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Production of a Capsular Polysaccharide by a Marine Filamentous Fungus

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The spent seawater medium of 4-day-old-cultures of the filamentous marine fungus *Leptosphaeria albopunctata* had a high viscosity after the fungus was collected by high-speed centrifugation. Microscopic examination of uncentrifuged mycelium suspended in India ink revealed that the viscosity resulted from capsular material. These capsules became disassociated from the mycelium during centrifugation. Precipitation of the medium of centrifuged cultures with 95% ethyl alcohol yielded a highly anthrone-positive polysaccharide material, composed of large amounts of glucose and minute amounts of mannose. Time course studies of the nutritional requirements for capsular polysaccharide production revealed that the capsular material was produced in large amounts, and on a wide variety of sugars, during the period of rapid growth, but was quickly degraded and presumably remetabolized in older cultures. The amount of capsular material produced was enhanced by NaCl concentrations above that of artificial seawater, and KCl could be substituted for NaCl. The salts $MgCl_2$ and $CaCl_2$ were also required for capsule production by *L. albopunctata*, although growth was obtained in cultures without added amounts of these constituents. The possible role of these salts in the metabolism of the fungus is discussed.

There are numerous reports concerning the production of extracellular polysaccharides by fungi (3, 11, 15). Reports describing the organization of extracellular polysaccharides into well-defined capsules are rare, restricted to the literature of yeasts such as *Hansenula*, *Cryptococcus*, *Rhodotorula*, and *Lipomyces* (5, 13). This report describes the formation of capsular polysaccharide by the marine ascomycete *Leptosphaeria albopunctata* (Westendorph) Saccardo. Some of the carbohydrate and salt requirements necessary for the production of the capsular polysaccharide by *L. albopunctata* are also described. To our knowledge, this is the first report describing capsular polysaccharide production by a marine filamentous fungus.

MATERIAL AND METHODS

Organism and cultural procedures. Cultures of *L. albopunctata* (Ko. 49 from *Spartina*, originally obtained from J. Kohlmeyer, University of North Carolina, Institute of Marine Sciences, Morehead City) were grown for inocula in 100-ml quantities of standard inoculum medium (SIM) consisting of 10.0 g of glucose, 1.0 g of yeast extract (BBL), and 1 liter of artificial seawater (9). The *p*H of the medium was adjusted to 7.5 before autoclaving. After 6 days of incubation at 26 to 28 C on a New Brunswick G50 gyrotory shaker rotating at 200 rev/min, the SIM cultures were blended at high speed for 1 min in a cold Waring Blendor microcup, and 1-ml quantities of the suspension were used to inoculate 100 ml-quantities of experimental media in 250-ml Erlenmeyer flasks. The experimental media were of similar composition to the SIM with components varied or omitted to determine the carbohydrate and salt requirements for capsular polysaccharide production. Duplicate experimental cultures, shaken and incubated as the inocula cultures, were examined daily by the following procedures over a 10- to 12-day period for polysaccharide production, which was used as an index of capsule formation. Yield of mycelium, *p*H changes, and, in some instances, glucose utilization were also determined from these cultures.

Experimental procedures. Each experimental culture was blended for 30 sec at high speed and was centrifuged at 18,000 × *g* for 10 min at 4 C to free most of the capsular polysaccharide from the vegetative mycelium. The supernatant fluid was decanted, the *p*H was measured, and the supernatant fluid volume was brought to 100 ml with distilled water. The mycelial pellet was washed by blending with

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100 ml of distilled water for 10 sec, and the resulting suspension was recentrifuged. The supernatant fluid was combined with the spent medium of the first centrifugation. Polysaccharide production was determined gravimetrically after precipitation with 95% alcohol (3:1, v/v), collection by filtration on tared membrane filter discs (0.45 μm), and drying to a constant weight at 103 C. A maximal correction factor accounting for the salts of artificial seawater which are precipitable with 95% ethyl alcohol was subtracted from all polysaccharide weights, except those obtained from cultures prepared with distilled water instead of artificial seawater. Addition of KCl to the supernatant fluids from cultures grown in distilled water medium did not increase the amount of precipitable polysaccharide. Growth yield was determined gravimetrically after the mycelium was rewashed and collected on tared Whatman no. 1 filter paper discs and dried to a constant weight at 103 C. Glucose utilization was determined by glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.) using broth samples obtained prior to the initial blending.

Preliminary chemical analysis. Capsular material to be subjected to preliminary chemical analysis was obtained in large amounts by culturing the fungus for 4 days at 21 C with agitation in 1,000-ml quantities of SIM in 2,500-ml low form flasks. After high-speed centrifugation of the cultures, the broth was combined with 95% ethyl alcohol (1:2, v/v) to precipitate the polymer. The polymer was removed from the alcohol solution, dissolved in distilled water, and centrifuged to clarify the solution. The solution was then exhaustively dialyzed against distilled water at 4 C to remove salts. After dialysis, a small amount of the material was tested for hexose with anthrone reagent (16), and the remainder was lyophilized. The monosaccharide composition of the polysaccharide was determined by use of the hydrolysis and chromatographic procedures for sugars outlined by Szaniszlo and Gooder (17), whereas nitrogen was determined by the method of Koch and McMeekin (8). Ash weights were determined on dialyzed and undialyzed samples. Glucose identification was verified with glucostat reagent and galactostat reagent (Worthington Biochemical Corp.). Optical rotation of the partially purified polymer was determined with a Perkin-Elmer 141 polarimeter.

RESULTS

Preliminary experiments conducted on the growth of a number of terrestrial and marine *Leptosphaeria* revealed that a high degree of viscosity was associated with the spent media of 4-day-old cultures of the marine fungus *L. albopunctata* after collection of the mycelium by centrifugation. Upon microscopic examination of uncentrifuged and centrifuged mycelium (Fig. 1, 2, 3), it was apparent that the viscosity of the medium previously noted resulted from capsular material which became disassociated from the mycelium after high-speed centrifugation. An investigation was thus initiated to determine some of the cultural requirements for capsular polysaccharide production and also to determine, in a preliminary fashion, the chemical nature of the polymer.

Preliminary chemical analysis. A single, highly anthrone-positive capsular product obtained from 4-day-old, nonblended SIM cultures was used in all the preliminary chemical analyses. The product was initially soluble in hot water after precipitation from the culture filtrates with 95% ethyl alcohol, but the extensively dialyzed and freeze-dried product was largely insoluble. The degree of solubility of the product decreased with the length of time of dialysis. It was gravimetrically determined that the initial product had about 25% ash content, whereas the dialyzed product had an ash content of 2.31%. The nitrogen content of the dialyzed product was determined to be 0.2%, and the $[\alpha]_D^{20}$ was determined to be greater than +200. The accuracy of the latter figure, however, might have been greatly influenced by the 2.3% ash, if the ash still represented dialyzable salts. Acid hydrolysis of the polymer yielded only large amounts of glucose and minute amounts of mannose, as determined by paper chromatography. Whether the mannose was an integral part of the capsular material has not yet been determined. The glucostat and galactostat tests confirmed that glucose was the sugar being detected in major amounts.

Capsule production in SIM. The course of a typical experiment with SIM is shown in Fig. 4a. The initial 10 g of glucose was utilized within 4 days with about 15% converted to capsular material and about 45% converted to mycelium. After the depletion of the glucose, the rate of mycelial production decreased as did capsular polysaccharide yield. Maximal mycelial yields were obtained after capsular polysaccharide yields decreased. This phenomenon strongly suggested that the capsular material was being degraded and reutilized by *L. albopunctata*. The *p*H immediately began to decrease during the period of rapid growth but quickly rose and stabilized at 6.5 to 6.8. This subsequent rise in *p*H correlated well with increasing capsule production. When 0.1 M Trisma Base (Sigma Chemical Co., St. Louis, Mo.) was added to the medium, the course (Fig. 4b) of growth and capsule production was depressed with respect to rate and maximal yield, even though the buffer at 0.1 M concentration did not have the capacity to stabilize the culture medium at the initial *p*H of 7.5. Since both experiments revealed that the capsular material was quickly degraded and presumably reutilized by the fungus, it was judged important that most of the subsequent experiments be

of a time course nature. All the subsequent series of time course experiments included cultures grown in SIM so that comparisons could be made accurately, since yields were dependent on inoculum size and various other cultural factors. Trisma Base was not used because higher concentrations would be necessary to maintain the initial *p*H and because Trisma Base addition

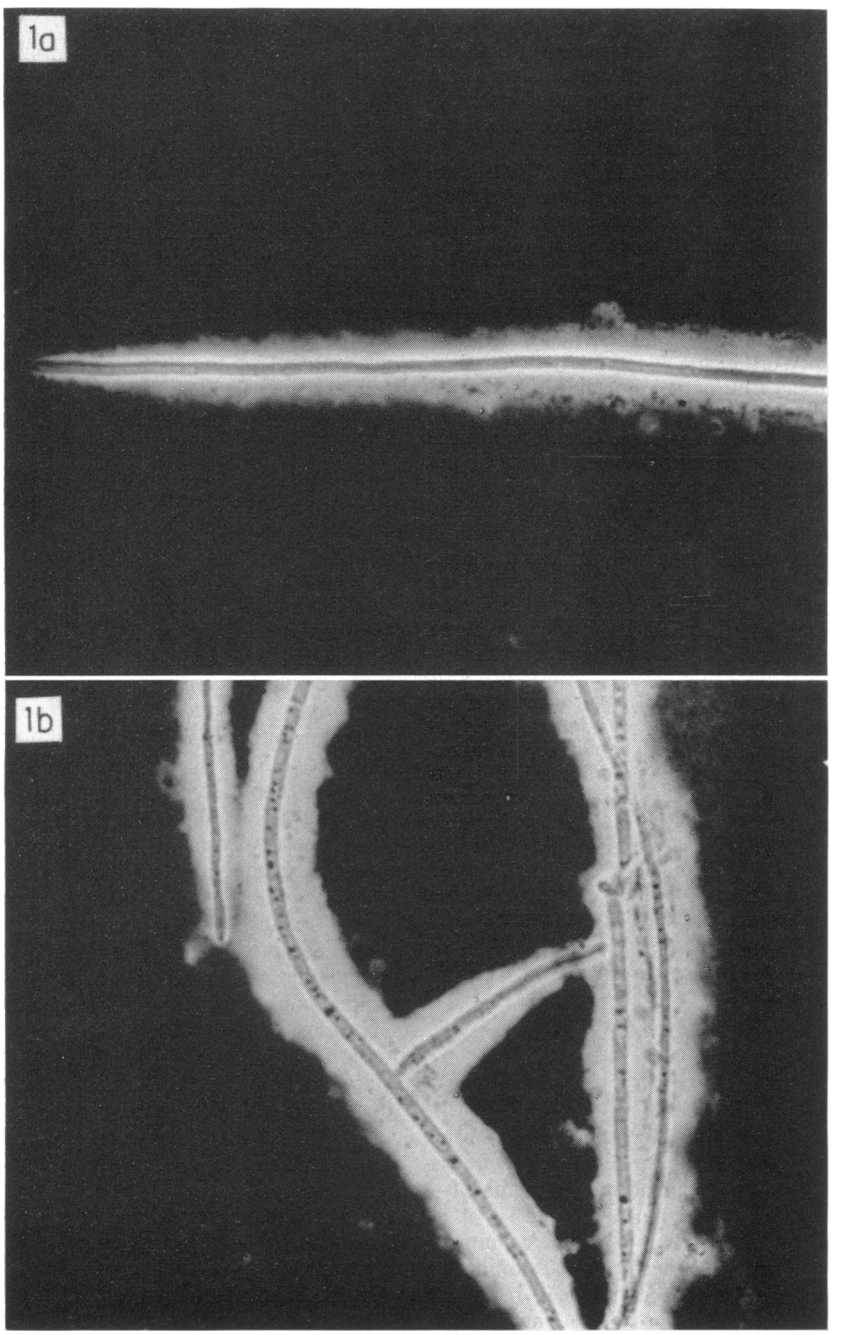

FIG. 1. *Encapsulated hyphae of L. albopunctata in India ink. (a) Hyphal tip under bright field. × 1,600. (b) Vegetative hyphae under phase contrast. × 1,500.*

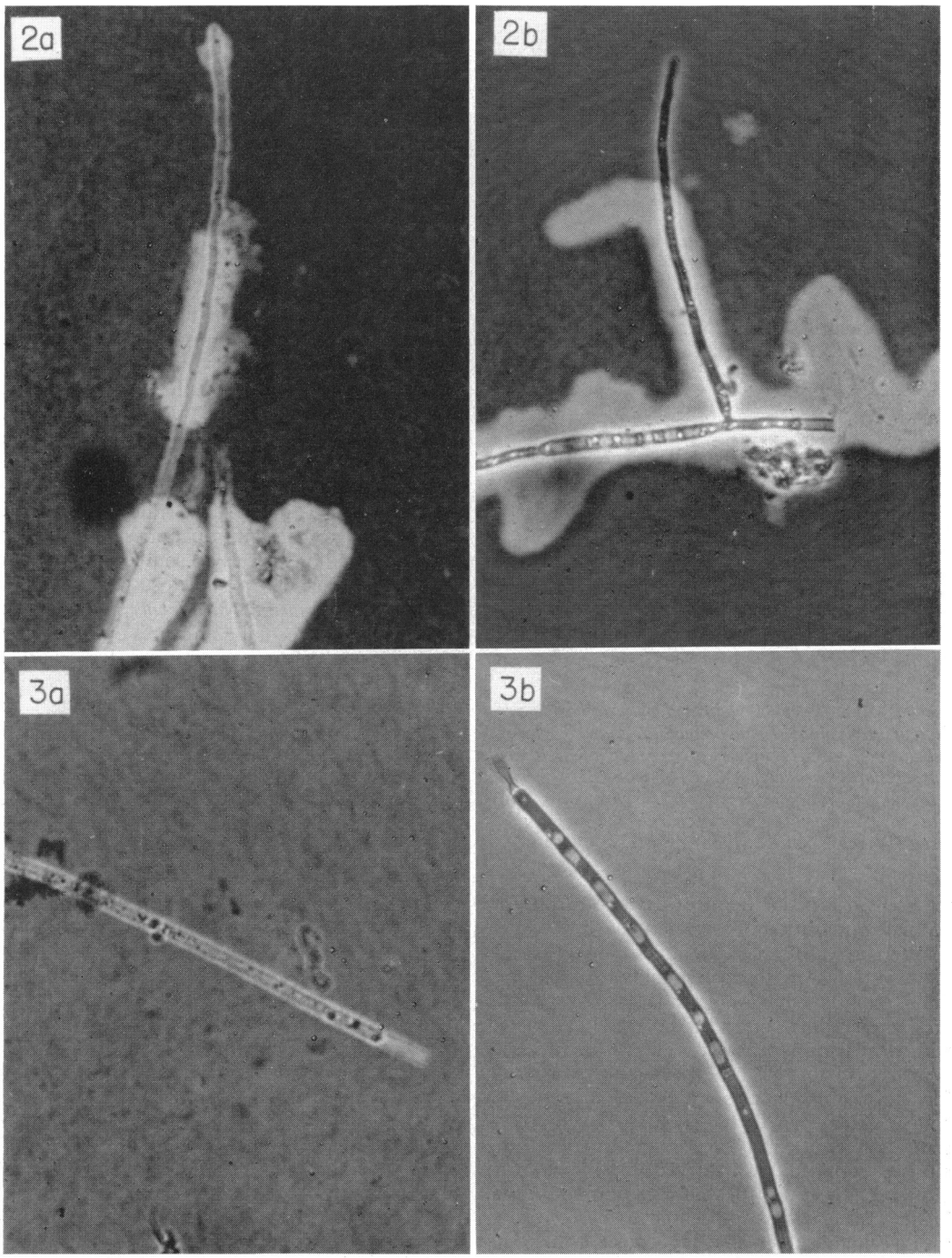

FIG. 2. *Hyphae of L. albopunctata in India ink after first blending and centrifugation showing partially dissociated capsules. (a) Bright field. × 1,600. (b) Phase contrast. × 1,500.*

FIG. 3. *Hyphae of L. albopunctata in India ink after second blending and centrifugation showing the absence of capsules. (a) Bright field. × 1,600. (b) Phase contrast. × 1,500.*

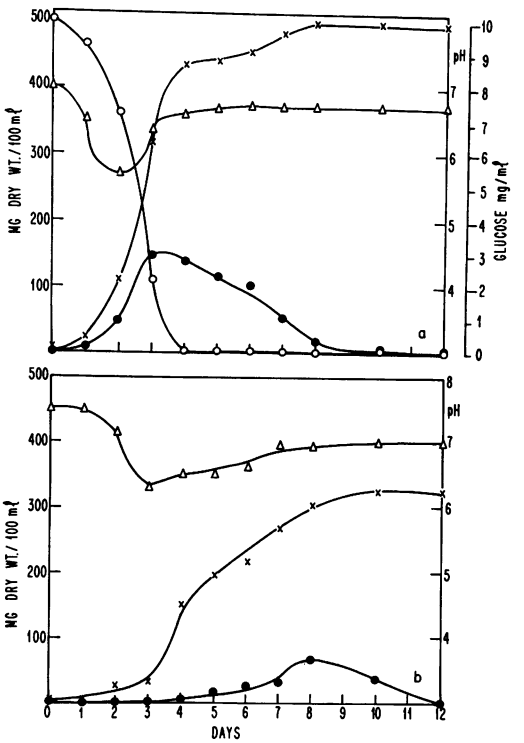

FIG. 4. *(a) Mycelial yield and polysaccharide production in SIM by L. albopunctata, showing glucose utilization and pH changes. (b) Mycelial yield and polysaccharide production in SIM with 0.1 M Trisma Base added, showing pH changes. Symbols: ×, mycelial dry weight; ●, polysaccharide dry weight; ○, residual glucose concentration; △, pH.*

represented a major point of deviation from the normal composition of seawater.

Effect of glucose concentration. Since the aim of this study was the determination of the medium requirements for capsular polysaccharide production, time course studies were conducted with increasing glucose concentrations (Fig. 5). With regard to *p*H and capsular polysaccharide formation, the courses are all similar to the course obtained with the standard glucose concentration (Fig. 5b). All or almost all of the glucose supplied was utilized by the fungus. However, most of the glucose utilized in the higher concentrations appeared to be converted to mycelium rather than to capsular material (Fig. 5c–f). In no instance in this series did the maximal percentage of glucose recovered as capsular polysaccharide exceed 10% of the total glucose initially available to the fungus. The most efficient conversion (8.7) of substrate to capsular product was obtained in SIM (Fig. 5b), and three- to fivefold increases in glucose concentration were necessary before an actual doubling of polysaccharide product occurred (Fig. 5d–f).

Effect of carbon source. In addition to glucose, the carbon sources galactose, mannose, arabinose, xylose, fructose, maltose, cellobiose, sucrose, rhamnose, lactose, alginic acid, agarose, soluble starch, and cellulose (presented as washed, shredded Whatman no. 1 filter paper) were tested as substrates for capsule formation. All the substrates, except cellulose, allowed some growth. Galactose, sucrose, mannose, xylose, fructose, maltose, cellobiose, and soluble starch allowed growth about equal to or approaching that obtained when glucose was supplied as the carbon source. Significant amounts of capsular material were produced by the fungus when it was cultured on all of the sugars that allowed good growth, except for galactose and soluble starch. Time course studies showed which carbon sources produced significant amounts of capsular material (Fig. 6). The fungus produced approximately the same quantities of capsular material on all of the carbon sources tested, with the possible exception of fructose (Fig. 6f).

Effect of increasing NaCl concentration. The effects of various osmotic pressures on the ability of the fungus to form capsular polysaccharide were tested by increasing or decreasing the standard concentration of NaCl (23.48 g/liter) in SIM. Increasing NaCl concentrations in SIM had a pronounced effect on capsular polysaccharide production (Fig. 7). Without NaCl, and with NaCl (11.74 g/liter), both growth and capsular material increased toward (but did not reach) the values obtained for the SIM concentration of NaCl (Fig. 7a–c). However, at NaCl concentrations of 46.96 g/liter (Fig. 7d), the rate of growth and maximal yield of mycelium decreased, whereas polymer weight increased. In SIM with 70.44 g of NaCl per liter (Fig. 7e), there was more capsular material being produced than mycelium by weight at the time of maximal polysaccharide production. The maximal amount of polysaccharide was produced in SIM with 93.92 g of NaCl per liter (Fig. 7f), with a maximum of about 30% of the initial glucose being converted to polysaccharide product. Mycelial production at this NaCl concentration and in SIM with 117.40 g of NaCl per liter (Fig. 7g) continued to be depressed with regard to both growth rate and final yield.

Effects of seawater constituents. The influence of the various salts in artificial seawater was studied because *L. albopunctata* grew in a glucose (10 g), yeast extract (1 g), distilled water (1 liter) medium (GYB), as well as in SIM from which the NaCl was omitted, but it did not produce significant amounts of capsular material.

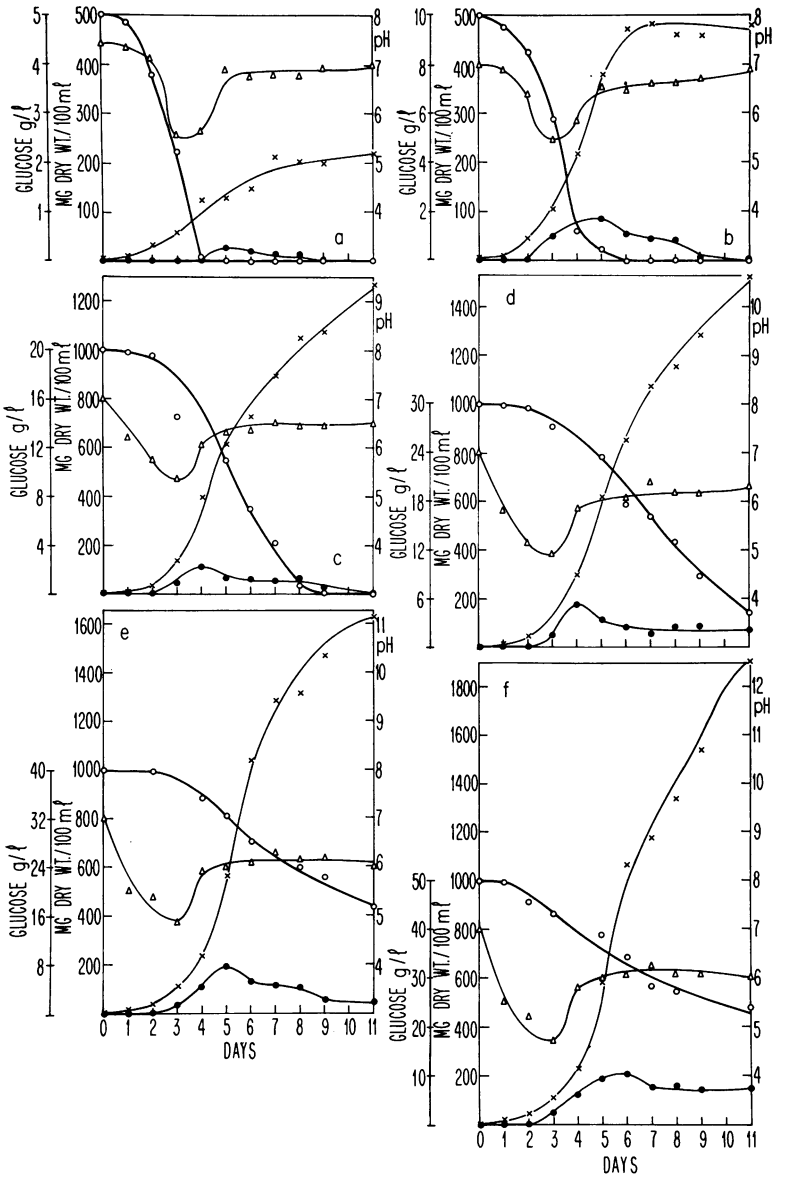

FIG. 5. *Effect of initial glucose concentration on mycelial yield and polysaccharide production by L. albopunctata in SIM. The tested glucose concentrations were (a) 0.5%, (b) 1.0%, (c) 2.0%, (d) 3.0%, (e) 4.0%, and (f) 5.0%. Symbols: ×, mycelial dry weight; ●, polysaccharide dry weight; >, residual glucose concentration; △, pH.*

Capsular material was produced in GYB in the presence of the chlorides of artificial seawater ($NaCl$, $MgCl_2$, $CaCl_2$, KCl, $SrCl_2$). The addition of any one of the other components of artificial seawater to this basal chloride medium did not greatly enhance growth or capsule production. The fungus was next cultured in GYB containing only one of the chlorides alone or one of the chlorides with NaCl. Only minute amounts of capsular material were produced when one of the chlorides was present in the medium. However, high yields of growth and capsular polysaccharide were observed when $MgCl_2$ or $CaCl_2$ was added to the medium in combination with NaCl. High yields of growth and capsular polysaccharide were also obtained when KCl was supplied

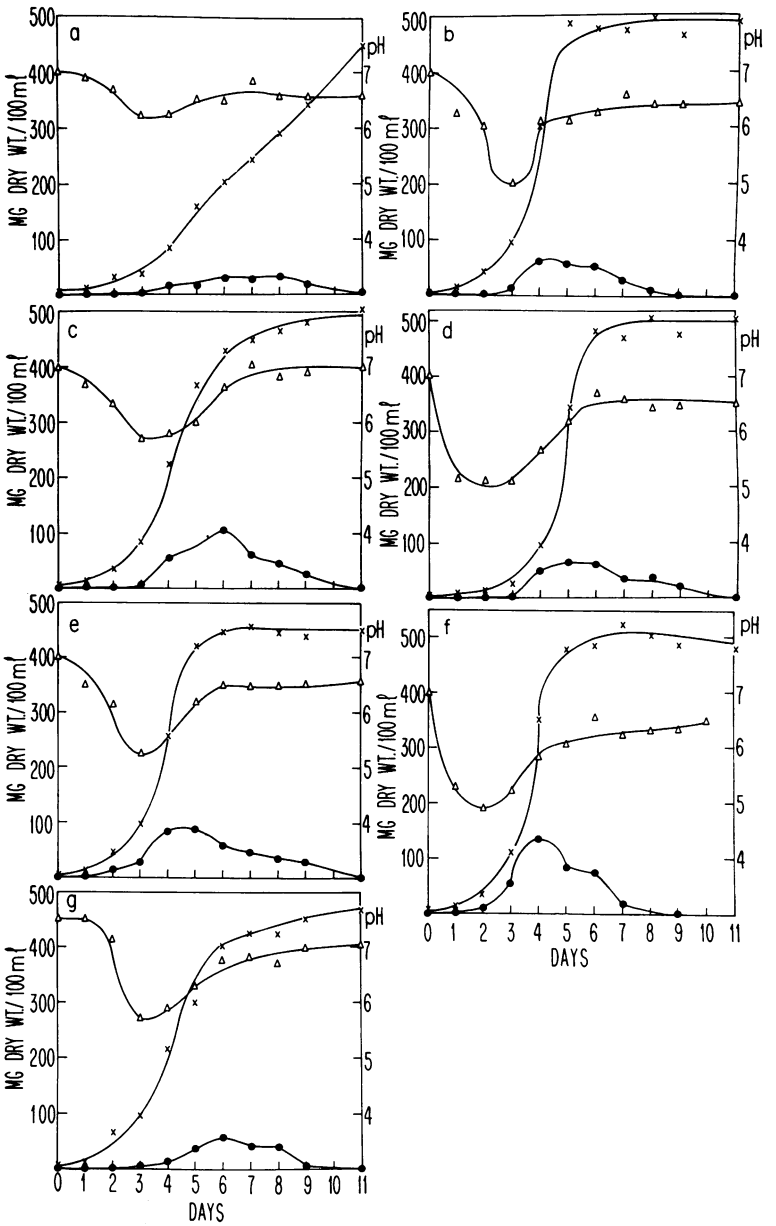

FIG. 6. *Effect of carbohydrate source on mycelial yield and polysaccharide production by L. albopunctata in SIM. The carbohydrates tested were (a) cellobiose, (b) mannose, (c) maltose, (d) xylose, (e) glucose, (f) fructose, and (g) sucrose. Initial concentration of all sugars was 1.0. Symbols: ×, mycelial dry weight; ●, polysaccharide dry weight; △, pH.*

with $MgCl_2$ or $CaCl_2$ at a K^+ concentration equal to that of Na^+ of artificial seawater. The yield of mycelium and capsular material was about the same in the cultures with $MgCl_2$ or $CaCl_2$, even though the Mg^{+2} was supplied at five times the concentration of Ca^{+2}. The concentration of the individual salts used in these tests was the same as the concentration of that salt in artificial seawater.

DISCUSSION

It is apparent from the preliminary chemical studies that the capsular material produced by the marine ascomycete *L. albopunctata* is, in all

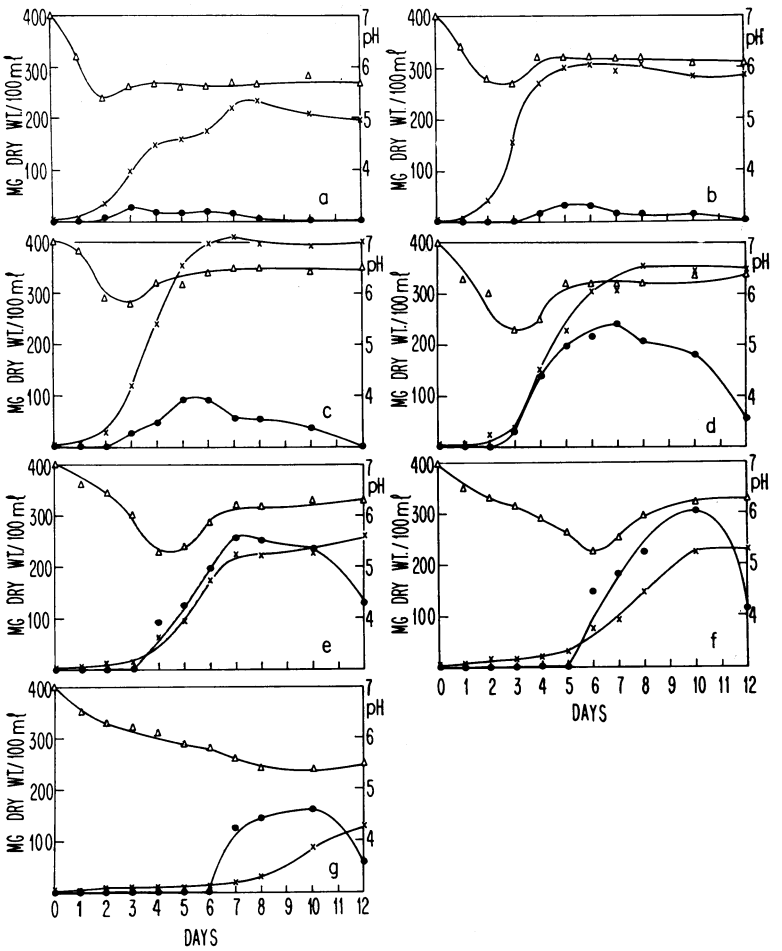

FIG. 7. *Effect of increasing NaCl concentrations on mycelial yield and polysaccharide production by L. albopunctata in SIM. The tested NaCl concentrations (grams per liter) were (a) 0, (b) 11.74, (c) 23.48, (d) 46.96, (e) 70.44, (f) 93.92, and (g) 117.40. Symbols: ×, mycelial dry weight; ●, polysaccharide dry weight; △, pH.*

probability, chemically similar to other extracellular polysaccharides recently found in the culture filtrates of *Claviceps fusiformis* (2), *Pullularia pullulans* (1), and *Plectania occidentales* and *Helotium* sp. (19). In many respects, the physiology of capsular polysaccharide production by *L. albopunctata* is also similar to the physiology of polysaccharide production by the above organisms as indicated by time course of production, ability to utilize similar carbon sources as substrates, and utilization patterns of the fungus grown in increasing concentrations of substrate. However, in most instances, the maximal yields of capsular material in terms of weight or percentage of glucose conversion, obtained from cultures of *L. albopunctata*, are not as high as the maximal yields of polysaccharide reported for cultures of *Helotium* sp., *Plectania occidentalis*, or *Claviceps fusiformis*. The fact that the polysaccharide produced by *L. albopunctata* is in the form of a well-defined capsule probably contributes to the lower yield. Also, the maximal yield of polymer from *L. albopunctata* cultures is greatly affected by the rapidity of polysaccharide degradation. Although degradation of polysaccharide has occurred in cultures of *Helotium* sp. and *Plectania occidentalis* (6), the degradation occurred much earlier in the cultures of *L. albopunctata*. In contrast, Buck et al. (2) reported that the polysaccharide produced by *Claviceps fusiformis* showed little or no decline in quantitv for at least 14 days. Apparently, an extracellular enzyme is quickly produced by *L. albopunctata*, either as a constitutive enzyme or as an induced enzyme formed in response to the production of the extracellular polysaccha-

ride capsular material. Reutilization of the capsular material by *L. albopunctata* apparently occurs, since glucose does not reappear in the culture filtrates following the disappearance of the polymer, and mycelial yields continue to increase after the depletion of glucose.

Polysaccharide production by *Claviceps fusiformis* and *Plectania occidentalis* was observed by Buck et al. (2) and Davis, Rhodes, and Shulke (6), respectively, to be independent within a fairly wide range of the amount of substrate supplied. This is also true for *L. albopunctata*, at least in terms of maximal yield of polysaccharide obtained as product. Stimulation of the final yield of polysaccharide above 10% of the initial glucose supplied was not achieved, although twice as much polysaccharide product could be isolated in 4 and 5% glucose concentrations than in a 1% glucose concentration. Such data would indicate that polysaccharide production by *L. albopunctata* is also fairly independent of substrate concentration. However, since degradation must significantly affect polysaccharide yield, the per cent conversion of initial glucose to product may not reflect accurately the amount of glucose converted to capsular polysaccharide. Evidence for this supposition is provided in Fig. 5d–f. It appears that polymer production and degradation are occurring at rates that allow the product to remain essentially in a steady state, at least as long as there is excess glucose available. If capsule formation were not still occurring, it would seem that such a steady state would be impossible.

Similar capacities for the utilization of various carbon sources are found for *L. albopunctata* when compared to *Helotium* sp. and *Claviceps fusiformis*. One notable exception, however, is observed with regard to each of these fungi. Davis, Rhodes, and Shulke (6) have reported that no polysaccharide was produced by *Helotium* sp. when grown with maltose as the carbon source, whereas Buck et al. (2) reported that *Claviceps fusiformis* produced no polysaccharide when xylose was used as the carbon source. *L. albopunctata* produces capsular material from both maltose and xylose, as well as from any other sugar previously found to stimulate significant polysaccharide production in *Helotium* sp. and *Claviceps fusiformis*.

The marine nature of *L. albopunctata* is reflected in the physiology of capsule formation. Apparently, major amounts of polysaccharides are only obtained when *L. albopunctata* is cultured in a medium containing NaCl or KCl along with $MgCl_2$ or $CaCl_2$. By use of increasing concentrations of NaCl, it is possible to significantly enhance the production of capsular material, as measured by both percentages of conversion of initial glucose and maximal yield. In fact, the results show that a doubling of the NaCl concentration of artificial seawater more than doubles polysaccharide production, whereas higher concentrations of NaCl allow higher percentages of initial glucose to be converted to polysaccharide and less to mycelium at the time of maximal polysaccharide production. This may indicate that the NaCl at concentrations above that of artificial seawater simply depresses growth to the extent that large amounts of glucose can be converted to extracellular polysaccharide. The same results may also indicate that the degradative process is so depressed in time that larger amounts of capsular polysaccharide can accumulate before its degradation begins. Alternatively or in addition, the results may indicate the NaCl is facilitating increased substrate penetration. The involvement of Na^+ and K^+ in this role has been established by Payne (12), Drapeau and MacLeod (7), and Rhodes and Payne (14) for marine bacteria.

Although this last hypothesis is very appealing, it does not help explain the necessity of having $MgCl_2$ or $CaCl_2$ in the medium together with NaCl to obtain excellent growth and capsule production. Since good, but not excellent, growth is obtained in a glucose, yeast extract, distilled water, NaCl medium, it is apparent that quantities of Mg^{+2} and Ca^{+2} sufficient to support growth are being supplied to the fungus as contaminants. Larger amounts of the Mg^{+2} or Ca^{+2} supplied as their chlorides not only allow excellent growth when supplied together with NaCl but also allow capsular polysaccharide production. This may mean that it is the larger amounts of Mg^{+2} or Ca^{+2} which are facilitating substrate uptake by activating an active transport system such as that proposed by Cirillo (4) for yeasts. In a system in which Mg^{+2} or Ca^{+2} may be promoting increased substrate uptake, it may be possible to suggest that Na^+ at increasing concentrations causes increasing rates of substrate oxidation. Such a role for Na^+ and K^+ has also been established for marine bacteria by Tomlinson and MacLeod (18) and MacLeod et al. (10).

In conclusion, this paper describes, for the first time, the formation of well-defined polysaccharide capsules by a marine filamentous fungus. The nutritional requirements for the production of the capsular polysaccharide by *L. albopunctata* in shake cultures appear very similar to the nutritional requirements for the production of extracellular polysaccharide products by other fungi. The constituents of artificial seawater, however, seem to exert a definite influence on *L. albopunctata* with regard to cap-

sule production. This phenomenon possibly indicates that the capsules formed in artificial cultures by *L. albopunctata* are not cultural artifacts but are structures having a true physiological and ecological significance. However, before such a significance can be ascribed to capsule formation by *L. albopunctata*, more studies must be conducted dealing with the mechanisms of capsule formation, and, perhaps more importantly, in situ studies must be carried out to determine whether capsules are ever formed by *L. albopunctata* in its marine habitat.

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