaller than that observed in the *pbr1*Δ disruptant strain (Table 4); by contrast, *cwh53-1* is hypersensitive to papulacandin B, whereas *pbr1-1* is clearly resistant to it. It seemed reasonable to assume that *cwh53-1* has lost the functionality of the *CWH53*/*PBR1*-encoded protein in a way similar to that which occurs with the *pbr1*Δ disruptant. However, *cwh53-1* shows no defect in the in vitro (1,3)β-D-glucan synthase activity level, in contrast with the *pbr1* disruptant (this work) and *fks1* and *fks*Δ*1* strains (15).

It is curious that the *pbr1*Δ strain is sensitive to papulacandin B in comparison with the *pbr1-1* mutant. Our observation of an additional sequence that cross-hybridizes with a *PBR1* probe under low-stringency conditions is consistent with the report on the existence of an *FKS1*/*ETG1* homologous gene named *FKS2* (15, 26), whose transcription depends on calcineurin. Therefore, there might be a second papulacandin B-susceptible target, which would explain the sensitivity to papulacandin B or echinocandin in *pbr1*Δ or *fks1*Δ strains, respectively. Simultaneous disruption of *FKS1* and *FKS2* genes is lethal (26), and it is possible that these genes might be encoding structural subunits of redundant (1,3)β-D-glucan synthases, as is the case for chitin synthases (59, 67), although rigorous confirmation of this would demand further experimental evidence.

A plasmid construction carrying the *S. cerevisiae PBR1* gene under the control of the *nmt* (no message in thiamine) promoter from *S. pombe* (38) was used to complement the *S. pombe pbr1-8* mutation, either in the presence or absence of thiamine. In view of the apparently similar behavior of *S. cerevisiae* and *S. pombe* mutants and the similar location of the papulacandin B resistance in the particulate fraction of the synthase, it was surprising to find out that *PBR1* did not complement the *S. pombe* papulacandin B resistance phenotype. However, expression of *PBR1* in *S. pombe* has not been confirmed by Western blot. An alternative explanation would be that the *S. pombe pbr1*$^+$ gene might be homologous not to *S. cerevisiae FKS1*/*PBR1* but rather to *FKS2* or another, similar (1,3)β-D-glucan synthase-related gene; in that case, the possible functional redundancy between both genes might not be so extensive, and therefore complementation between them would not be mandatory. Finally, it is also possible that *S. pombe pbr1*$^+$ may define a papulacandin B target, which is β-glucan related, different from *S. cerevisiae PBR1* (for instance, the homolog to the recently clone *GNS1* gene [19]), and therefore, new efforts to clone it are currently in progress.

ACKNOWLEDGMENTS

We thank P. Perez and C. Roncero for a critical reading of the manuscript, C. Belinchón for photographic work, and N. Skinner for correcting the English.

This research was supported by grants from the Comisión Interministerial de Ciencia y Tecnología (BIO91-0437 and BIO93-0161).

REFERENCES

1. **Algranati, I. D., N. Behrens, H. Carminatti, and E. Cabib.** 1966. Mannan synthetase from yeast. Methods Enzymol. **8:**411–416.
2. **Awald, P. D., D. Frost, R. R. Drake, and C. P. Selitrennikoff.** 1994. (1,3)β-Glucan synthase activity of *Neurospora crassa*: identification of a substrate-binding protein. Biochim. Biophys. Acta **1201:**312–320.
3. **Baguley, B. C., G. Rommele, J. Gruner, and D. M. Wehrli.** 1979. Papulacan-

din B: an inhibitor of glucan synthesis in yeast spheroplasts. Eur. J. Biochem. **97:**345–351.
4. **Blagoeva, J., G. Stoev, and P. Venkov.** 1991. Glucan structure in a fragile mutant of *Saccharomyces cerevisiae*. Yeast **7:**455–461.
5. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
6. **Boone, C., S. S. Sommer, A. Hensel, and H. Bussey.** 1990. Yeast *KRE* genes provide evidence for a pathway of cell wall β-glucan assembly. J. Cell Biol. **110:**1833–1843.
7. **Broach, J. R.** 1983. Construction of high copy yeast vectors using 2-μm circle sequences. Methods Enzymol. **101:**307–325.
8. **Brown, J. L., and H. Bussey.** 1993. The yeast *KRE9* gene encodes an O-glycoprotein involved in cell surface β-glucan assembly. Mol. Cell. Biol. **13:** 6346–6356.
9. **Brown, J. L., Z. Kossaczka, B. Jiang, and H. Bussey.** 1993. A mutational analysis of killer toxin resistance in *S. cerevisiae* identifies new genes involved in cell wall (1,6)-β-glucan synthesis. Genetics **133:**837–849.
10. **Bulawa, C. E.** 1992. *CSD2*, *CSD3* and CSD4, genes required for chitin synthesis in *Saccharomyces cerevisiae*: the *CSD2* gene product is related to chitin synthases and to developmentally regulated proteins in *Rhizobium* species and *Xenopus laevis*. Mol. Cell. Biol. **12:**1764–1776.
11. **Cabib, E., and R. Roberts.** 1982. Synthesis of the yeast cell wall and its regulation. Annu. Rev. Biochem. **51:**763–793.
12. **Carmichael, G. G., and G. K. McMaster.** 1980. The analysis of nucleic acids in gel using glyoxal and acridine orange. Methods Enzymol. **65:**380–391.
13. **Chu, G., D. Volrath, and R. W. Davis.** 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science **234:** 1582–1585.
14. **Diaz, M., Y. Sanchez, T. Bennett, C. R. Sun, C. Godoy, F. Tamanoi, A. Duran, and P. Perez.** 1993. The *Schizosaccharomyces pombe cwg2*$^+$ gene codes for the β subunit of a geranylgeranyltransferase type I required for β-glucan synthesis. EMBO J. **12:**5245–5254.
15. **Douglas, C. M., F. Foor, J. A. Marrinan, N. Morin, J. B. Nielsen, A. M. Dahl, P. Mazur, W. Baginsky, W. Li, M. El-Sherbeini, J. A. Clemas, S. M. Mandala, B. R. Frommer, and M. B. Kurtz.** 1994. The *Saccharomyces cerevisiae FKS1* (*ETG1*) gene encodes an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase. Proc. Natl. Acad. Sci. USA **91:**12907–12911.
16. **Douglas, C. M., J. A. Marrinan, W. Li, and M. B. Kurtz.** 1994. A *Saccharomyces cerevisiae* mutant with echinocandin-resistant 1,3-β-D-glucan synthase. J. Bacteriol. **176:**5686–5696.
17. **Duran, A., and E. Cabib.** 1978. Solubilization and partial purification of yeast chitin synthetase. J. Biol. Chem. **253:**4419–4425.
18. **El-Sherbeini, M., and J. A. Clemas.** 1995. Nikkomycin Z supersensitivity of an echinocandin-resistant mutant of *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. **39:**200–207.
19. **El-Sherbeini, M., and J. A. Clemas.** 1995. Cloning and characterization of *GNS1*: a *Saccharomyces cerevisiae* gene involved in synthesis of 1,3-β-glucan in vitro. J. Bacteriol. **177:**3227–3234.
20. **Enderlin, C. S., and C. P. Selitrennikoff.** 1994. Cloning and characterization of a *Neurospora crassa* gene required for (1,3)β-glucan synthase activity and cell wall formation. Proc. Natl. Acad. Sci. USA **91:**9500–9504.
21. **Eng, W.-K., L. Faucette, M. M. McLaughlin, R. Cafferkey, Y. Koltin, R. A. Morris, P. R. Young, R. K. Johnson, and G. P. Livi.** 1994. The yeast *FKS1* gene encodes a novel membrane protein, mutations in which confer FK506 and cyclosporin A hypersensitivity and calcineurin-dependent growth. Gene **151:**61–71.
22. **Farkas, I., T. A. Hardy, A. A. DePaoli-Roach, and P. J. Roach.** 1990. Isolation of the *GSY1* gene encoding yeast glycogen synthase and evidence for the existence of a second gene. J. Biol. Chem. **265:**20879–20886.
23. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
24. **Flores da Cuhna, M.** 1970. Mitotic mapping of *Schizosaccharomyces pombe*. Genet. Res. **16:**127–144.
25. **Font de Mora, J., R. Gil, R. Sentandreu, and E. Herrero.** 1991. Isolation and characterization of *Saccharomyces cerevisiae* mutants resistant to aculeacin A. Antimicrob. Agents Chemother. **35:**2596–2601.
26. **Foor, F., C. M. Douglas, M. El-Sherbeini, N. Morin, J. A. Marrinan, J. A. Clemas, S. M. Mandala, B. R. Frommer, J. B. Nielsen, A. M. Dahl, P. Mazur, W. Baginsky, J. Williamson, C. Bonfiglio, and M. B. Kurtz.** 1994. *FKS1* and *FKS2* of *S. cerevisiae* encode highly similar integral membrane proteins which are likely subunits of distinct 1,3-β-D-glucan synthases, abstr. 116A, p. 116. *In* Abstracts of the 1994 Yeast Genetics and Molecular Biology Meeting. The Genetics Society of America, Seattle, Washington.
27. **Gonzalez-Ros, J. M., M. Llanillo, A. Paraschos, and M. M. Martinez-Carrion.** 1982. Lipid environment of acetylcholine receptor from *Torpedo californica*. Biochemistry **21:**3467–3473.
28. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
29. **Hong, Z., P. Mann, N. H. Brown, L. E. Tran, K. J. Shaw, R. S. Hare, and B. DiDomenico.** 1994. Cloning and characterization of *KNR4*, a yeast gene involved in (1,3)-β-D-glucan synthesis. Mol. Cell. Biol. **14:**1017–1025.
30. **Hutchins, K., and H. Bussey.** 1983. Cell wall receptor for yeast killer toxin: involvement of (1,6)β-D-glucan. J. Bacteriol. **154:**161–169.
31. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
32. **Kang, M. S., and E. Cabib.** 1986. Regulation of fungal cell wall growth: a guanine nucleotide-binding, proteinaceous component required for activity of (1,3)-β-D-glucan synthase. Proc. Natl. Acad. Sci. USA **83:**5808–5812.
33. **Kasahara, S., H. Yamada, T. Mio, Y. Shiratori, C. Miyamoto, T. Yabe, T. Nakajima, E. Ichishima, and Y. Furuichi.** 1994. Cloning of the *Saccharomyces cerevisiae* gene whose overexpression overcomes the effects of HM-1 killer toxin, which inhibits β-glucan synthesis. J. Bacteriol. **176:**1488–1499.
34. **Kates, M.** 1972. Techniques in lipidology. Isolation, analysis and identification of lipids, p. 360–361. *In* T. S. Work and E. Work (ed.), Laboratory techniques in biochemistry and molecular biology. American Elsevier Publishing Co., New York.
35. **Kohli, J., H. Hottinger, P. Munz, A. Strauss, and P. Thuriaux.** 1977. Genetic mapping in *Schizosaccharomyces pombe* by mitotic and meiotic analysis and induced haploidization. Genetics **87:**474–489.
36. **Kozhina, T., L. Stateva, and P. Venkov.** 1979. Genetic analysis of an osmotic sensitive *Saccharomyces cerevisiae* mutant. Mol. Gen. Genet. **170:**351–355.
37. **Mahrenholz, A. M., Y. Wang, and P. J. Roach.** 1988. Catalytic site of rabbit glycogen synthase isozymes. J. Biol. Chem. **263:**10561–10567.
38. **Maundrell, K.** 1993. Thiamine repressible expression vectors *pREP* and *pRIP* for fission yeast. Gene **123:**127–130.
39. **Meaden, P., K. Hill, J. Wagner, D. Slipetz, S. S. Sommer, and H. Bussey.** 1990. The yeast *KRE5* gene encodes a probable endoplasmic reticulum protein required for (1,6)-β-D-glucan synthesis and normal cell growth. Mol. Cell. Biol. **10:**3013–3019.
40. **Miyata, M., T. Kanbe, and K. Tanaka.** 1985. Morphological alterations of the fission yeast *Schizosaccharomyces pombe* in the presence of aculeacin A: spherical wall formation. J. Gen. Microbiol. **131:**611–622.
41. **Miyata, M., J. Kitamura, and H. Miyata.** 1980. Lysis of growing fission-yeast cells induced by aculeacin A, a new antifungal antibiotic. Arch. Microbiol. **127:**12–16.
42. **Mizoguchi, J., T. Saito, K. Mizuno, and K. Hayano.** 1977. On the mode of action of a new antifungal antibiotic, aculeacin A: inhibition of cell wall synthesis in *Saccharomyces cerevisiae*. J. Antibiot. **30:**308–313.
43. **Mol, P. C., H.-M. Park, J. T. Mullins, and E. Cabib.** 1994. A GTP-binding protein regulates the activity of (1,3)β-glucan synthase, an enzyme directly involved in yeast cell wall morphogenesis. J. Biol. Chem. **269:**31267–31274.
44. **Moreno, S., A. Klar, and P. Nurse.** 1991. Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. **194:**793–823.
45. **Notario, V., H. Kawai, and E. Cabib.** 1982. Interaction between yeast β-(1,3) glucan synthetase and activating phosphorylated compounds. A kinetic study. J. Biol. Chem. **257:**1902–1905.
46. **Percival-Smith, A., and J. Segall.** 1984. Isolation of DNA sequences preferentially expressed during sporulation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4:**142–150.
47. **Perez, P., I. Garcia-Acha, and A. Duran.** 1983. Effect of papulacandin B on the cell wall and growth of *Geotrichum lactis*. J. Gen. Microbiol. **129:**245–250.
48. **Perez, P., R. Varona, I. Garcia-Acha, and A. Duran.** 1981. Effect of papulacandin B and aculeacin A on β-(1,3)glucan-synthase from *Geotrichum lactis*. FEBS Lett. **129:**249–252.
49. **Perkins, D. D.** 1949. Biochemical mutants of the smut fungus *Ustilago maydis*. Genetics **34:**607–626.
50. **Peterson, G. L.** 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal. Biochem. **83:**346–356.
51. **Ram, A. F. J., S. S. C. Brekelmans, L. J. W. M. Oehlen, and F. M. Klis.** 1995. Identification of two cell cycle regulated genes affecting the β (1,3)-glucan content of cell walls in *Saccharomyces cerevisiae*. FEBS Lett. **358:**165–170.
52. **Ribas, J. C., M. Diaz, A. Duran, and P. Perez.** 1991. Isolation and characterization of *Schizosaccharomyces pombe* mutants defective in cell wall (1-3)β-D-glucan. J. Bacteriol. **173:**3456–3462.
53. **Roemer, T., and H. Bussey.** 1991. Yeast β-glucan synthesis: *KRE6* encodes a predicted type II membrane protein required for glucan synthesis *in vivo* and for glucan synthase activity *in vitro*. Proc. Natl. Acad. Sci. USA **88:**11295–11299.
54. **Roemer, T., S. Delaney, and H. Bussey.** 1993. *SKN1* and *KRE6* define a pair of functional homologs encoding putative membrane proteins involved in β-glucan synthesis. Mol. Cell. Biol. **13:**4039–4048.
55. **Roncero, C., M. H. Valdivieso, J. C. Ribas, and A. Duran.** 1988. Isolation and characterization of *Saccharomyces cerevisiae* mutants resistant to calcofluor white. J. Bacteriol. **170:**1950–1954.
56. **Rose, M. D., P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink.** 1987. A *Saccharomyces cerevisiae* genomic plasmid band based on a centromeric-containing shuttle vector. Gene **60:**237–243.
57. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
58. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

59. **Shaw, J. A., P. C. Mol, B. Bowers, S. J. Silverman, M. H. Valdivieso, A. Duran, and E. Cabib.** 1991. The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. J. Cell Biol. **114:**111–123.
60. **Shematek, E. M., J. A. Braatz, and E. Cabib.** 1980. Biosynthesis of the yeast cell wall. I. Preparation and properties of β(1-3)glucan synthetase. J. Biol. Chem. **255:**888–894.
61. **Shematek, E. M., and E. Cabib.** 1980. Biosynthesis of the yeast cell wall. II. Regulation of β(1-3)glucan synthetase by ATP and GTP. J. Biol. Chem. **255:**895–902.
62. **Sherman, F., G. R. Fink, and B. Hicks.** 1979. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
63. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
64. **Struhl, K., D. T. Stinchcomb, S. Scherer, and R. W. Davis.** 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA **76:**1035–1039.
65. **Szaniszlo, P. J., M. S. Kang, and E. Cabib.** 1985. Stimulation of β(1,3)glucan synthetase of various fungi by nucleoside triphosphates: generalized regulatory mechanism for cell wall biosynthesis. J. Bacteriol. **161:**1188–1194.
66. **Traxler, M., J. Gruner, and J. A. L. Auden.** 1977. Papulacandins, a new family of antibiotics with antifungal activity. 1. Fermentation, isolation, chemical and biological characterization of papulacandins A, B, C, D and E. J. Antibiot. **30:**289–296.
67. **Valdivieso, M. H., P. C. Mol, J. A. Shaw, E. Cabib, and A. Duran.** 1991. *CAL1*, a gene required for activity of chitin synthase 3 in *Saccharomyces cerevisiae*. J. Cell Biol. **114:**101–109.
68. **Varona, R., P. Perez, and A. Duran.** 1983. Effect of papulacandin B on β-glucan synthesis in *Schizosaccharomyces pombe*. FEMS Microbiol. **20:**243–247.
69. **Vrati, S., D. A. Mann, and K. C. Reed.** 1987. Alkaline Northern blots: transfer of RNA from agarose gels to Zeta-Probe membrane in dilute NaOH. Mol. Biol. Rep. **1:**1–4.
70. **Yamaguchi, H., T. Hiratani, K. Iwata, and Y. Yamamoto.** 1982. Studies on the mechanism of antifungal action of aculeacin A. J. Antibiot. **35:**210–219.
71. **Yamamoto, T., T. Hiratani, H. Hirata, M. Imai, and H. Yamaguchi.** 1986. Killer toxin from *Hansenula mrakii* selectively inhibits cell wall synthesis in sensitive yeast. FEBS Lett. **197:**50–54.