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Comparison of *Coccidioides immitis* Arthrospore, Mycelium, and Spherule Cell Walls, and Influence of Growth Medium on Mycelial Cell Wall Composition

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Comparative lipid content, cell wall yield, neutral monosaccharide, glucosamine, and protein (amino acid) contents of arthrospores, mycelia, and spherules of *Coccidioides immitis* Cash were studied. Cellular lipid contents were found in the decreasing order: spherules, arthrospores, mycelia. Lipid content of mycelia did not reach the level of arthrospores or spherules even when mycelia were grown on relatively rich media. Cell wall yields of spherules were lower than for mycelia when grown on comparable media. Cell walls of arthrospores, mycelia, spherules, and spherule culture filtrate all contained 3-*O*-methylmannose, mannose, and glucose, but in varying amounts. Cell wall yield and cell wall glucose content increased in mycelia grown in increasingly rich media, whereas mannose content either decreased or remained constant.

Little is known of the comparative compositions of cells and cell walls of spherules, mycelia, and arthrospores of *Coccidioides immitis*. Most analyses reported pertain to the carbohydrate and nitrogen content of coccidioidin preparations, whether from mycelia, mycelial culture filtrates (i.e., coccidioidin) contain autolysates (13). Mycelial autolysates and culture filtrates (i,e., coccidioidin) contain ethanol-precipitable polymers composed predominantly of mannose, glucose, 3-*O*-methylmannose, and protein (2, 11, 15–18), but the presence of galacturonic acid (11, 15) was not confirmed (2, 16, 17). Spherule autolysates of a variety of strains were reported to contain carbohydrate as mannose, protein, and a high content of nonprotein nitrogen (e.g., 13). The presence of chitin in arthrospore, mycelia, and spherule cell walls has been reported (e.g., references 4, 28), although, as pointed out (28), both heat-volatile nitrogen components and alkali-soluble polysaccharide and alkali-insoluble glycans also occur (e.g., 24, 27, 28). An excellent in-depth comparison of lipid composition of arthrospores and mycelia of wild-type strain RS and an avirulent auxotrophic mutant strain derived from it has also been reported (1).

The present work was initiated for two primary reasons. First, we offer comparative composition data for cell walls of arthrospores, mycelia, and spherules of *Coccidioides immitis* Cash, as base line data for comparable studies on other strains. Second, our curiosity was aroused by reports of thickened cell walls of *C. immitis* cells grown in tissues compared with cells grown in vitro (e.g., references 5, 8, 22). This raised the possibility that nutritional conditions in vitro may not reflect conditions *C. immitis* meets in vivo, which may also affect virulence or pathogenesis, as has been suggested by Smith (e.g., reference 20). Therefore, because *C. immitis* has no fastidious growth requirements (e.g., reference 10), we wished to compare the cell wall compositions of spherules and mycelia grown on simple, monosaccharide-salts media, and in addition, we wished to determine whether the composition of in vitro-grown mycelial cells remained constant or changed in response to increasingly rich media, including mannitol-salts, glucose-yeast extract, and brain heart infusion media.

MATERIALS AND METHODS

Organism. *C immitis* Cash M11 was obtained from Edwin P. Lowe, Fort Detrick, Frederick, Md.

Production and harvest of aerial arthrospores, cultured on Sabouraud glucose agar, and of spherules, grown on the Tamol-N-glucose-ammonium acetate-salts liquid medium of Converse, were as previously described (6, 7, 28). Mycelial phase was grown on 2% mannitol-Converse medium without Tamol-N (12), on 2% glucose plus dialysate of 5% yeast extract (16, 19), or on a dialysate of 5% brain heart infusion. Incubation was for 2 to 5 days at 34°C, and cultures were killed with either 0.01%

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merthiolate or by suspension in 80% acetone and checked for viability by dilution (>1:1,000) into fresh medium or by plating on Sabouraud glucose agar and incubation at 34°C for 1 week before use. Culture filtrate and cells were separated by filtration and lyophilized separately.

Defatting. Difficulty experienced in preparing evenly distributed aqueous suspensions of arthrospores was alleviated after partial defatting by acetone extraction. For uniformity, all cell preparations were therefore treated as follows. The cell mass was dehydrated by suspension in acetone in a Waring blender (controlled by a Variac) at gradually increasing speed, and the resulting homogenate was poured into excess of acetone with stirring. The mixture was filtered on a Büchner funnel and washed with dithyl ether, and the cell cake was air dried. A suspension of the acetone-ether-dried material was then further sequentially defatted with 20 volumes each of methanol, chloroform-methanol (2:1), acetone, and diethyl ether. The lipid extracts were combined and filtered, solvents were removed under reduced pressure, and the residue was weighed, resuspended in a known volume of chloroform-methanol (3:1), and stored under dry nitrogen at 5°C.

Cell wall preparations. Defatted cells were broken by shaking at 4,000 rpm for 1 to 6 min at 0 to 10°C in a Braun MSK cell fractionator by mixing 12 g of 0.25- to 0.5-mm glass (or polystyrene) Ballotini beads per g of cell materials, suspended in 20 ml of water. Cell suspensions and washings decanted from Ballotini beads were further separated from Ballotini beads by centrifugation. Mycelia and spherules were optionally broken in a refrigerated Ribi-Sorvall cell fractionator at pressures up to 50,000 lb/in^2. Optimal cell disruption and cell wall separation procedures were determined by monitoring fractions by phase microscopy. Cell walls were recovered by differential centrifugation. Arthrospore walls were recovered at 6,000 × *g* for 30 min at 0 to 5°C, mycelial walls were recovered at 10,000 × *g* for 20 min at 0 to 5°C, and spherule walls were collected at 1,000 × *g* for 20 min. Endospores were separated from spherule cytoplasmic solubles by centrifugation at 27,000 × *g*. Cell fractions were then lyophilized.

The arthrospore preparation appeared, by light microscopy, to contain a minimum of free mycelium. However, since conversion of mycelium to arthrospores usually involves alternate cells, arthrospore preparations of *C. immitis* necessarily contain some hyphal material. The arthrospore cell wall preparation was fragmented to the point that only occasionally could nearly complete cell wall fragments be recognized by light microscopy, and no morphological differentiation between hyphal and arthrospore walls was possible. The mycelial preparations were composed of uniform hyphal strands, and the cell wall preparations made from them were fragmented to the point that little recognizable morphology remained. The spherule preparation, which was filtered through cheesecloth to remove hyphal strands, contained approximately 98% spherules. Breakage of spherules in the Ribi-Sorvall refrigerated cell fractionator allowed controlled breakage, which resulted in the release of apparently intact endospores and recognizable spherule wall fragments, which were easily separated by differential centrifugation.

Cell wall fractionation. One gram of dry cell walls was extracted twice for 30 min each in 250 ml of 2% aqueous sodium dodecyl sulfate (SDS) at room temperature and recovered by centrifugation at 10,000 × *g* for 30 min. The pellet was washed four times with distilled water and lyophilized. SDS wall preparations, 50 mg/ml, were treated with 0.05% Pronase (B grade, Calbiochem) in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.8, under toluene, at 37°C for 24 h. Cell wall pellets were recovered by centrifugation at 10,000 × *g* for 30 min at 0 to 5°C. After washing, the pellets were lyophilized, and portions were extracted with 44% aqueous phenol by the procedure of Westphal and Jann (25). Phenol-insoluble components were extensively washed with water to remove phenol before lyophilization.

Analyses. (i) Saponification of lipids. Samples, 1.0 mg/0.5 ml of 0.5 M methanolic potassium hydroxide, were heated at 80°C for 4 h in Teflon-lined screw-cap vials and cooled, 1 to 2 ml of water was added, and the mixture was extracted five times with 2 ml of petroleum ether to remove steroids. The aqueous phase was acidified with H_2SO_4 to pH 3 to 5 and again extracted with petroleum ether to obtain fatty acids.

(ii) Fatty acid esterification. Petroleum ether solutions of fatty acids were dried under a stream of nitrogen, the residue was weighed, and the fatty acids were converted to methyl esters by addition of diazomethane in diethylether. The ether was evaporated under a stream of nitrogen to remove excess diazomethane, and the methyl esters were dissolved in 0.5 ml of hexane. Portions were brominated to detect unsaturated fatty acids.

Reference standards were obtained from Applied Science Laboratories, College Park, Pa.

(iii) Polysaccharide hydrolyses. For thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) of neutral sugars, samples (10 mg/2 ml) were heated in 1 N H_2SO_4 at 100°C for 4 to 24 h in evacuated sealed tubes. Hydrolysates were neutralized with Dowex-1-bicarbonate, filtered, dried, and redissolved at the concentration desired. For release of neutral sugars, samples were hydrolyzed in 88% formic acid for 4 h, followed by hydrolysis in 1 N HCl for 1 h at 100°C and repeated pervaporation under reduced pressure to remove acids. To effect uronic acid release, samples were treated with 72% sulfuric acid in unsealed tubes for 1 to 4 h at room temperature, diluted with water to 1 N H_2SO_4, and heated in sealed, evacuated tubes for 10 h at 100°C. The hydrolysates were neutralized with $BaCO_3$, residues were removed, and the supernatant solution and washings were pervaporated; then the residues were redissolved in a minimum of water for TLC and high-voltage electrophoresis. Because of insolubility of barium uronides, $Ca(OH)_2$ was used for hydrolysate neutralization if uronic acids were to be isolated.

(iv) Amino sugars and amino acids. Samples, 10 mg/ml, were treated with 6 N HCl for 24 h in evacuated sealed tubes at 100°C to release amino sugars

and amino acids. HCl was removed by repeated pervaporation, and the residue was dissolved in water at the desired concentration.

(v) **TLC of lipids and fatty acids.** TLC was carried out on Silica Gel G, 0.25 mm, activated at 110°C for 1 h. Solvents used were (i) petroleum ether-diethyl ether-acetic acid (90:10:1), used for polar compounds; (ii) petroleum ether-diethyl ether-acetic acid (30:70:1), used for separation of polar compounds such as monoglycerides, glyceryl ethers, and phospholipids; and (iii) water-saturated *n*-butanol for separation of acylhydroxamates. Spots were detected by spraying with 2,7-dichlorofluorescein (0.2% in ethanol), (3 g of ammonium molybdate in 50 ml of water, 5 ml of 6 N HCl, and 13 ml of 70% $HClO_4$) or by iodine vapor. Standards used included palmitic acids, cholesterol, ergosterol, DL-α-lecithin, glycerol dipalmitate, lecithin, lysolecithin, phosphatidyl-L-serine, and sphingomyelin, obtained from Applied Science Laboratories, College Park, Pa.

(vi) **TLC of sugars, uronic acids, and amino acids.** Microcrystalline cellulose or Machery and Nagel MN 300 cellulose (Brinkman and Co., Westbury, N.Y.) was used. Solvents used were (i) *n*-butanol–glacial acetic acid–water (5:1:2, vol/vol/vol), and (ii) *n*-butanol–pyridine–water (6:4:3). Ninhydrin was used for detection of amino acids and amino sugars; alkaline silver nitrate was used for detection of reducing compounds; and *p*-anisidine hydrochloride and aniline phthalate were used to detect and differentiate various reducing sugars. *Ortho*-aminobiphenyl was used for the specific detection of pentoses and uronic acids (23).

(vii) **GLC.** An F & M 400 chromatograph (Hewlett-Packard) was used. Fatty acids were chromatographed on an ethylene-glycol-succinate/Chromosorb-P column at 178°C; He flow rate was 70 ml/min. Steroids were separated on a 3% QF-1/Chrom-Q column at 230°C; He flow rate was 20 ml/min. Sugars were separated on a 12-foot (ca. 366-cm) column of 3% ECNSS-M on Gas-Chrom Q at 190 to 210°C; He flow rate was 70 ml/min, as alditol acetates, according to the procedure of Sawardeker et al. (19), as described previously (2, 18).

(viii) **Analyses.** Amino sugars and amino acids were identified on an amino acid analyzer modified to detect reducing and ninhydrin reactive groups simultaneously as previously described (21). Neutral sugars were determined by GLC, using added pentoses, i.e., ribose or xylose as internal standards. Glucose was also measured independently in hydrolysates by use of glucose oxidase (Glucostat, Worthington Biochemicals Corp., Freehold, N.J.). Ester and amide-bound acyl groups were converted to hydroxamates and detected by TLC by spraying with 3% $FeCl_3$ in water-saturated *n*-butanol, using ethyl esters of formic, acetic, propionic, and butyric acids as reference standards (9). Uronic acids were quantified colorimetrically by reaction with carbazole (3).

RESULTS

(i) Lipid composition. Differences in lipid content and composition were observed between arthrospore, mycelia, and spherules, the smallest amount of lipid being found in mycelia (Table 1). Mycelial lipid levels did not reach the levels of arthrospores or spherules even when mycelia were grown in the two richer media. Fatty acid content, used as an index to reflect the total of free fatty acids, glycerides, phospholipids, and glycolipids (Table 1), also indicates a range of variability. However, as shown in Table 2, only quantitative differences in fatty acid composition are apparent. These results essentially corroborate the much more extensive fatty acid analyses recently reported by Anderes et al. (1).

An unusual steroid was observed in the nonsaponifiable fraction of arthrospores and mycelia, but not in that of spherules. This material chromatographed on TLC Silica Gel G plates similarly to cholesterol. Also seen on chromatograms were several phospholipids, neutral fats, and fatty acids. Some 10 compounds were separable, of which most (about 50%) could be identified as glycerides by mobility on TLC and staining and by analysis after elution from preparative TLC. Free fatty acids accounted for some 15 to 25% of total lipid. However, because cells were killed by long-term incubation in 80% acetone or in 0.01% merthiolate, it is possible that degradation of lipids occurred, releasing free fatty acids. Therefore, no attempt was made to completely identify all compounds or to determine percentages of the different components. However, a comparison by TLC of lipid content of mycelia relative to growth medium indicated a variation in quantitative lipid composition.

The steroid or cholesterol-like material in the non-saponifiable alkaline petroleum ether extracts was examined further. The compound yielded a positive Lieberman-Burchard reaction for steroids. When isolated from crude lipid extracts from arthrospores or mycelia, the compound appeared to be almost homogeneous on thin-layer silica gel plates stained with iodine. Saponification caused no change in mobilities, indicating it to be a free, nonesterified component. By preparative TL-silica gel chromatog-

TABLE 1. *Lipid and fatty acid content of Coccidioides immitis*

Growth medium	Total free lipid (% dry wt of cells)	Fatty acids (% total lipid)
Whole arthrospores (Sabouraud dextrose agar)	16.2	58.0
Whole spherules (glucose-Converse)	23.0	21.5
Whole mycelium		
Mannitol-Converse	10.8	59.0
Glucose-yeast extract	9.1	68.0
Brain heart infusion	14.6	74.0

TABLE 2. *Tentative identification and major fatty acid esters obtained from various Coccidioides immitis growth forms*

Fatty acid ester	Lipid source[a] (wt % of total fatty acid esters)				
	A	B	C	D	E
Myristate	0.3	0.5	0.3	0.4	0.2
Uk. (sat.)[b]	0.5	0.5	0.4	0.7	0.4
Palmitate	14.1	19.1	15.3	15.5	18.5
Palmitoleate (?)	0.6	0.5	0.8	0.6	1.3
Uk. (sat.)	1.5	1.8	2.2	1.2	1.9
Uk. (unsat.)	0.6	1.3	1.5	0.6	0.8
Stearate	16.2	9.4	7.2	8.5	5.5
Oleate	56.8	16.8	39.7	37.4	26.9
Linoleate	8.1	50.0	32.6	32.5	44.5
Uk.	0.7	0.0	Trace	1.8	Trace
Uk.	0.5	0.0	Trace	0.7	Trace

[a] Lipid sources: A, whole arthrospores; B, whole spherules (glucose-Converse); C, whole mycelium (mannitol-Converse); D, whole mycelium (glucose-yeast extract); E, whole mycelium (brain heart infusion).

[b] UK., Unknown; sat., saturated; unsat., unsaturated.

raphy, 3 mg of the steroid was isolated. The isolated material was examined by infrared spectroscopy and found to give absorption for R-OH but not for carbonyl group function. When chromatographed on GLC, the compound had a longer retention time than did ketosteroids, cholesterol, and ergosterol. The same compound was observed in both arthrospore and mycelial extracts. By use of a GLC stream-splitter, we obtained a sample of some 50 μg of pure material, which was analyzed by mass spectrometry in an MS-9 instrument (AEI Ltd., Manchester, Great Britain). The compound volatilized at 130°C, resulting in a peak mass of 394.3226 for the parent compound. This corresponds to a theoretical mass of 394.3260 for $C_{28}H_{40}O$. No Cl, Br, S, or N peaks were observed, but peaks of mass 15 (—OH group) and mass 18 ($—CH_3$) were observed. These data indicate an unusual steroid of four double bonds and a single hydroxyl group. It is probable that two of the double bonds resulted from degradative elimination either during GLC or mass spectrometry.

Analysis of cell walls. The yields of cell walls and cell wall fractions are given in Table 3. Comparison of lipid yield from whole cells (Table 1) agrees well reciprocally with defatted cell weight recovery shown in Table 3. The most obvious difference between the three forms of *C. immitis* is the small yield of cytoplasm and large yield of cell wall from mycelium as compared with spherules and arthrospores. Perhaps surprising is the large amount of material extracted by detergent (SDS) from all cell walls.

Amino acid and amino sugar composition of cell walls. Cell wall preparations (Table 5) were assayed for amine components after SDS treatment and Pronase digestion. As shown in Tables 4 and 5, protein concentration, based on amino acid content per milligram, decreased significantly in contrast to a relative increase of glucosamine content per milligram, even though total glucosamine also fell 30, 50, and 56%, respectively, in arthrospores, mycelia, and spherule walls, upon treatment with Pronase. Some protein in the cell walls of all growth phases was apparently protected from Pronase digestion, although spherules appeared to contain less of this type of protein than did arthrospores or mycelium.

Carbohydrate composition. Cell walls and spherule culture filtrates were found to have the same sugar components as whole cells and whole defatted cells (e.g., Fig. 1). The same sugars were previously reported in mycelial culture filtrates (e.g., references 2, 16–18). Approximate relative quantities of neutral sugars in the cell wall fractions were determined as alditol acetates by GLC (Table 6). The highest concentration of 3-methoxymannose occurred in spherule and arthrospore cell walls as compared with mycelial cell walls. The two major monosaccharide components, glucose and mannose, also varied in the different growth forms,

TABLE 3. *Coccidioides immitis cell wall yields from whole cells*

Cell fraction	Lipid source[a] (% dry wt)				
	A	B	C	D	E
Defatted cells	81	73	86	88	82
Walls	27	26	71		
Cytoplasm	45	33	7	9	3
Cell walls after SDS	19	18	53		
Walls after SDS and Pronase	12	12	24	40	50

[a] Lipid sources: A, arthrospores; B, spherules (glucose-Converse); C, mycelium (mannitol-Converse); D, mycelium (glucose-yeast extract); E, mycelium (brain heart infusion).

TABLE 4. *Effect of Pronase digestion on amino acid and glucosamine content of Coccidioides immitis cell walls*[a]

Source of cell wall material	Effect of Pronase (% dry wt) on:			
	Amino acids		Glucosamine	
	Before	After	Before	After
Arthrospore	37	28	17	20
Mycelium[b]	60	18	46	33
Spherule	52	9	35	28

[a] Analyses done on an amino acid analyzer (21).

[b] Grown in mannitol-Converse medium.

TABLE 5. *Coccidioides immitis: comparative glucosamine and amino acid content of detergent-extracted, Pronase-digested cell walls*

Compound	μmol/μmol of aspartic acid[a]		
	Spherules	Mycelia	Arthrospores
Aspartic acid[a]	1.0	1.0	1.0
Threonine	0.8	1.1	0.6
Serine	0.8	0.1	0.6
Glutamic acid	1.0	1.0	0.8
Proline	1.0	1.1	0.1
Glycine	1.2	1.1	1.2
Alanine	1.0	0.8	0.8
Glucosamine	21.0	12.5	4.4
Valine	0.5	0.6	0.9
Cystine	0.2	0.9	0.0
Methionine	0.2	0.0	0.0
Isoleucine	0.5	0.5	1.0
Leucine	0.8	0.7	1.5
Tyrosine	0.2	0.7	0.0
Phenylalanine	0.7	0.4	1.0
Ammonia	7.2	3.6	3.0
Lysine	0.8	0.3	0.3
Histidine	0.3	0.2	0.3
Arginine	0.7	0.4	0.3

[a] Aspartic acid (micromoles per milligram): spherules, 0.06; mycelium, 0.12; arthrospores, 0.21. Glucosamine (micromoles per milligram): spherules, 1.27; mycelium, 1.51; arthrospores, 0.92.

and apparently diminished levels of 3-*O*-methylmannose occurred in mycelia cell walls grown in brain heart infusion. The following components appear to be common to all three growth phases, although in differing concentrations, as indicated above, and were identified or characterized from mycelia as follows.

(i) Glucosamine. This was obtained by hydrolysis in 4 N HCl for 20 h, isolated by cation exchange chromatography, and crystallized as glucosamine-hydrochloride. It was characterized as glucosamine, $[\alpha]_D^{+72}$, by ninhydrin degradation to arabinose, and by mixed-melting-point analysis with an authentic carbobenzoxy-glucosamine derivative at 208 to 213°C.

(ii) *N*-acetylglucosamine. This was obtained by chitinase digestion. Identification was done by R_f values on paper chromatograms.

(iii) Glucose. Glucose was identified by R_f values on paper chromatography, TLC, and GLC as the alditol acetate and characterized and quantified as glucose by assay with glucose oxidase.

(iv) Mannose. Mannose was obtained by hydrolysis in 2 N HCl for 3 h at 100°C. It was identified by TLC and GLC and characterized after isolation by paper chromatography by preparing the phenylhydrazone derivative (mixed melting point, 201°C). The infrared spectrum of the phenylhydrazone derivative also was identical to that of authentic sample. Mannose was also identified by mobility and differential staining on paper chromatograms using alkaline silver and *p*-anisidine staining.

(v) 3-*O*-methylmannose. This compound was identified by thin-layer cellulose chromatography and by GLC of the alditol acetate, using chemically synthesized reference compound as previously reported (18).

(vi) Uronic acid. This could not be isolated, although the colorimetric carbazole assay indicated 3, 5.8, and 13.3% uronic acid in mycelial cell wall, arthrospore cell wall, and spherule cell wall, respectively, using galacturonic acid as a standard. By the use of several different hydrolytic procedures and subsequent ionophoresis, chromatography, and specific staining re-

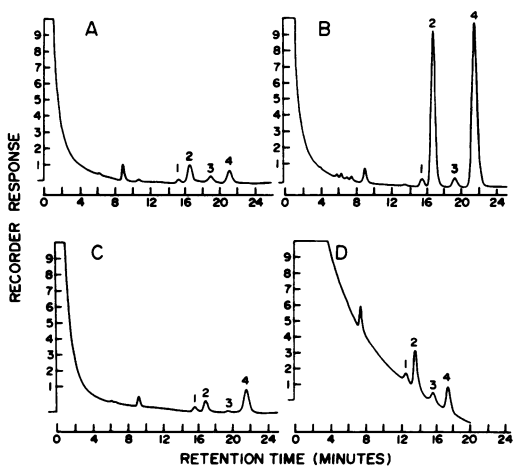

FIG. 1. *GLC of neutral sugar alditol acetate derivatives prepared from hydrolysates of Coccidioides immitis cell walls: peak 1,3-O-methylmannose; peak 2, mannose; peak 3, galactose; peak 4, glucose. (A) Arthrospores, (B) mycelia (mannitol-salts), (C) spherules, (D) dialyzed spherule culture filtrate.*

TABLE 6. *Comparison of relative neutral sugar composition of Coccidioides immitis mycelial, arthrospore, and spherule cell walls*

Sugar	% of total					
	A[a]	B	C	D	E	F
3-Methoxy-mannose	7	8	10	2	5	Trace
Mannose	42	27	46	42	35	35
Galactose	17	Trace	12	3	3	3
Glucose	35	66	33	55	58	62

[a] Cell wall preparations from: A, arthrospore cell walls; B, spherule cell walls (glucose-Converse); C, spherule culture supernatant solution; D, mycelium cell walls (mannitol-Converse); E, mycelium cell walls (glucose-yeast extract); F, mycelium cell walls (brain heart infusion).

actions, we could not isolate uronic acid. On the other hand, uronic acid could easily be isolated by these procedures from the bacterial lipopolysaccharides of *Proteus mirabilis* and *Chromobacterium violaceum* in which uronic acid was assayed colorimetrically at only 1 to 6%.

(vii) Acetyl. Acetyl groups were detected as the hydroxamate derivative on TLC plates in all three growth forms, i.e., arthrospores, mycelia, and spherules. Formyl and propionyl groups were not detected. The acetyl groups presumably are present as amide acyl groups in *N*-acetylglucosamine, but could also be present as *O*-acetyl groups.

DISCUSSION

After completion of the present work, a much more detailed report appeared in which lipids of arthrospores and mycelia of a mouse-virulent wild-type strain, RS, and an auxotrophic mutant, strain RS-95, of *C. immitis* were compared (1). Our results are reported to support the lipid levels and major fatty acid components identified in lipids of arthrospores and mycelia by Anderes et al., including a previously unidentified sterol. The report by Anderes et al. should be consulted for details of composition (1). In addition to their observations, we observed that this steroid can be separated and differentiated from ergosterol, cholesterol, and 17-keto ketosteroids and has an hydroxyl function. We did not find this compound in the spherule lipid preparation we examined, but this point needs verification.

Our choice of media for the comparison of mycelial cell wall compositions grown in three media of increasing complexity was based in part on the following three observations. First, Levine (12) reported that conversion of mycelium to arthrospores or spherules was suppressed when mannitol was substituted for glucose in the chemically defined Converse liquid medium of glucose, ammonium acetate, and salts used for spherule growth. Use of Converse medium with either glucose or mannitol, therefore, offered the opportunity to compare these growth forms when grown in chemically defined media. The second mycelial growth medium, glucose-yeast extract, was chosen because Levine also indicated (12) that, in this medium, mycelial growth was enhanced some 10-fold over that of spherules. This is also the same medium that has been used for the production of the mycelial autolysate coccidioidin (16, 17). And finally, brain heart infusion was chosen as a very nutritionally rich growth medium, which also would contain "host" components and one which also might contain the fatty acids that have been reported by Lones et al. (14) to cause reversion of spherules to mycelia, thus, hopefully, ensuring no conversion of mycelia to spherules.

In comparing arthrospore, spherule, and mycelial walls, it is of interest to note that mycelial walls make up more of the SDS-insoluble, protease-resistant fraction than do arthrospores or spherules. Even so, arthrospore walls retained 1.5 times more protein than mycelial walls and 3 times more protein than spherule walls. Mycelial and spherule walls, on the other hand, retained more glucosamine than arthrospore walls. Together, protein and *N*-acetylglucosamine comprised 40 to 50% of the isolated cell walls, and polysaccharides comprised the remainder. Specific differences in amino acid composition were observed. Arthrospore walls contained no sulfur amino acids, no tyrosine, and almost no proline, whereas mycelial walls contained cystine, no methionine, and little serine. Both mycelial and spherule walls contained higher levels of proline than did arthrospore walls. However, no amino acid appeared in disproportionate amounts. Because some glucosamine was lost during Pronase digestion of all three growth phases, it is possible that glucosamine is present in a form other than chitin, or, alternatively, in a chitin-peptide cross-linked form, which becomes soluble when a portion or all of the peptide is digested by protease. Glucosamine, glucose, and mannose appear to be the major monosaccharide components of *C. immitis* cell walls, whereas 3-*O*-methylmannose appears to be a minor constituent.

In comparing the effect of growth media on the variation of mycelial composition, it is of interest to note that the major variation appears to be in an increase in the insoluble glycan portion, which increases with richer media and which can be accounted for as an increase in glucose. Also of interest were the indications that a higher relative mannose concentration occurred in mannitol-grown cells, whereas the relative mannose content appeared to decrease reciprocally with glucose increase in mycelia grown in richer media. These results indicate that host components may also affect the cell wall composition of *C. immitis* when grown in vivo.

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