INFECTION AND IMMUNITY, Feb. 1989, p. 413–419
0019-9567/89/020413-07$02.00/0

Vol. 57, No. 2

Analysis of Mannans of Two Relatively Avirulent Mutant Strains of *Candida albicans*

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Received 21 September 1988/Accepted 29 October 1988

We previously reported the isolation of two cerulenin-resistant mutant strains of *Candida albicans* 4918 that differ in adherence properties and are less virulent than the parental strain. In addition, biochemical characterization demonstrated significant differences in both protein and polysaccharide composition of cell wall material between the mutant and wild-type strains. These observations prompted studies concerning the chemical structure of mannans in these strains. After extraction and subsequent purification by ion-exchange chromatography, mannan fractions were subjected to either mild acid hydrolysis, alkali hydrolysis, or acetylation followed by acetolysis. Acid- and alkali-modified mannans were studied by proton magnetic resonance spectroscopy, and released products were analyzed by high-performance liquid chromatography on an Aminex HPX-42A column. The results demonstrated quantitative and qualitative differences between mannooligosaccharides of the wild-type and mutant strains in the identity of released oligosaccharides as well as in linkage of the oligosaccharides to the protein backbone.

Candida albicans is a member of the indigenous human flora and is recognized as an important opportunistic pathogen. In this respect, individuals compromised by predisposing conditions, such as immunodeficiencies, malignancies and diabetes mellitus, are at risk for *C. albicans* infections ranging in severity from the superficial to the life threatening.

The ability of *C. albicans* to adhere efficiently to host tissues apparently plays an important role in the establishment of infection. Evidence from several laboratories suggests that mannan components of the cell wall help to mediate adherence (9, 12, 17). We have previously described two *C. albicans* mutant strains (designated 4918-2 and 4918-10), isolated on the basis of resistance to the antilipogenic agent cerulenin (3), that do not adhere readily to epithelial cells. In addition, the mutant strains are less virulent than the parental strain (*C. albicans* 4918 [13]) in the rabbit model of endocarditis (1) and have proven deficient in ability to promote vaginal infection (10). Initial characterization of the mutant strains revealed several biochemical differences between the cell walls of the mutant and the wild-type strains (7). These investigations have been expanded to include characterization of mannan structure in these strains, and we report the results in this communication.

MATERIALS AND METHODS

Strains and culture conditions. *C. albicans* 4918 (13) was the wild-type strain used in all experiments. Two cerulenin-resistant *C. albicans* isolates (4918-2 and 4918-10) were obtained in our laboratory as previously described (3). Yeast cells were grown at 23°C with shaking at 150 rpm in Phytone-peptone broth (BBL Microbiology Systems, Cockeysville, Md.) containing 0.1% glucose. The cells were harvested by centrifugation, washed with 0.1 M phosphate-buffered saline (pH 7.2), and stored at −20°C.

General methods. Carbohydrate was determined by the phenol-sulfuric acid method of Dubois et al. (4), using D-mannose as the standard. Protein was determined by the method of Peterson (16), with bovine serum albumin as the standard. Phosphate was estimated by the method of Chen et al. (2). Complete acid hydrolysis of mannan was carried out with 1 M H_2SO_4 at 100°C for 2.5 h. After neutralization with barium carbonate, samples were analyzed by high-performance liquid chromatography (HPLC). Thin-layer chromatography of mannooligosaccharides was carried out with precoated silica gel plates (Kiesel gel, without fluorescent indicator; 0.25-mm thickness; 5 by 20 cm; E. Merck AG, Darmstadt, Federal Republic of Germany) and a mixture of 1-butanol–ethanol–water (5:3:2, vol/vol/vol) as the solvent (15). The plates were sprayed with orcinol reagent to detect the sugars (23). HPLC analysis of monosaccharides and mannooligosaccharides was performed with an HPLC system (Waters Associates, Inc., Milford, Mass.) equipped with a Shodex RI SE-51 refractive index detector and a single-channel recorder integrator. Samples were chromatographed on an Aminex HPX-42A column (7.8 by 300 mm; Bio-Rad Laboratories, Richmond, Calif.) at 85°C with water as the eluant at a flow rate of 0.6 ml/min. Standard mannooligosaccharides from *Saccharomyces cerevisiae* were a gift from C. E. Ballou.

Proton magnetic resonance (PMR) spectra of the H-1 region of mannans were recorded by means of a Bruker AM300WB spectrometer with an external standard of 3-(trimethylsilyl)-propane sulfonic acid. Samples were lyopholized, and 2% solutions were made with 99.9% D_2O. Resolution was 0.18 Hz per data point.

Mannan isolation. Peptidoglucomannan was extracted from yeast cells of wild-type and mutant strains by autoclaving whole cells (100 g [wet weight]) for 90 min at 120°C in 500 ml of 0.02 M citrate buffer, pH 7.0, according to the method of Peat et al. (15). After cooling, the gelatinous solid was recovered by centrifugation and reextracted as before. The extracts were combined and lyophilized. After lyophilization, the extract was dissolved in 100 ml of water, neutralized with 6 N NaOH, and precipitated with 3 volumes of cold ethanol. The precipitate was recovered by centrifugation at 890 × *g* for 20 min, dissolved in 100 ml of water, dialyzed overnight against water, and lyophilized to yield a powder.

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Fractional precipitation of the crude extract of whole cells with hexadecyltrimethylammonium bromide (Cetavlon) was carried out essentially according to the method of Lloyd (11). Fraction B, which contained peptidoglucomannan (1.3 to 1.58 g), was further purified by DEAE-Sephacyl (Pharmacia, Uppsala, Sweden) chromatography. A portion of fraction B (250 mg) was then dissolved in 15 ml of 10 mM Tris hydrochloride, pH 7.5, and loaded on a DEAE-Sephacyl column (1.5 by 100 cm) equilibrated in the same buffer. The column was run at a flow rate of 25 ml/h, and 20-ml fractions were collected. After the column was washed with 400 ml of equilibration buffer, mannan was eluted with 500 ml of a linear gradient of 0 to 500 mM NaCl in 10 mM Tris hydrochloride, pH 7.5. Carbohydrate-containing fractions were then pooled, dialyzed against water, and lyophilized to yield 125 mg of purified mannan. Apparent molecular weights of purified mannan fractions were determined by gel filtration chromatography on a Protein Pak 300 SW column (7.5 mm by 30 cm; Waters Associates), using 50 mM Tris hydrochloride, pH 7.5, containing 100 mM NaCl as the eluant. The column was calibrated by using the following molecular weight standards: thyroglobulin (669,000), ferritin (440,000), catalase (232,000), and aldolase (158,000).

Acid degradation of mannans. Mannans from wild-type and mutant cells were subjected to mild acid hydrolysis by first dissolving 4 to 5 mg of sample in 0.5 ml of 10 mM HCl and then heating the preparations at 100°C for 1 h as described elsewhere (14). Samples were neutralized with AG1-X8 (OH^-) resin and analyzed by HPLC on an Aminex HPX-42A column. The eluate was monitored with a refractive index detector, and the oligosaccharide-containing peaks were identified by comparing their retention times with those of D-mannose, D-glucose, and mannooligosaccharides from *S. cerevisiae*. Mannooligosaccharides were also identified by thin-layer chromatography.

Alkali degradation of mannans. Mild alkali hydrolysis of mannans was carried out by dissolving 5 mg of each sample in 750 μl of 100 mM NaOH, with subsequent incubation at 25°C for 24 h (6). The samples were neutralized with Amberlite IR-120-P (H^+) resin and analyzed by HPLC on an Aminex HPX-42A column as described above. Oligosaccharides were also identified by thin-layer chromatography.

Acetylation and acetolysis of mannans. Mannan acetylation and acetolysis were carried out by the method of Kocourek and Ballou (8) as modified by Suzuki and Fukazawa (21). Mannans (10 mg) were acetylated in a mixture containing 0.5 ml of dry pyridine and 0.5 ml of acetic anhydride at 100°C for 8 h. The solvent was removed under nitrogen; the residue was suspended in a mixture containing 0.5 ml of acetic anhydride, 0.5 ml of acetic acid, and 50 μl of concentrated sulfuric acid and heated at 40°C for 13 h. The reaction was stopped with 2 ml of anhydrous pyridine, and the solvent was removed under nitrogen. The residue was extracted with a mixture of 2.5 ml of chloroform and 2.5 ml of water, and the chloroform extract was then evaporated to dryness. The residue was dissolved in 0.4 ml of anhydrous methanol, and the solution was made alkaline by the addition of 0.5 M sodium methoxide in methanol. After 20 min at room temperature, the precipitate was centrifuged at 12,000 × *g* for 10 min, washed once with anhydrous methanol, suspended in 0.4 ml of water, and neutralized with Amberlite IR-120-P (H^+) resin before being analyzed on the Aminex HPX-42A column.

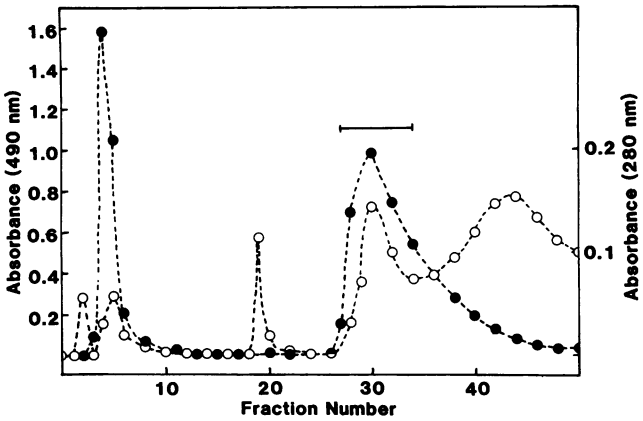

FIG. 1. DEAE-Sephacyl chromatography of fraction B from *C. albicans* 4918-10. A 200-mg amount of fraction B was dissolved in 15 ml of 10 mM Tris hydrochloride, pH 7.5, and loaded on a column (1.5 by 100 cm) equilibrated in the same buffer. Mannan was eluted with 500 ml of a linear gradient of 0 to 500 mM NaCl in 10 mM Tris hydrochloride, pH 7.5, which was initiated beginning with fraction 25. The column was run at a flow rate of 20 ml/h, and 20 ml fractions were collected. Carbohydrate (●) and protein (○) concentrations are shown. Mannan-containing fractions indicated by the bar were combined, dialyzed against water, and lyophilized.

RESULTS

Mannan isolation. Mannans from mutant and wild-type cells were isolated by fractional precipitation of the crude extract of whole cells with Cetavlon as described elsewhere (11). Three fractions were obtained from each extract, and in each case fraction B (fraction of high mannose content) was precipitated with Cetavlon at pH 8.8 in the presence of boric acid. This was determined to be the major mannan-containing fraction. Major mannan fractions from each strain were then used for further studies after purification by anion-exchange chromatography on a DEAE-Sephacyl column. A representative elution profile of this purification is shown in Fig. 1. The major carbohydrate-containing fractions which eluted at the beginning of the salt gradient were pooled, dialyzed, and lyophilized. When analyzed on an Aminex HPX-42A column, the purified mannan fractions from strains 4918, 4918-2, and 4918-10, designated F-man, F-man-2, and F-man-10, eluted as single peaks at 5.48, 5.75, and 5.76 min, respectively (Fig. 2).

Properties of wild-type and mutant mannans. Purified mannan samples of strains 4918, 4918-2, and 4918-10 subjected to gel filtration eluted at 4.99, 4.95, and 5.06 min, respectively. Comparison with the elution profile of the standards indicated that the apparent molecular weights of these samples were all in excess of 600,000. The results of chemical analyses of wild-type and mutant mannans are shown in Table 1. Thin-layer chromatography as well as HPLC analyses of acid hydrolysates of all three mannans revealed mannose as the major (99%) and glucose as a minor sugar component.

Acid hydrolysis studies. To examine the presence of acid-labile oligomannosyl residues in mannans isolated from wild-type and mutant strains, the purified mannan fractions were treated with 10 mM HCl at 100°C for 1 h. After neutralization, samples were analyzed on an Aminex HPX-42A column which resolved the acid-modified mannans and released mannooligosaccharides (Fig. 3). The percentage compositions of released mannooligosaccharides are sum-

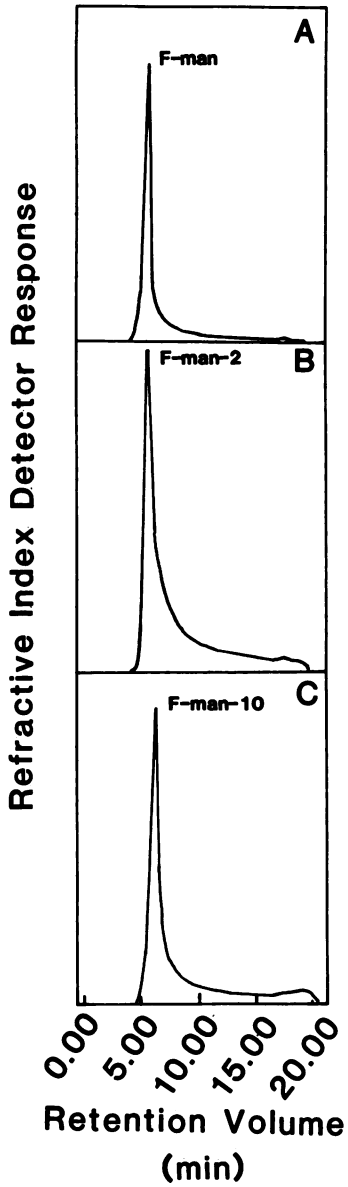

FIG. 2. HPLC analysis of purified wild-type and mutant mannans on an Aminex HPX-42A column. Mannan samples purified by DEAE-Sephacyl chromatography were analyzed on an Aminex HPX-42A column. Elution was effected with water at a flow rate of 0.6 ml/min and a temperature of 85°C. Carbohydrate-containing peaks were detected by monitoring the eluate with a refractive index detector. (A) Native wild-type mannan from *C. albicans* 4918; (B) native mutant mannan from *C. albicans* 4918-2; (C) native mutant mannan from *C. albicans* 4918-10.

marized in Table 2. Of mannan obtained from the wild-type strain, 15% was acid labile. In contrast, 56 and 36% of mannan from 4918-2 and 4918-10, respectively, was found to be acid labile.

The released products consisted of mannose and of oligosaccharides of up to five hexose residues as determined by HPLC. Further analysis demonstrated that acid-modified mannans F-man-2-a and F-man-10-a eluted with a longer retention time of 6.039 min than did the parent mannans F-man-2 and F-man-10, which eluted at 5.75 and 5.76 min, respectively (Fig. 2). However, acid treatment of F-man

TABLE 1. Chemical composition of purified wild-type and mutant mannans

Fraction	Composition (%)		
	Protein[a]	Carbohydrate[b]	Phosphate[c]
F-man	9.3	69.1	0.39
F-man-2	13.8	62.5	0.25
F-man-10	6.7	52.5	2.34

[a] Determined by the method of Peterson (16).
[b] Determined by the phenol-sulfuric acid method (4).
[c] Determined by the method of Chen et al. (2).

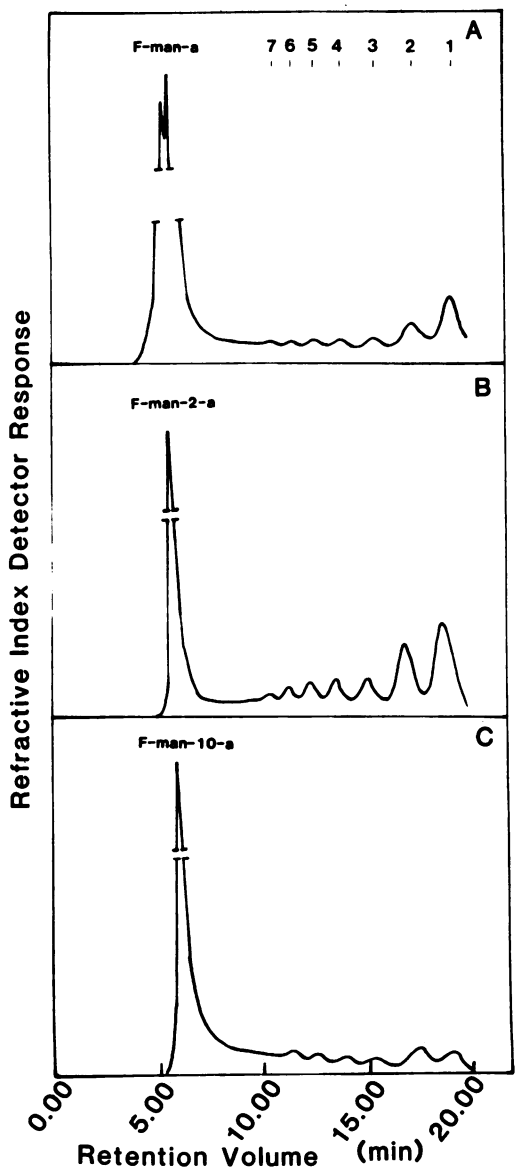

FIG. 3. HPLC separation of the acid-labile products from wild-type and mutant mannans. Purified mannan samples from wild-type strain 4918 (A) and mutant strains 4918-2 (B) and 4918-10 (C) were treated with 10 mM HCl at 100°C for 1 h, neutralized with AG1-X8 (OH^-) resin, and analyzed on an Aminex HPX-42A column. Peaks were detected and identified as described in Materials and Methods.

TABLE 2. Composition of released mannooligosaccharides from wild-type and mutant mannans

Treatment	Peak no.	Retention time (min)	Released oligosaccharide and mannose (% by wt)		
			F-man	F-man-2	F-man-10
10 mM HCl	1	18.9	5.95	17.20	6.24
	2	17.02	2.91	12.67	9.50
	3	15.18	0.97	6.67	4.01
	4	13.67	1.04	6.20	4.08
	5	12.5	1.34	5.49	4.89
	6	11.33	1.17	4.38	3.89
	7	10.49	1.10	3.08	4.18
100 mM NaOH	1	18.9	2.59	8.69	4.14
	2	17.5		3.30	6.48
	3	15.28	8.98	29.36	24.40
	4	12.84	3.79	11.74	8.88
	5	10.33		10.58	8.14
	6	9.49		9.59	17.90
Acetylation-acetolysis	1	18.9	3.96	3.76	2.85
	2	17.02	2.82	2.26	1.37
	3	15.18	6.30	4.48	4.13
	4	12.84	7.31	7.17	6.20
	5	11.33	12.54	14.21	14.0
	6	10.33	14.84	17.34	15.25
	7	9.49	25.84	38.01	43.73

yielded equal amounts of two species of F-man-a which eluted at 5.65 and 5.92 min.

Alkali hydrolysis studies. To examine the presence of alkali-labile oligosaccharide side chains in the mannans from wild-type and mutant strains, mannans were subjected to mild alkali hydrolysis in the presence of 100 mM NaOH at 25°C for 24 h. After neutralization, the products of β-elimination reactions were separated on an Aminex HPX-42A column (Fig. 4). The percentage compositions of released oligosaccharides are summarized in Table 2. The alkali-modified mannan from wild-type cells (F-man-b) eluted with a retention time of 5.92 min, which is similar to the time for one of the acid-modified mannans (Fig. 3). The alkali-modified mannans from mutant strains (F-man-2-b and F-man-10-b) eluted with a retention time of 6.15 min. Mannan from the wild-type strain proved to be least affected by alkali treatment, since only 15% of wild-type mannan was alkali labile whereas 73 and 60% of mannan from strains 4918-2 and 4918-10, respectively, was alkali labile. Although several qualitative as well as quantitative differences were observed between the mannooligosaccharides released from mannans of mutant and wild-type cells, the major product was mannobiose. Mannose, mannobiose, and mannotriose were the only products of the β-elimination reaction for wild-type mannan. However, alkali treatment of mannans from 4918-2 and 4918-10 released oligosaccharides of up to six mannose residues.

Acetolysis studies. Mannans from wild-type and mutant strains were subjected to acetolysis, and the deacetylated acetolysis products were fractionated on an Aminex HPX-42A column. The oligosaccharides were identified by comparing their retention times with those of standard mannooligosaccharides. The maximum length of acetolysis products was hexasaccharide (retention time of 9.49 min) for mutant as well as wild-type mannans (Fig. 5). However, mutant mannans differed from the wild-type mannan in the amount of mannohexaose released upon acetolysis (Table 2).

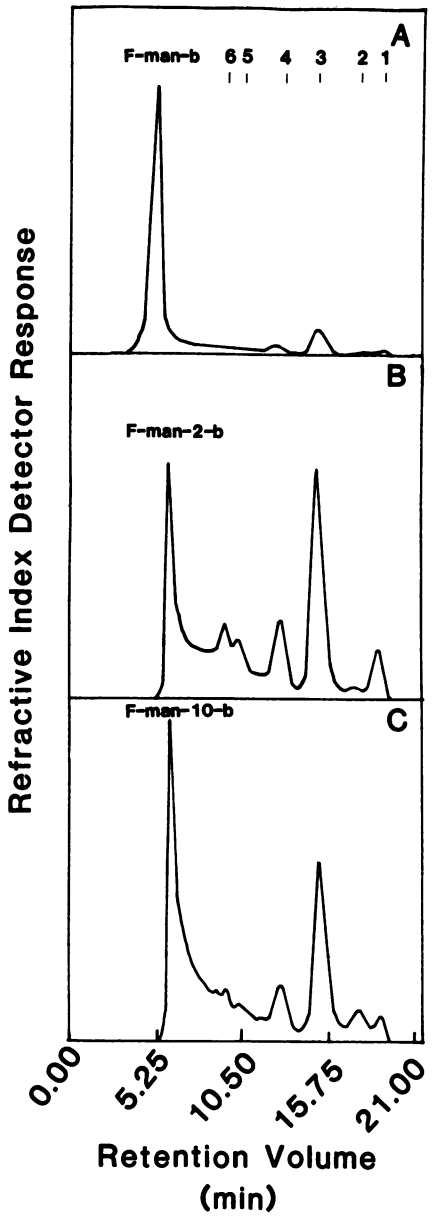

FIG. 4. HPLC separation of the β-elimination products from wild-type and mutant mannans. Purified mannan samples from wild-type strain 4918 (A) and mutant strains 4918-2 (B) and 4918-10 (C) were treated with 100 mM NaOH at 25°C for 24 h, neutralized with Amberlite IR-120-P (H^+) resin, and analyzed on an Aminex HPX-42A column. Peaks were detected and identified as described in Materials and Methods.

PMR spectra of wild-type and mutant mannans. The high-resolution PMR spectra of the anomeric protons of purified wild-type and mutant mannans are shown in Fig. 6A, D, and G. As is evident from the figure, the resolutions of spectra were enhanced in comparison with those reported in earlier studies (18, 19). The signals ranging from 4.7 to 4.45 ppm signified the presence of β-D linkages in these mannans (5). Qualitative (5.45 to 5.43 and 5.05 to 5.01 ppm) as well as quantitative (4.85 and 4.92 ppm) differences in the chemical shifts between wild-type and mutant mannans were also suggested. The presence of a signal from 5.55 to 5.52 ppm indicates that these mannans contained 1-*O*-α-glycosylated

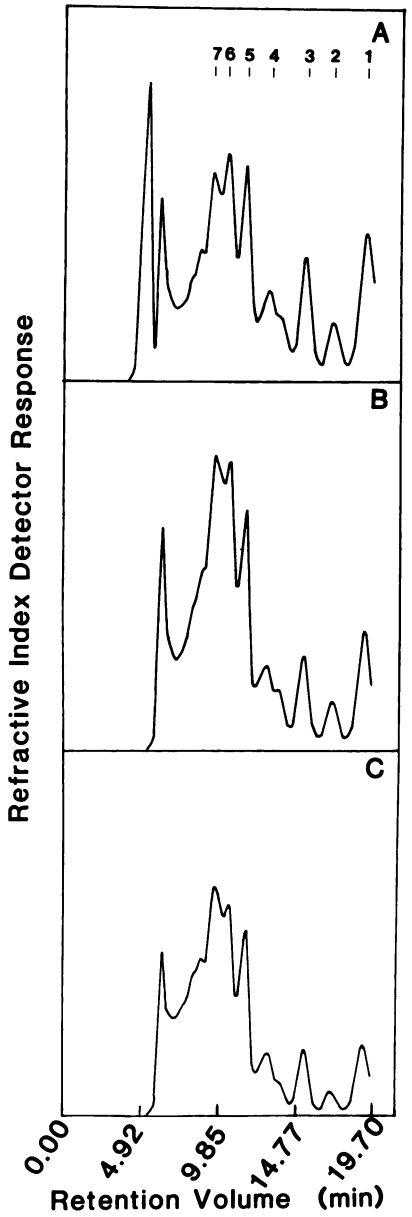

FIG. 5. HPLC separation of the acetolysis products from wild-type and mutant mannans. Purified mannan samples from wild-type strain 4918 (A) and mutant strains 4918-2 (B) and 4918-10 (C) were subjected to acetylation followed by acetolysis, and the deacetylated acetolysis products were fractionated on an Aminex HPX-42A column. Peaks were detected and identified as described in Materials and Methods.

phosphate residues (22). Comparison of these spectra with those of corresponding acid-modified wild-type and mutant mannans (Fig. 6B, E, and H) indicates the disappearance of the chemical shift of the α-anomeric proton of 1-*O*-α-glycosylated phosphate residues upon mild acid hydrolysis. Furthermore, acid treatment of wild-type and mutant mannans resulted in a decrease in chemical shifts corresponding to phosphate-bound β linkages (4.92 to 4.77 ppm), similar to results presented in earlier reports concerning phosphomannans of *Kloeckera brevis* (22), *S. cerevisiae* (14), and *C. albicans* (18, 19). The PMR spectra of alkali-modified wild-type and mutant mannans (Fig. 6C, F, and I) indicated that 1-*O*-α-glycosylated phosphate residues (5.55 to 5.52 ppm) as well as phosphate-bound β-1,2-linked oligomannosyl residues (4.92 ppm) in F-man and F-man-2 were stable to the action of aqueous alkali at room temperature, in agreement with results of an earlier study on *C. albicans* (18). However, such linkages in F-man-10 were labile to the action of alkali, which again demonstrates differences in mannan structure among the strains.

DISCUSSION

We previously reported the isolation of two ceruleninresistant mutant strains of *C. albicans* 4918, designated 4918-2 and 4918-10 (3). These two strains were less virulent than the parental strain in the rabbit model of endocarditis, and both strains exhibited significantly less ability to adhere to human buccal epithelial cells, as well as to fibrin-platelet matrices, than did wild-type cells (1, 7). In addition, quantitative differences in cell wall polysaccharide components of mutant and wild-type strains have been reported (7). These observations, coupled with evidence suggesting the involvement of mannans in cell-cell recognition processes (9, 12, 17), prompted us to investigate the chemical properties of mannans in mutant and wild-type cells.

To investigate possible structural differences in mannans from mutant and wild-type strains, mannans were isolated by hot-water extraction of whole cells followed by Cetavlon precipitation and were then analyzed. These mannans, designated F-man, F-man-2 and F-man-10, had apparent molecular weights in excess of 600,000 and were similar to each other in protein, mannose, and phosphate contents. However, F-man eluted with a retention time of 5.48 min, whereas F-man-2 and F-man-10 eluted at 5.75 and 5.76 min, respectively, when analyzed by HPLC on an Aminex HPX-42A column. Similarly, PMR analysis demonstrated differences between the various mannan preparations. These results suggested that the structures of F-man-2 and F-man-10 differ from that of F-man.

Treatment of F-man, F-man-2, and F-man-10 with 10 mM HCl at 100°C for 1 h yielded acid-modified fractions F-man-a, F-man-2-a, and F-man-10-a, respectively, and a mixture of mannooligosaccharides which were resolved into various species by HPLC. The identities of peaks eluting at 18.9, 15.18, and 11.33 min were established as mannose, mannobiose, and mannotetraose by comparing their retention times with those of standard mannooligosaccharides from *S. cerevisiae*. In addition, analysis of acid-hydrolyzed samples by thin-layer chromatography resolved them into five species corresponding to mannose, mannobiose, mannotriose, mannotetraose, and mannopentaose. The R_fs of these species were indistinguishable from those of standard mannooligosaccharides. Therefore, the species eluting at 10.49 min was probably mannopentaose, and those eluting at 12.5 and 13.67 min corresponded to mannotriose. However, these two mannotriose species must be structurally different from standard mannotriose, which eluted at 12.84 min. The species eluting at 17.02 min could not be definitely identified, but we do know that it did not contain phosphorus and was distinct from glucose, glucosamine, *N*-acetylglucosamine, ribose, and galactose. These results revealed significant quantitative differences in the phosphate-bound oligomannosyl residues of wild-type and mutant mannans and also indicated that although F-man-a comprised 85% (by weight) of mannan when subjected to acid hydrolysis, F-man-2-a and F-man-10-a constituted 44 and 64%, respectively, of this fraction. Since F-man-a, F-man-2-a, and F-man-10-a elute at

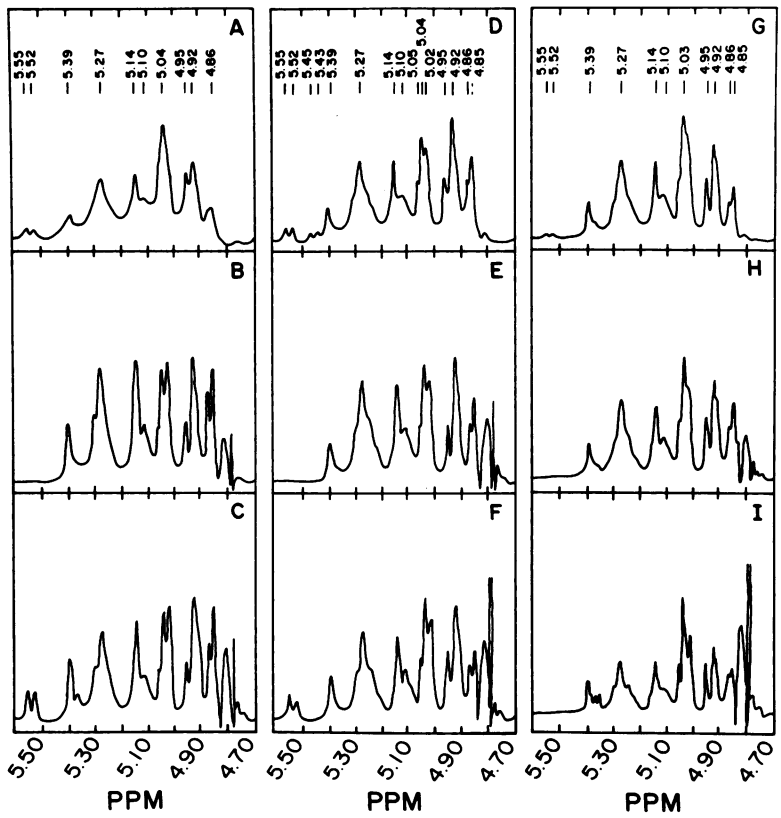

FIG. 6. PMR spectra of anomeric regions of wild-type and mutant mannans in D_2O at 26°C. Chemical shifts are expressed as parts per million. (A) F-man; (B) F-man-a; (C) F-man-b; (D) F-man-2; (E) F-man-2a; (F) F-man-2b; (G) F-man-10; (H) F-man-10a; (I) F-man-10b.

different retention times, they are structurally distinct from one another. The increased susceptibility of mutant mannans to acid hydrolysis could indicate changes in number or size or both of mannose side chains attached to the mannan backbone via phosphate bonds compared with the wild type.

Treatment of F-man, F-man-2, and F-man-10 with 100 mM NaOH at 25°C for 24 h yielded alkali-modified fractions F-man-b, F-man-2-b, and F-man-10-b, respectively, and a mixture of mannooligosaccharides which were resolved by HPLC. The released products from F-man eluted at 18.9, 15.28, and 12.84 min and were identified as mannose, mannobiose, and mannotriose. These results are in close agreement with those of earlier studies on *C. albicans* even though different strains were used (19). In the cases of F-man-2 and F-man-10, however, three additional products were observed which eluted with retention times of 17.5, 10.33, and 9.49 min and were identified as glucose, mannopentaose, and mannohexaose by comparing their retention times and R_fs with those of standard mannooligosaccharides. These results demonstrate several qualitative as well as quantitative differences in *O*-glycosidically peptide-linked oligomannosyl residues of wild-type and mutant mannans. Although 85% (by weight) of wild-type mannan was resistant to alkali treatment, only 27 and 40% of mannans from 4918-2 and 4918-10, respectively, were resistant to this treatment. The increased susceptibility of mutant mannans to alkali hydrolysis may be the result of an increase in number as well as size of mannose side chains attached to serine and threonine residues in the peptide backbone, although other explanations are also possible.

Acetolysis of wild-type and mutant mannans yielded complex acetolysis patterns that were very similar to one another. The released products included mannose as well as mannooligosaccharides (from di- to hexasaccharide), as was reported earlier (20, 21). The most dramatic difference observed was in the amount of hexasaccharide released from the three mannans. The content of this fraction was 25% (by weight) for wild-type mannan, compared with 40% for mutant mannan. However, a peak at 5.34 min comprising 15% (by weight) of mannan and corresponding to an acetolysis-stable product was observed only for wild-type mannan. The other acetolysis-stable product eluted at 6.15 min and comprised 10 to 12% (by weight) of mannan in all three cases.

In summary, the chemical properties of mannans isolated from wild-type and cerulenin-resistant mutants of *C. albicans* suggest that differences may reside not only in mannan side chain and backbone structure but in peptide structure as well. Since mannans are thought to function as surface adhesins, it is possible that these differences in mannan structure are, at least in part, responsible for the reduced adherence as well as relative avirulence of these strains. Further structural characterization of mannooligosaccharide side chains and the peptide backbone are now in progress to identify determinants which may be involved in these processes.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant DE07168 from the National Institutes of Health. R.L.C. is the recipient of Public Health Service research career development award DE00189 from the National Institutes of Health.

The technical assistance of Gail Feser and Cynthia Hale is greatly appreciated. We thank Richard Calderone and W. Van for their

suggestions and criticisms and Adriana Cabrales for preparation of the manuscript.

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