JOURNAL OF BACTERIOLOGY, June 1995, p. 2971–2976
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology
Vol. 177, No. 11

D-Arabitol Metabolism in *Candida albicans*: Construction and Analysis of Mutants Lacking D-Arabitol Dehydrogenase

BRIAN WONG,[1]* SANDRA LEESON,[1] SUZANNE GRINDLE,[2] BEATRICE MAGEE,[2] ELWOOD BROOKS,[3] AND PAUL T. MAGEE[2]

Division of Infectious Diseases, Department of Internal Medicine,[1] and Department of Chemistry,[3] University of Cincinnati, Cincinnati, Ohio, and Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota[2]

Received 13 December 1994/Accepted 21 March 1995

***Candida albicans* produces large amounts of the acyclic pentitol D-arabitol in culture and in infected animals and humans, and most strains also grow on minimal D-arabitol medium. An earlier study showed that the major metabolic precursor of D-arabitol in *C. albicans* was D-ribulose-5-PO$_4$ from the pentose pathway, that *C. albicans* contained an NAD-dependent D-arabitol dehydrogenase (ArDH), and that the ArDH structural gene (*ARD*) encoded a 31-kDa short-chain dehydrogenase that catalyzed the reaction D-arabitol + NAD <=> D-ribulose + NADH. In the present study, we disrupted both *ARD* chromosomal alleles in *C. albicans* and analyzed the resulting mutants. The *ard* null mutation was verified by Southern hybridization, and the null mutant's inability to produce ArDH was verified by Western immunoblotting. The *ard* null mutant grew well on minimal glucose medium, but it was unable to grow on minimal D-arabitol or D-arabinose medium. Thus, ArDH catalyzes the first step in D-arabitol utilization and a necessary intermediate step in D-arabinose utilization. Unexpectedly, the *ard* null mutant synthesized D-arabitol from glucose. Moreover, ^{13}C nuclear magnetic resonance studies showed that the *ard* null mutant and its wild-type parent synthesized D-arabitol via the same pathway. These results imply that *C. albicans* synthesizes and utilizes D-arabitol via separate metabolic pathways, which was not previously suspected for fungi.**

Many fungi produce large amounts of the acyclic pentitol D-arabitol (13), and two fungal D-arabitol biosynthetic pathways have been described. The osmotolerant yeast species *Saccharomyces mellis* (25), *Zygosaccharomyces rouxii* (12), and *Debaryomyces hansenii* (14) convert glucose to D-ribulose-5-PO$_4$ via the pentose pathway, dephosphorylate D-ribulose-5-PO$_4$, and then reduce D-ribulose to D-arabitol with an NADP-dependent pentitol dehydrogenase. In contrast, another strain of *Z. rouxii* (4) and the marine fungus *Dendryphiella salina* (18) convert glucose to D-xylulose-5-PO$_4$ via the pentose pathway, dephosphorylate D-xylulose-5-PO$_4$, and then reduce D-xylulose to D-arabitol with an NAD-dependent pentitol dehydrogenase.

The human pathogen *Candida albicans* produces large amounts of D-arabitol in culture and in infected mammalian hosts (3, 7, 15, 27), and most strains also grow on minimal D-arabitol medium (3). Although many groups have investigated the diagnostic implications of D-arabitol production by *C. albicans*, little is known about the metabolic pathways by which *C. albicans* synthesizes and utilizes D-arabitol, the functions of D-arabitol in *C. albicans*, or the role that D-arabitol may play in virulence or pathogenesis. Therefore, Wong et al. (28) studied D-arabitol metabolism in *C. albicans* and found that most of the D-[^{14}C]arabitol produced by *C. albicans* B311 from [2-^{14}C]glucose was labeled at position C-1; this implied on structural grounds that D-ribulose-5-PO$_4$ from the pentose pathway was the principal D-arabitol metabolic precursor. The same study also established that *C. albicans* lysates contained an NAD-dependent D-arabitol dehydrogenase (ArDH) and that the ArDH structural gene (*ARD*) encoded a 30,643-Da member of the short-chain dehydrogenase enzyme family that catalyzed the reaction D-arabitol + NAD <=> D-ribulose + NADH. On the basis of these results, it was proposed (i) that *C. albicans* synthesizes D-arabitol by dephosphorylating D-ribulose-5-PO$_4$ derived from the pentose pathway and then reducing D-ribulose to D-arabitol with ArDH and (ii) that ArDH also catalyzes the first step in D-arabitol utilization by *C. albicans.*

The evidence supporting the conclusions that fungi metabolize D-arabitol via the pathways described above is indirect, consisting mostly of ^{14}C and ^{13}C metabolic labeling data and the presence in cell extracts of enzymes with the expected catalytic activities. To our knowledge, it has not yet been shown for any fungus that a defined mutation or a specific enzyme inhibitor interferes with either biosynthesis or utilization of D-arabitol. Therefore, we sought to ascertain more directly the metabolic functions of ArDH in *C. albicans*. Specifically, we tested the hypothesis that ArDH catalyzes the last step in D-arabitol biosynthesis and the first step in D-arabitol utilization by disrupting both chromosomal alleles of *ARD* and by analyzing the resulting null mutants.

MATERIALS AND METHODS

Strains, media, and plasmids. *C. albicans* 1161 (*arg4 lys1 ser57 MPA1 ura3 gal1*) was obtained from S. Scherer (University of Minnesota) (9) and was cultured in yeast extract-peptone supplemented with 1% glucose (YEPD), 1% D-arabitol (YEP/D-arabitol), 1% D-arabinose (YEP/D-arabinose), or no sugar (YEP) (10) or in 0.67% yeast nitrogen base without amino acids (Difco, Detroit, Mich.) supplemented with arginine, lysine, and serine to which 1% glucose (minimal glucose), 1% D-arabitol (minimal D-arabitol), or 1% D-arabinose (minimal D-arabinose) was added. *C. albicans* transformants were selected on minimal glucose medium plus 1 M sorbitol, and uracil auxotrophs (Ura$^-$) were selected on minimal glucose medium supplemented with 625 mg of 5-fluoroorotic acid (FOA) and 100 mg of uridine per liter (FOA medium). Agar (1.5 to 2%) was included as needed.

Escherichia coli strains DH5α (Gibco BRL, Gaithersburg, Md.) and JM109 (29) were used as plasmid hosts. Cultures were grown in Luria-Bertani medium (20), supplemented with ampicillin (50 μg/ml) and/or agar (2%) as needed. *E.*

* Corresponding author. Mailing address: Department of Internal Medicine, University of Cincinnati College of Medicine, Box 670560, Cincinnati, Ohio 45267-0560. Phone: (513) 558-4707. Fax: (513) 558-2089. Electronic mail address: wongb@ucunix.san.uc.edu.

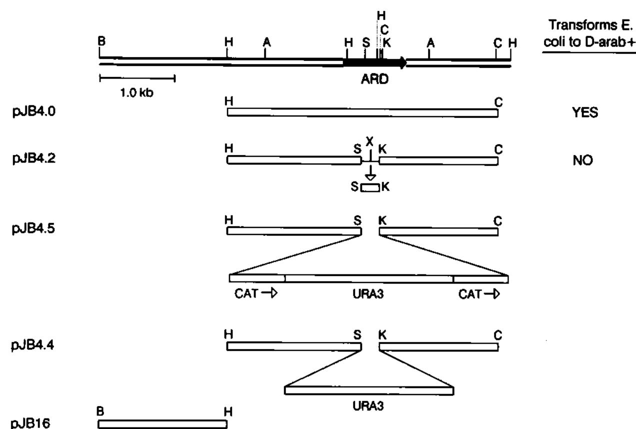

FIG. 1. Restriction maps. Plasmid pJB4.0 contains the *ARD* coding sequence (arrow) and expresses functional ArDH in *E. coli. ARD* was disrupted by replacing the 225-bp *Sal*I-*Kpn*I fragment with an artificial *Sal*I-*Xho*I-*Kpn*I linker; this yielded pJB4.2, which did not express ArDH. Two *ARD* gene-targeting cassettes were constructed by ligating into the new *Xho*I site of pJB4.2 the *Bam*HI-linkered Cat*URA3*Cat cassette from pCat*URA3*Cat (yielding pJB4.5) or the *Bam*HI-linkered *URA3* gene from p*URA3*/Bam (yielding pJB4.4). pJB16 was used as a hybridization probe. Abbreviations: A, *Acc*I; B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I, S, *Sal*I; X, *Xho*I.

coli BW31M is an *araC*(Con) derivative of *E. coli* JM109 that utilizes D-arabitol (D-arab$^+$) when transformed with plasmids expressing ArDH. Transformants were tested for the D-arab$^+$ phenotype on MacConkey agar (Difco) plus 1% D-arabitol, as previously described (28).

Plasmids. Plasmid pJB4 consists of a 3.8-kb *Hin*dIII fragment of *C. albicans* genomic DNA containing *ARD* in pBluescriptII SK$^+$ (Stratagene, La Jolla, Calif.). Plasmid pJB4.0 consists of a 3.6-kb *Hin*dIII-*Cla*I fragment of pJB4 in pBluescriptII SK$^+\Delta$KSX (a version of pBluescriptII SK$^+$ lacking the *Kpn*I, *Sal*I, and *Xho*I restriction sites). Plasmid pJB16 consists of the 1.7-kb *Bam*HI-*Hin*dIII fragment of *C. albicans* genomic DNA immediately 5′ (with respect to the *ARD* coding sequence) to the insert in pJB4.0, in pBluescriptII SK$^+$ (28) (Fig. 1). Plasmid p1123 consists of *C. albicans URA3* in pBR322, and plasmid p1041 contains *URA3* and replicates autonomously in *C. albicans* (9) (from S. Scherer). Plasmid pWC31 contains a gene disruption cassette (Cat*GAL1*Cat) consisting of *C. albicans GAL1* flanked by direct repeats of the bacterial chloramphenicol acetyltransferase gene (Cat), all in pUC19 (obtained from G. Livi, SmithKline Beecham, King of Prussia, Pa.) (8).

DNA constructions. When an integrated selectable marker is flanked by directly repeated DNA, recombinations between the flanking repeats can "loop out" the selectable marker, thereby restoring the original auxotrophy. Alani et al. (1) used this phenomenon to disrupt multiple *Saccharomyces cerevisiae* genes with a single selectable marker, and Gorman et al. (8) and Fonzi and Irwin (6) adapted this mutagenesis strategy to *C. albicans.*

To disrupt *C. albicans ARD* by this approach, we first constructed a *Bam*HI-linkered gene disruption cassette consisting of *C. albicans URA3* flanked by directly repeated Cat DNA (Cat*URA3*Cat). *URA3* was excised from p1123 with *Xba*I and *Eco*RV, the *Xba*I overhang was filled in with Klenow fragment, *Bam*HI linkers were added, and the 2.7-kb *URA3*-containing fragment was ligated into *Bam*HI-digested pBluescriptII SK$^+$, thereby yielding p*URA3*/Bam. Next, Cat*GAL1*Cat was excised from pWC31 with *Sma*I, *Bam*HI linkers were added, and the resulting fragment was ligated into *Bam*HI-digested pUC19, yielding pWC31.2. Next, *GAL1* was excised from pWC31.2 with *Mlu*I and *Bss*HII, blunt ends were created with Klenow fragment, *Xho*I linkers were added, and the ends of the plasmid were ligated together, yielding pCatXCat. Lastly, the 2.7-kb *Bam*HI-linkered insert from p*URA3*/Bam was ligated into the *Xho*I site of pCatXCat by performing 2-base fill-in reactions. This yielded pCat*URA3*Cat, which consists of a 4.2-kb *Bam*HI-linkered Cat*URA3*Cat gene disruption cassette in pUC19.

Next, an essential portion of the *ARD* coding sequence was deleted and replaced with a short artificial polylinker. pJB4.0 was digested with *Sal*I and *Kpn*I, which removed 225 bp from the *ARD* coding region. The plasmid was recircularized by being ligated with the *Kpn*I-*Xho*I-*Sal*I fragment from the polylinker of pBluescriptII SK$^+$, which yielded plasmid pJB4.2. pJB4.0 transformed *E. coli* BW31M to a D-arab$^+$ phenotype, whereas pJB4.2 did not, thereby verifying the gene disruption. Lastly, two selectable *ARD* gene disruption cassettes were constructed by performing 2-base fill-in reactions to insert into the new *Xho*I site in pJB4.2 either (i) the 4.2-kb *Bam*HI-linkered Cat*URA3*Cat cassette from pWC31.2 or (ii) the 2.7-kb *Bam*HI-linkered insert from p*URA3*/Bam. These steps yielded (i) pJB4.5, whose insert consists of an *ard*Δ::Cat*URA3*Cat gene disruption cassette, and (ii) pJB4.4, whose insert consists of an *ard*Δ::*URA3* gene disruption cassette (Fig. 1).

***C. albicans* transformations.** To transform *C. albicans*, overnight cultures in YEPD were diluted into 50 ml of fresh YEPD and shaken at 30°C until the cell density was approximately 3×10^7/ml ($A_{600} = 4.0$). The cells were washed in 20 ml of water and then in 20 ml of 1 M sorbitol, they were suspended in 20 ml of SPEM (1 M sorbitol, 10 mM sodium phosphate [pH 7.5], 10 mM EDTA, 30 mM β-mercaptoethanol), Zymolyase 20T (9 U) was added, and the suspension was shaken slowly at 30°C until spheroplast formation reached 80 to 90% (approximately 20 min). The spheroplasts were collected by centrifugation at 200 to 300 $\times g$, washed once in 20 ml of STC (1 M sorbitol, 10 mM Tris [pH 7.5], 10 mM $CaCl_2$), and resuspended in 2 ml of STC. Five micrograms of transforming DNA was added to 100 μl of spheroplasts, the cells were allowed to stand at room temperature for 10 min, 1 ml of polyethylene glycol solution (20% polyethylene glycol 8000, 10 mM Tris [pH 7.5], 10 mM $CaCl_2$) was added, and the contents were gently inverted several times and allowed to stand at room temperature for 10 min. The cells were sedimented by centrifugation at 200 to 300 $\times g$ for 4 min, the supernatant was carefully removed, and the cells were resuspended in 150 μl of SOS (1 M sorbitol, 6.5 mM $CaCl_2$, 0.25% yeast extract, 0.5% peptone). The cells were incubated at 30°C for 30 to 40 min without shaking and then plated on minimal glucose plus 1 M sorbitol. The plates were incubated at 30°C for 5 to 7 days. Controls were transformed with 5 μg of plasmid p1041 DNA or with no DNA.

Southern hybridizations. Genomic DNA was extracted from *C. albicans* by standard methods (10), and restriction enzyme digests were separated by electrophoresis in 0.9% agarose, transferred to nylon filters (Magnagraph; Micron Separations, Inc., Westboro, Mass.), and hybridized at 65°C overnight with DNA probes labeled with digoxigenin-labeled dUTP by the random primers method. The filters were washed in 2× SSC (300 mM NaCl, 30 mM sodium citrate [pH 7]) plus 0.1% sodium dodecyl sulfate (SDS) (twice for 5 min at 21°C) and in 0.5× SSC (75 mM NaCl, 7.5 mM sodium citrate [pH 7]) plus 0.1% SDS (twice for 15 min at 65°C), and the probes were detected with alkaline phosphatase-conjugated antibodies to digoxigenin (Genius system; Boehringer, Indianapolis, Ind.).

ArDH assays. *C. albicans* lysates were tested for ArDH catalytic activity as previously described (28). Briefly, reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was measured spectrophotometrically (A_{578}) in cuvettes containing 12.5 mM D-arabitol, 35 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 8.5), 0.2 mM NAD or NADP, 0.2 mM phenazine methosulfate, 0.4 mM MTT, and the diluted cell supernatant.

To test for immunoreactive ArDH, proteins in *C. albicans* lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (12% polyacrylamide, denaturing conditions) and transferred to nitrocellulose by electroelution. Immunoreactive ArDH was detected with 1:5,000 dilutions of rabbit serum containing polyclonal antibodies to purified *Candida tropicalis* ArDH (which is 95% identical to *C. albicans* ArDH) and then with alkaline phosphatase-conjugated goat antibody to rabbit immunoglobulin, as previously described (19).

Phenotypic analyses. Growth curves were determined by inoculating uridine-supplemented minimal glucose, minimal D-arabitol, and minimal D-arabinose media with the strains of interest, shaking them at 30°C, and measuring the A_{600} at various intervals thereafter.

C. albicans strains were tested for the ability to synthesize D-arabitol from glucose by harvesting log-phase cultures in uridine-supplemented minimal glucose medium, heating them (100°C, 10 min) to release intracellular polyols, and analyzing the supernatants for D-arabitol by gas chromatography. Briefly, α-methylmannoside and α-methylglucoside were added as internal standards, proteins were removed by acetone precipitation, and the trimethylsilyl ether derivatives were formed by adding trimethylsilylimidazole (Pierce Chemical Co., St. Louis, Mo.) in dimethyl formamide (1:1 by volume), extracted into dry hexane, and analyzed by using a fused silica SPB-5 capillary gas chromatography column (0.32 mm by 60 m) (Supelco, Supelcoport, Pa.) and by flame ionization detection, as previously described (27).

^{13}C NMR. The ^{13}C nuclear magnetic resonance spectroscopy (^{13}C NMR) method described by Jovall et al. (14) was adapted to study the metabolic pathway by which *C. albicans* synthesizes D-arabitol. The *C. albicans* strains of interest were cultured to mid-late log phase in uridine-supplemented yeast nitrogen base containing 1% [2-^{13}C]glucose (99 mol%; Sigma Chemical Co., St. Louis, Mo.), the cell suspensions were heated to 100°C for 10 min to release intracellular polyols, and the supernatants were concentrated in vacuo and resuspended in deionized water. ^{13}C NMR spectra were recorded on a Bruker AC-250 spectrometer operating at a field frequency of 62.89 MHz. A 5.4-μs 60° pulse, and a 3-s delay time with a 16,000 datum point acquisition block size were used. The spectra were collected by using a 9.8-kHz spectral window employing a gated composite pulse decoupling sequence, thus decoupling ^{1}H from ^{13}C with nuclear Overhauser enhancement. An internal standard of dimethyl sulfoxide (ca. 2.5 μl) was used, and the resonance was assigned to a chemical shift value of 39.50 ppm. We collected 256 transients for the D-arabitol solutions and 1,024 transients for the *C. albicans* extracts. An exponential multiplication corresponding to 1-Hz line broadening was applied to the free induction decay prior to the Fourier transform.

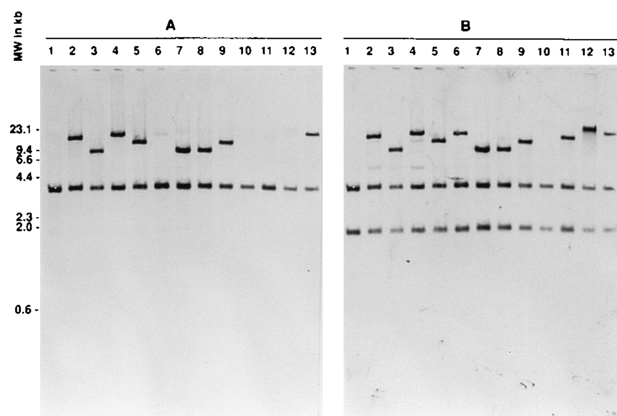

FIG. 2. Disruption of the first *ARD* allele. Genomic DNA from *C. albicans* 1161 (lanes 1) and 12 clones transformed to a Ura$^+$ phenotype with the linearized insert from pJB4.5 (lanes 2 to 13) was digested with *Bam*HI and *Cla*I and probed with the labeled inserts from pJB16 (A) or pJB4.0 (B). The pJB16 insert hybridized with the 3.7-kb *Bam*HI-*Cla*I fragment from the wild-type *ARD* locus in all strains tested and with one extra band in eight transformants. The pJB4.0 insert hybridized with the 3.7-kb *Bam*HI-*Cla*I and 1.6-kb *Cla*I fragments from the wild-type *ARD* locus in all strains tested, with the eight extra bands hybridized by the pJB16 probe, and with one extra band in three additional transformants. Since the *ard*Δ::Cat*URA3*Cat gene-targeting construct contained insert DNA from pJB4.0 but not from pJB16 (Fig. 1), these results implied that the construct integrated homologously within an *ARD* locus in eight transformants, integrated ectopically in three transformants, and did not integrate in one transformant. The size of the smallest extra *ARD* loci (lanes 3, 7, and 8) was as expected for simple replacements of *ARD* by *ard*Δ::Cat*URA3*Cat; the larger extra loci presumably resulted from more-complex recombination events. Molecular sizes (MW) in kilobases are shown on the left.

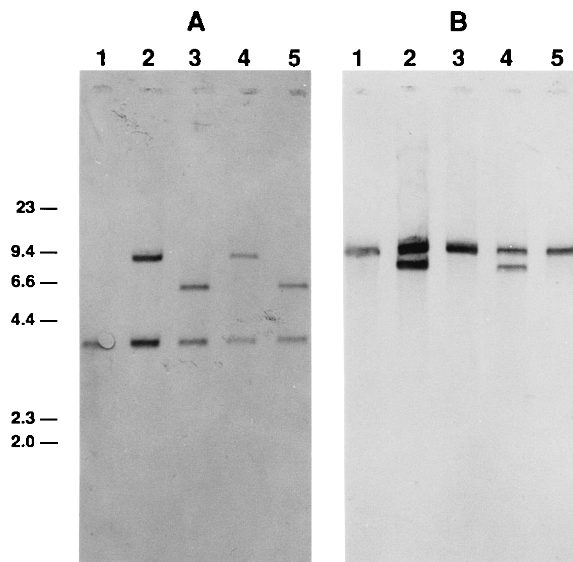

FIG. 3. Loss of *URA3*. Genomic DNA from *C. albicans* 1161 (lanes 1) and from *ARD*/*ard*Δ::Cat*URA3*Cat mutants *C. albicans* 1161.5.6 (lanes 2) and 1161.5.7 (lanes 4) and their respective Ura$^-$ derivatives *C. albicans* 1161.5.6.6 (lanes 3) and 1161.5.7.7 (lanes 5) was digested with *Bam*HI and *Cla*I and hybridized with the labeled inserts from pJB16 (A) or p*URA3*/Bam (B). The mutant *ard* loci in both Ura$^-$ strains were smaller than those in their respective Ura$^+$ parents, and these smaller loci were not hybridized by *URA3*. Thus, recombinations between the flanking Cat repeats looped out the intervening *URA3* DNA, and the genotypes of *C. albicans* strains 1161.5.6.6 and 1161.5.7.7 were both *ARD*/*ard*Δ::Cat. Molecular sizes in kilobases are shown on the left.

RESULTS

Disruption of the first *ARD* allele of *C. albicans* 1161. To disrupt the first *ARD* chromosomal allele, *C. albicans* 1161 spheroplasts were transformed with 5 μg of *Bam*HI- and *Cla*I-digested pJB4.5 DNA. Approximately 150 Ura$^+$ transformants were obtained, and 12 of these were purified and analyzed. Genomic Southern hybridizations showed that the pJB4.5 insert (which consists of *ard*Δ::Cat*URA3*Cat) integrated homologously within the *ARD* locus in eight transformants, integrated ectopically in three transformants, and did not integrate in one transformant (Fig. 2).

Restoration of uracil auxotrophy. The *ARD*/*ard*Δ::Cat*URA3*Cat strains 1161.5.6 and 1161.5.7 (Fig. 2, lanes 7 and 8) were expanded in YEPD to permit intramolecular Cat-Cat recombinations, and uracil auxotrophs were selected on FOA medium. Genomic Southern hybridization analyses showed that FOA-resistant clones 1161.5.6.6 and 1161.5.7.7 had smaller mutant *ard* loci than did their respective Ura$^+$ parents and that these smaller *ard* loci did not contain *URA3* DNA (Fig. 3). Thus, the genotypes of *C. albicans* strains 1161.5.6.6 and 1161.5.7.7 were both *ARD*/*ard*Δ::Cat.

Disruption of the second *ARD* allele. To disrupt the second *ARD* allele, *C. albicans* 1161.5.7.7 was transformed with 5 μg of *Bam*HI- and *Cla*I-digested pJB4.5 or pJB4.4 DNA. Genomic Southern analyses of representative Ura$^+$ transformants showed that the gene-targeting constructs either (i) replaced the previously mutated *ard*Δ::Cat allele, (ii) replaced the wild-type *ARD* allele, or (iii) did not integrate homologously. Results for an *ard*Δ::Cat/*ard*Δ::Cat*URA3*Cat null mutant (*C. albicans* 1161.5.7.7.5) are shown in Fig. 4.

ArDH expression. There was no detectable ArDH catalytic activity in glass-bead lysates of minimal glucose- or minimal D-arabitol-grown *C. albicans* 1161, 1161.5.7.7 (*ARD*/*ard*Δ::Cat), or 1161.5.7.7.5 (*ard*Δ::Cat/*ard*Δ::Cat*URA3*Cat). Therefore, we tested lysates of these *C. albicans* strains for immunoreactive ArDH by Western immunoblotting. Polyclonal rabbit antibodies to *C. tropicalis* ArDH recognized affinity-purified *C. albicans* recombinant ArDH, as was expected because the deduced

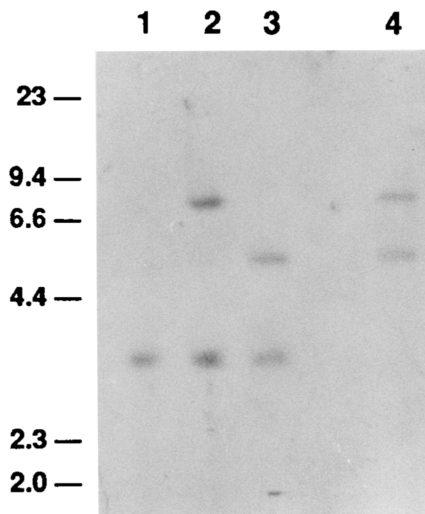

FIG. 4. Disruption of the second *ARD* allele. Genomic DNA from *C. albicans* strains 1161 (wild type; lane 1), 1161.5.7 (*ARD*/*ard*Δ::Cat*URA3*Cat; lane 2), 1161.5.7.7 (*ARD*/*ard*Δ::Cat; lane 3), and 1161.5.7.7.5 (obtained by transforming *C. albicans* 1161.5.7.7 to a Ura$^+$ phenotype with linearized pJB4.5 DNA; lane 4) was digested with *Bam*HI and *Cla*I and hybridized with the labeled insert from pJB16. The hybridization pattern showed that *C. albicans* 1161.5.7.7.5 had one *ard*Δ::Cat allele, one *ard*Δ::Cat*URA3*Cat allele, and no wild-type *ARD* allele. Molecular sizes in kilobases are shown on the left.

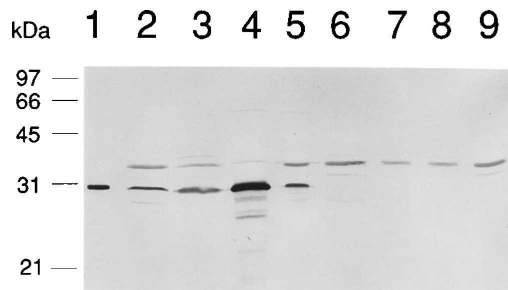

FIG. 5. Expression of ArDH. Wild-type *C. albicans* strain 1161 (lanes 2 to 5) and *ard* null mutant *C. albicans* 1161.5.7.7.5 (lanes 6 to 9) were grown to mid-late log phase in YEPD (lanes 2 and 6), YEP/D-arabitol (lanes 3 and 7), YEP/D-arabinose (lanes 4 and 8), or YEP (lanes 5 and 9), and glass-bead lysates were separated by SDS-PAGE, electroeluted to nitrocellulose, and probed with polyclonal antibodies to *C. tropicalis* ArDH. The antibodies recognized *C. albicans* recombinant ArDH that had been produced in *E. coli* and affinity purified as described for *C. tropicalis* recombinant ArDH by Murray et al. (19) (lane 1) and a protein of the same molecular size in all of the *C. albicans* 1161 lysates. In contrast, the antibodies did not detect the 31-kDa protein in any of the *C. albicans* 1161.5.7.7.5 lysates (10 ng of protein in lane 1 and 10 μg of protein in lanes 2 to 9; Coomassie blue staining). Molecular sizes in kilodaltons are shown on the left.

amino acid sequences of the ArDHs of *C. albicans* and *C. tropicalis* are 95% identical (19). The antibodies also recognized a 31-kDa protein in lysates of D-arabitol-grown *C. albicans* 1161 cells and lesser amounts of the same protein in D-arabitol-grown *C. albicans* 1161.5.7.7. In contrast, the 31-kDa protein was not detected in lysates of *C. albicans* 1161.5.7.7.5 (data not shown). Because the differing abilities of these strains to grow on minimal D-arabitol medium may have influenced the results, we also analyzed lysates of *C. albicans* 1161 and 1161.5.7.7.5 cells that had been grown to mid-late log phase in YEPD, YEP/D-arabitol, YEP/D-arabinose, or YEP. The 31-kDa protein was present in all of the *C. albicans* 1161 lysates and in none of the *C. albicans* 1161.5.7.7.5 lysates (Fig. 5).

Phenotypic consequences of disruption of *ARD*. *C. albicans* strains 1161 and 1161.5.7.7.5 (*ard*Δ::Cat/*ard*Δ::Cat*URA3*Cat) both grew well in uridine-supplemented minimal glucose medium. *C. albicans* 1161 also grew in uridine-supplemented minimal D-arabitol and D-arabinose media, but to lower final cell densities than in minimal glucose medium. In contrast, *C. albicans* 1161.5.7.7.5 did not grow in minimal D-arabitol or D-arabinose medium (Fig. 6).

Despite their differing abilities to utilize D-arabitol and D-arabinose, *C. albicans* 1161 and 1161.5.7.7.5 both produced large amounts of D-arabitol when grown in uridine-supplemented minimal glucose medium. In one experiment, *C. albicans* 1161 and 1161.5.7.7.5 were cultured in uridine-supplemented YNB plus 1% [2-^{13}C]glucose at 30°C until they reached mid-log phase (A_{600} = 0.94 and 1.01, respectively). Gas chromatographic analyses showed that the *C. albicans* 1161 and 1161.5.7.7.5 extracts contained, respectively, 255 and 103 μg of D-arabitol per ml of culture. ^{13}C NMR analyses of these culture extracts showed that most of the D-[^{13}C]arabitol produced from [2-^{13}C]glucose by both strains was labeled at position C-1 (Fig. 7). In a second experiment, *C. albicans* 1161.5.7.7.5 produced more D-arabitol from [2-^{13}C]glucose than did *C. albicans* 1161, and the ^{13}C labeling patterns were the same (data not shown). Thus, the inability to produce ArDH affected neither the ability of *C. albicans* to synthesize D-arabitol from glucose nor the metabolic pathway by which D-arabitol biosynthesis was accomplished.

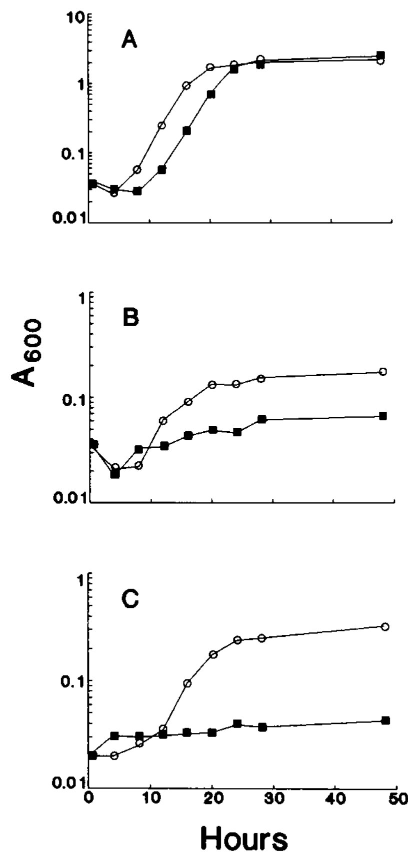

FIG. 6. Growth curves. Wild-type *C. albicans* 1161 (open circles) and *ard* null mutant *C. albicans* 1161.5.7.7.5 (filled squares) were shaken at 30°C in uridine-supplemented minimal glucose (A), minimal D-arabitol (B), or minimal D-arabinose (C), and A_{600} was measured at the intervals shown. Both strains grew well in minimal glucose, but the *ard* null mutant did not grow in minimal D-arabitol or minimal D-arabinose.

DISCUSSION

Since *C. albicans* has a diploid genome and does not reproduce sexually, classical genetic approaches cannot be used to define the functions of specific genes or their products (16, 21). Therefore, the most practical way to ascertain the functions of ArDH was to use targeted gene disruption to construct ArDH-deficient *C. albicans* mutants. To accomplish this, we constructed *ARD* gene disruption cassettes in which 225 bp of essential ArDH-encoding DNA was deleted and replaced with a Cat*URA3*Cat cassette or with *URA3* alone. We substituted *URA3* for *GAL1* in the Cat*GAL1*Cat cassette constructed by Gorman et al. (8) because we found that transformation of *C. albicans* with *URA3* was easier and more efficient than was transformation with *GAL1* (unpublished data). The Cat*URA3* Cat cassette that we used is very similar to a *URA3*-containing gene disruption cassette recently described by Fonzi and Irwin (6). Once the *ARD* gene-targeting plasmids were constructed, the gene disruptions were straightforward. Linearized *ard*Δ::Cat*URA3*Cat DNA integrated into the homologous *ARD* chromosomal locus in 8 of 12 transformants analyzed. Moreover, two of two FOA-resistant clones derived from *ARD*/*ard*Δ::Cat *URA3*Cat heterozygotes lost their ectopic *URA3* genes by intramolecular Cat-Cat recombinations, as expected. Lastly, transformation of an *ARD*/*ard*Δ::Cat*URA3*Cat heterozygote with linearized *ard*Δ::Cat*URA3*Cat or *ard*Δ::*URA3* DNA yielded the predicted homozygous *ard* null mutants.

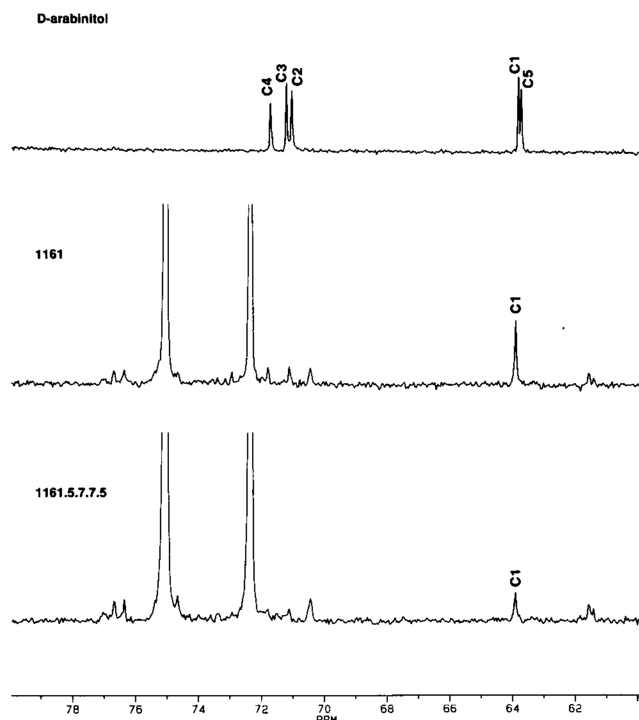

FIG. 7. ^{13}C NMR spectra. An aqueous solution of D-arabitol (1 M) and concentrated extracts of [2-^{13}C]glucose-grown *C. albicans* strains 1161 (*ARD*/*ARD*) and 1161.5.7.7.5 (*ard*/*ard*) were analyzed by ^{13}C NMR. Both strains converted [2-^{13}C]glucose to D-[1-^{13}C]arabitol (C1), which implied that D-ribulose-5-PO_4 from the pentose pathway was the major D-arabitol metabolic precursor. NMR peaks representing ^{13}C in each of the carbon atoms of D-arabitol are labeled C1 to C5. Peak identifications for the *C. albicans* extracts were confirmed by adding D-arabitol and reanalyzing (data not shown).

Since earlier studies showed that ArDH was unstable in *C. albicans* B311 lysates (28), it was not surprising that ArDH catalytic activity was not demonstrable for *C. albicans* 1161 lysates. Therefore, it was necessary to use Western immunoblotting to assess the abilities of *C. albicans* 1161 and its derivatives to express ArDH. Lysates of wild-type *C. albicans* 1161 cells grown in several defined or rich media contained a protein of the expected molecular size that was recognized by polyclonal antibodies to *C. tropicalis* ArDH. Lysates of D-arabitol-grown *C. albicans* 1161.5.7.7 (*ARD*/*ard*) contained reduced amounts of this protein, and lysates of *C. albicans* 1161.5.7.7.5 (*ard*/*ard*) cells grown on several different media contained none. Since the results summarized above established that *C. albicans* 1161.5.7.7.5 had two disrupted *ARD* alleles and expressed no detectable ArDH, we used this mutant to assess the metabolic functions of ArDH.

The inability of *C. albicans* 1161.5.7.7.5 to grow in minimal D-arabitol medium implies that the first step in the *C. albicans* D-arabitol utilization pathway is oxidation of D-arabitol to D-ribulose by ArDH. The subsequent metabolic fate of D-ribulose is not known; one possibility is that it is phosphorylated at C-5 by a ribulokinase, which would permit its entry into the pentose pathway. The inability of *C. albicans* 1161.5.7.7.5 to grow on minimal D-arabinose medium also implies that D-arabitol is a necessary intermediate in the *C. albicans* D-arabinose utilization pathway. Presumably, *C. albicans* utilizes D-arabinose by reducing D-arabinose to D-arabitol, after which D-arabitol is oxidized to D-ribulose by ArDH. The conclusion that ArDH catalyzes a key step in D-arabinose utilization is further supported by the observation that *C. albicans* 1161 expressed larger amounts of immunoreactive ArDH when cultured on YEP/D-arabinose than it did when cultured on YEPD, YEP/D-arabitol, or YEP alone (Fig. 5).

Since the final steps in all previously described fungal D-arabitol biosynthetic pathways are catalyzed by NAD- or NADP-dependent pentitol dehydrogenases (4, 12, 18, 24, 27), the observation that *C. albicans* 1161.5.7.7.5 retained the ability to synthesize D-arabitol from glucose was unexpected. To characterize the D-arabitol biosynthetic pathway further, we compared the labeling patterns in D-[^{13}C]arabitol produced from [2-^{13}C]glucose by wild-type *C. albicans* 1161 and by the *ard* null mutant. That *C. albicans* 1161 converted [2-^{13}C]glucose to D-[1-^{13}C]arabitol confirmed the results obtained earlier with [2-^{14}C]glucose-grown *C. albicans* B311 (28) and also established the feasibility of using ^{13}C NMR analysis of whole-cell extracts to study D-arabitol biosynthesis in *C. albicans*. That *C. albicans ard* null mutant 1161.5.7.7.5 also converted [2-^{13}C]glucose to D-[1-^{13}C]arabitol showed that D-ribulose-PO_4 from the pentose pathway was the principal D-arabitol metabolic precursor, even in the absence of ArDH. Thus, the earlier hypothesis of Wong et al. (28) that ArDH catalyzes the final step in D-arabitol biosynthesis by *C. albicans* was incorrect. Instead, it is now apparent that *C. albicans* utilizes and synthesizes D-arabitol via separate metabolic pathways. Since the existence of separate D-arabitol utilization and biosynthetic pathways was not previously suspected for fungi, our results with *C. albicans* suggest that the conclusions that pentitol dehydrogenases catalyze the final steps in D-arabitol biosynthesis in other fungi (4, 12, 14, 18, 25) also warrant reexamination.

The pathway by which *C. albicans* synthesizes D-arabitol is not yet known, but the fact that D-ribulose-5-PO_4 from the pentose pathway is the major D-arabitol metabolic precursor limits the possibilities. A single reduction and a single dephosphorylation are sufficient to convert D-ribulose-5-PO_4 to D-arabitol; thus, it is likely that D-arabitol is synthesized by one of two pathways. The first possibility is that D-ribulose-5-PO_4 is dephosphorylated, after which D-ribulose is reduced to D-arabitol by a second ArDH isoform. Alternatively, D-ribulose-5-PO_4 could be reduced to D-arabitol-5-PO_4 by a D-ribulose-5-PO_4 reductase, after which D-arabitol-5-PO_4 is dephosphorylated. We consider the second pathway more likely for two reasons. First, *C. albicans ard* null mutants would be expected to grow on minimal D-arabitol and minimal D-arabinose media if a second ArDH isoform had been present, and no such growth was observed. Second, hypothetical D-arabitol pathway 2 is very analogous to the pathways by which fungi and other eukaryotes synthesize other acyclic polyols. For example, *S. cerevisiae* (2, 17) and *Saccharomyces diastaticus* (24) synthesize glycerol (i) by reducing dihydroxyacetone phosphate to glycerol-3-PO_4 with glycerol-3-PO_4 dehydrogenase and then (ii) by dephosphorylating glycerol-3-PO_4. Similarly, many fungi (11), the protozoan *Eimeria tenella* (22), and transgenic tobacco plants expressing bacterial *mtlD* (23) all synthesize mannitol (i) by reducing fructose-6-PO_4 to mannitol-1-PO_4 with mannitol-1-PO_4 dehydrogenase and then (ii) by dephosphorylating mannitol-1-PO_4.

Since the cytoplasmic acid phosphatase of *C. albicans* dephosphorylates many sugar phosphates (5), the key enzymes in both hypothetical D-arabitol biosynthetic pathways described above are the oxidoreductases (i.e., a second ArDH isoform in pathway 1 and D-ribulose-5-PO_4 reductase in pathway 2). Therefore, future studies will focus on elucidating the *C. albicans* D-arabitol biosynthetic pathway, identifying and characterizing key enzymes in this pathway, and using molecular approaches to ascertain the consequences of mutations in this pathway. We also plan to introduce the *ard* null mutation into

a *C. albicans* strain with wild-type virulence properties so that the role of ArDH in virulence can be assessed directly. These studies should provide new insights about the biochemical, physiological, and pathogenetic significance of the metabolic pathways by which *C. albicans* and other fungi utilize and synthesize D-arabitol and other acyclic polyols.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service grants AI-28392 (to B.W.) and AI-16567 (to P.T.M.).

REFERENCES

1. **Alani, E., L. Cao, and N. Kleckner.** 1987. A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. Genetics **116:**541–545.
2. **Albertyn, J., S. Hohmann, J. M. Thevelein, and B. A. Prior.** 1994. *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell. Biol. **14:**4135–4144.
3. **Bernard, E. M., K. J. Christiansen, S. Tsang, T. E. Kiehn, and D. Armstrong.** 1982. Rate of arabinitol production by pathogenic yeast species. J. Clin. Microbiol. **146:**353–359.
4. **Blakeley, E. R., and J. F. T. Spencer.** 1962. Studies on the formation of D-arabitol by osmophilic yeasts. Can. J. Biochem. **40:**1737–1748.
5. **Chattaway, F. W., F. C. Odds, and A. J. E. Barlow.** 1971. An examination of the production of hydrolytic enzymes and toxins by pathogenic strains of *Candida albicans*. J. Gen. Microbiol. **67:**255–263.
6. **Fonzi, W. A., and M. Y. Irwin.** 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics **134:**717–728.
7. **Gold, J. W. M., B. Wong, E. M. Bernard, T. E. Kiehn, and D. Armstrong.** 1983. Serum arabinitol concentrations and arabinitol/creatinine ratios in invasive candidiasis. J. Infect. Dis. **147:**504–514.
8. **Gorman, J. A., W. Chan, and J. W. Gorman.** 1991. Repeated use of *GAL1* for gene disruption in *Candida albicans*. Genetics **129:**19–24.
9. **Goshorn, A. K., S. M. Grindle, and S. Scherer.** 1992. Gene isolation by complementation in *Candida albicans* and applications to physical and genetic mapping. Infect. Immun. **60:**876–884.
10. **Guthrie, C., and G. R. Fink (ed.).** 1991. Guide to yeast genetics and molecular biology. Academic Press, San Diego, Calif.
11. **Hult, K., A. Veide, and S. Gatenbeck.** 1980. The distribution of the NADPH regenerating mannitol cycle among fungal species. Arch. Microbiol. **128:** 253–255.
12. **Ingram, J. M., and W. A. Wood.** 1965. Enzymatic basis for D-arabitol production by *Saccharomyces rouxii*. J. Bacteriol. **89:**1186–1193.
13. **Jennings, D. H.** 1984. Polyol metabolism in fungi. Adv. Microb. Physiol. **25:**149–193.
14. **Jovall, P.-A., I. Tunblad-Johansson, and L. Adler.** 1990. ^{13}C NMR analysis of production and accumulation of osmoregulatory metabolites in the salt-tolerant yeast *Debaryomyces hansenii*. Arch. Microbiol. **154:**209–214.
15. **Kiehn, T. E., E. M. Bernard, J. W. M. Gold, and D. Armstrong.** 1979. Candidiasis: detection by gas-liquid chromatography of D-arabinitol, a fungal metabolite, in human serum. Science **206:**577–580.
16. **Kurtz, M. B., R. Kelly, and D. R. Kirsch.** 1990. Molecular genetics of *Candida albicans*, p. 21–73. *In* D. R. Kirsch, R. Kelly, and M. B. Kurtz (ed.), The genetics of *Candida*. CRC Press, Boca Raton, Fla.
17. **Larsson, K., R. Ansell, P. Ericsson, and L. Adler.** 1993. A gene encoding *sn*-glycerol 3-phosphate dehydrogenase (NAD^+) complements an osmosensitive mutant of *Saccharomyces cerevisiae*. Mol. Microbiol. **10:**1101–1111.
18. **Lowe, D. A., and D. H. Jennings.** 1975. Carbohydrate metabolism in the fungus *Dendryphiella salina*. V. The pattern of label in arabitol and polysaccharide after growth in the presence of specifically-labelled carbon sources. New Phytol. **74:**67–79.
19. **Murray, J. S., C. G. Miyada, L. Wong, A. C. Switchenko, T. C. Goodman, and B. Wong.** 1995. Isolation, characterization and expression of the gene that encodes D-arabinitol dehydrogenase in *Candida tropicalis*. Gene **155:**123–128.
20. **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.
21. **Scherer, S., and P. T. Magee.** 1990. Genetics of *Candida albicans*. Microbiol. Rev. **54:**226–241.
22. **Schmatz, D., W. F. Baginsky, and M. J. Turner.** 1989. Evidence for and characterization of a mannitol cycle in *Eimeria tenella*. Mol. Biochem. Parasitol. **32:**263–270.
23. **Tarczynski, M. C., R. G. Jensen, and J. H. Bohnert.** 1992. Expression of a bacterial *mtlD* gene in transgenic tobacco leads to production and accumulation of mannitol. Proc. Natl. Acad. Sci. USA **89:**2600–2604.
24. **Wang, H.-W., P. Rahaim, P. Robbins, and R. R. Yocum.** 1994. Cloning, sequence, and disruption of the *Saccharomyces diastaticus DAR1* gene encoding a glycerol-3-phosphate dehydrogenase. J. Bacteriol. **176:**7091–7095.
25. **Weimberg, R.** 1962. Mode of formation of D-arabitol by *Saccharomyces mellis*. Biochem. Biophys. Res. Commun. **8:**442–445.
26. **Wong, B., E. M. Bernard, J. W. M. Gold, D. Fong, A. Silber, and D. Armstrong.** 1982. Increased arabinitol levels in experimental candidiasis in rats: arabinitol appearance rates, arabinitol/creatinine ratios, and severity of infection. J. Infect. Dis. **146:**346–352.
27. **Wong, B., and K. L. Brauer.** 1988. Enantioselective measurement of fungal D-arabinitol in serum from normal adults and patients with candidiasis. J. Clin. Microbiol. **29:**1670–1674.
28. **Wong, B., J. S. Murray, M. Castellanos, and K. D. Croen.** 1993. D-Arabitol metabolism in *Candida albicans*: studies of the biosynthetic pathway and the gene that encodes NAD-dependent D-arabitol dehydrogenase. J. Bacteriol. **175:**6314–6320.
29. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.