JOURNAL OF BACTERIOLOGY, Nov. 1967, p. 1594-1602

Vol. 94, No. 5
Printed in U.S.A.

Role of a Cell-Wall Glucan-degrading Enzyme in Mating of *Schizophyllum commune*

JOSEPH G. H. WESSELS AND DONALD J. NIEDERPRUEM

Botanical Laboratory, University of Leiden, Leiden, The Netherlands, and Department of Microbiology, Indiana University Medical Center, Indianapolis, Indiana

Received for publication 7 August 1967

Mycelial enzyme extracts of *Schizophyllum commune* were prepared during vegetative growth matings leading to common-*A* and common-*B* heterokaryons and the dikaryon, and were examined for hydrolytic activity against an alkali-insoluble cell-wall glucan (R-glucan) isolated from this mushroom. In extracts from several individual homokaryotic mycelia the R-glucanase activity was low and did not increase when the cultures exhausted glucose in the medium. In common-*A* matings, a 30-fold increase in specific activity of intracellular R-glucanase was found even in the presence of glucose in the broth. An increase of this magnitude was not observed in the common-*B* mating nor in the fully compatible cross leading to the dikaryon. Extracts of the dikaryon did show elevated R-glucanase activity after exogenous glucose disappearance and subsequent fruiting. In none of these situations was an enzyme activity detected towards an alkali-soluble cell-wall glucan (S-glucan) prepared from *S. commune*. Changes in R-glucanase were not paralleled by identical changes in laminarinase, pustulanase, cellobiase, and *p*-nitrophenyl-β-D-glucosidase, but comparable increases in specific activities were found for hydrolysis of glycogen and maltose. After interaction of the various mycelia in mating combinations, the S-glucan/R-glucan ratio of the cell wall of the dikaryon was found to be similar to that of the homokaryons, but increased in the common-*B* interaction and was elevated almost threefold in the common-*A* heterokaryon.

In the heterothallic tetrapolar basidiomycete *Schizophyllum commune*, normal sexual morphogenesis (i.e., the establishment of a dikaryon typified by clamp connections and binucleate cells) occurs only if the two interacting homokaryons are heteroallelic for both the *A* and *B* incompatibility factors. Distinct features of the dikaryotization process appear to be controlled by each of these genetic factors (12). The *B*-morphogenetic sequence, operative when the *B* factors are different in a mating, involves nuclear migration from the donor mycelium into an established recipient mycelium, and also the final step in the completion of a clamp connection (i.e., the fusion of the hook cell with the subterminal cell of the hypha). The *A*-morphogenetic sequence, functional when the *A* factors are different, includes nuclear pairing, conjugate nuclear division, hook-cell initiation, and hook-cell septation. A precise interaction between the *A* and *B* sequence is suggested in the formation of the stable dikaryotic mycelium. A main body of evidence for the allocation of these particular events to the genetic activity of the *A* and *B* factors stems from the fact that these two morphogenetic sequences can be observed in isolation in heterokaryons heteroallelic for only one of the incompatibility factors. These comprise the so-called common-*A* and common-*B* heterokaryons of *S. commune* (10, 17).

The formation of normal sexual fruits is generally a regular feature of the dikaryon of *S. commune*. The expansion of the pileus in these structures occurs only in the absence of a rapidly metabolizable carbon source in the culture medium and is accompanied by degradation of one particular cell-wall glucan, termed R-glucan, containing β-(1→3) and β-(1→6) linkages (18). It was further demonstrated that the specific activity of the hydrolytic enzyme, R-glucanase, increases sharply owing to exhaustion of the external carbon supply (19). Common-*A* mycelia of *S. commune* have a peculiar hyphal morphology (10), and abundant simple septa are found (7) in addition to the complex septa commonly observed in basidiomycetes. High levels of a cell wall-hydrolyzing enzyme could be instrumental in causing these aberrancies. Therefore it was important to measure R-glucanase in homokaryons

and heterokaryons with allelic differences in one or both the incompatibility factors.

In *S. commune*, Raper and San Antonio (17) showed differences between common-*A* mycelia and homokaryons as well as dikaryons with respect to both growth rate and glucose utilization. However, apart from the demonstration of immunological and electrophoretic differences in general proteins from homokaryons and dikaryons of *S. commune* (4, 14), nothing is known about specific biochemical activities of the incompatibility genes. The present report offers data, already described briefly (Wessels and Niederpruem, Bacteriol. Proc., p. 124, 1967), which indicate that the regulation of R-glucanase might be one of these activities.

MATERIALS AND METHODS

Organism. In most experiments, prototrophic *S. commune* (Fr.) strains 699 *A41B41*, 845 *A51B51*, and 1 *A51B41* (F1 isolate from cross 699 × 845) were used. The latter strain as well as other prototrophic strains including 13 *A51B2*, 20 *A51B47*, 5h *A41B51*, and 14 *A2B51* were isolates obtained in our laboratory by Sharon Mishkin. Strains 699, 845, R367 *A41B4 nic-2*, R70 *A2B1 ura-1*, R667 *A2B2 arg-2*, S43 *A41B2 ura-1*, R593 *A2B1 ura nic-2 "puff major,"* and R144 *A1B1 ura-1 "streak,"* were obtained from Philip J. Snider, Department of Biology, University of Houston Houston, Tex. It is important to note that strains 1, 845, and 5h are predisposed to homokaryotic fruiting; the last strain also produced an aberrant common-*B* reaction (Mishkin and Niederpruem, *unpublished data*).

Culture conditions and matings. The strains of *S. commune* were grown and mated on minimal medium containing the following ingredients, per liter of distilled water: glucose, 20 g; L-asparagine·H_2O, 1.0 g; KH_2PO_4, 0.46 g; K_2HPO_4, 1.00 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; thiamine·HCl, 120 μg. For auxotrophic strains, this medium was supplemented with the requisite growth factors (10^{-3} M, final concentration).

To insure maximal contact between pregrown mycelia to be mated and also to avoid possible nutrient gradients, the following procedure was adopted. Homokaryotic mycelia, grown previously on minimal medium plus agar (2%), were fragmented in a Waring Blendor at full speed (2 min) and then cultivated in liquid minimal medium on a rotary shaker for 48 hr. The tiny mycelia were harvested by centrifugation under sterile conditions and washed with fresh minimal medium; then heavy mycelial suspensions of individual strains or mixtures were spread on top of sand-liquid minimal medium (20 ml) in 9-cm Pyrex petri dishes. Cultivation of *S. commune* on sand-liquid has been described elsewhere (18).

In other cases, matings were performed on minimal agar medium in the conventional manner. Dikaryons were prepared by isolating hyphal tips from compatible matings. Dikaryotic cultures were prevented from fruiting by incubation at 37 C. The homokaryons and heterokaryons were then fragmented in a Waring Blendor (30 sec), and mycelial fragments were transferred directly to sand-liquid medium.

All cultures were incubated at 25 C (±0.5) in an incubator (Precision Scientific, model 806) with continuous fluorescent illumination (100 ft-c ± 20). Residual glucose in the liquid medium was determined with the Glucostat reagents (Worthington Biochemical Corp., Freehold, N.J.).

Preparation of enzyme extracts. The mycelium and some adhering sand were collected by filtration through nylon cloth, washed first with distilled water and then with 0.04 M citrate-phosphate buffer, *p*H 5.5 (CP buffer). Mycelia were then stored at −15 C for varying periods not exceeding 1 week. The frozen mycelia were ground with the adhering sand and added CP buffer in a mortar for 6 min. The homogenate was squeezed through nylon cloth, and the filtrate was centrifuged at 7,000 × *g* for 20 min in an International model B-20 refrigerated centrifuge (International Equipment Co., Needham Heights, Mass.). The supernatant fluid was dialyzed overnight (4 C) against CP buffer and then used in the enzyme assays. In cases where extracellular enzyme activity was measured, the culture fluid was dialyzed as above and used directly in the enzyme assays.

Enzyme and protein assays. Hydrolysis of polysaccharides and disaccharides was measured by incubating 0.5-ml enzyme preparations with 0.5-ml substrates in CP buffer at 30 C. Enzyme and substrate blanks were always performed. The samples were then deproteinized by the addition of 1 ml of 0.3 N $Ba(OH)_2$ followed by 1 ml of 5% $ZnSO_4$. After centrifugation, the supernatant fluids were assayed for reducing sugar by the neocuproine procedure (5) or, in the case of hydrolysis of cellobiose or maltose, liberated glucose was measured by Glucostat reagents.

The R-glucan and S-glucan substrates were prepared from cell walls of *S. commune* K35 dikaryon as outlined previously (19). The R-glucan preparation contained at least 86% polyglucose and approximately 10% chitin. These glucans were used as substrates in a final concentration of 0.25%, and the incubation mixtures were continuously shaken for a period of 3 hr. Laminarin (K and K Laboratories, Jamaica, N.Y.) pustulan (a gift from E. T. Reese, U.S. Army Natick Laboratory, Natick, Mass.), glycogen (reagent grade, Fisher Scientific Co., Pittsburgh, Pa.), cellobiose and maltose, chemically pure, Pfanstiehl Chemical Corp., Waukegan, Ill.) were used in a final concentration of 0.1% and incubated with enzyme extracts for 1 hr. Enzyme activities are expressed in milligrams of glucose equivalents liberated per milligram of protein (or per milliliter of medium) during the time indicated. β-Glucosidase activity was measured by mixing 0.1 ml of enzyme preparation with 0.9 ml of a 0.05% solution of the chromogenic substrate *p*-nitrophenol-β-D-glucoside (PNPG). After 20 min at 30 C, the reaction was terminated by addition of 0.5 ml of 1 M tris(hydroxymethyl)aminomethane (*p*H 9.8) and the color was measured at 400 mμ. β-Glucosidase activity is expressed as millimoles of *p*-nitrophenol liberated per milligram of protein per 20 min. Protein was determined by the colorimetric procedure of Lowry et al. (9).

Determination of S- and R-glucans in the cell wall. The mycelial residue remaining after preparation of enzyme extracts was suspended in water up to 25 ml. A sample was heated at 100 C for 15 min to effect solubilization of any agar carry-over by the inoculum. The suspension was centrifuged, and the sediment was washed six times with water to remove a viscous water-soluble polysaccharide which was present in varying amounts. The S-glucan (i.e., alkali-soluble polysaccharide) was then extracted with KOH (1 N) for 18 hr at 25 C and subsequently was precipitated by adjusting the alkaline extract to *p*H 5 with acetic acid. The R-glucan (i.e., alkali-resistant polysaccharide) was determined as total glucan remaining after alkaline extraction. After solubilization of S-glucan in KOH (1 N) and R-glucan in hot formic acid, the amount of glucan was determined with the anthrone reagent. Details of these procedures have been presented elsewhere (19).

RESULTS

Changes in enzyme activities during growth and mating. To follow biochemical changes during matings resulting in the formation of the common-*A* heterokaryon ($A=B\neq$), the common-*B* heterokaryon ($A\neq B=$), and the dikaryon ($A\neq B\neq$), young (i.e., 48-hr) shake-culture mycelia of strains 1, 845, and 699 were spread in thick suspensions on top of sand-liquid medium either individually or in 1:1 (v/v) mixtures. The reason for using the particular strains 845 and 699 was that a cross between these strains yields a dikaryon with good fruiting capacity. It was anticipated that matings in the mixed mycelia would occur all over the surface of the culture in a more or less synchronous way. A quantitative conversion of the two component strains into a heterokaryon could only be expected in matings entailing extensive nuclear migration (i.e., in $A=B\neq$ and $A\neq B\neq$ matings). In an $A\neq B=$ cross, however, formation of the heterokaryon can be found only in a narrow line of contact between the two mycelia. With the present technique, this area of contact should be enlarged as much as possible, resulting in a higher proportion of heterokaryotic cells.

Figure 1 shows the gross morphological appearance of homokaryons and matings after incubation for 110 hr. The establishment of the $A=B\neq$ heterokaryon is immediately suggested by its flat appearance, showing few aerial hyphae. Microscopic observations revealed that most of the hyphae were irregularly shaped and sometimes highly inflated, as is typical of this reaction (10). The formation of the dikaryon was apparent from the regular presence of clamp connections on the hyphae and the fact that the whole surface of the culture was covered with tiny fruit body primordia which subsequently developed into normal fruiting bodies. Formation of the $A\neq B=$ heterokaryon was less evident; pseudoclamped hyphae were regularly observed, but no idea was gained about the relative occurrence of this type of mycelium.

Table 1 indicates the quantities of the two cell-wall glucans in the various mycelia of *S. commune* after 110 hr of incubation. It appears that especially the mycelium from the $A=B\neq$ mating and to a lesser extent also that from the $A\neq B=$ mating had an elevated S-glucan/R-glucan ratio in the cell walls as compared with the homokaryons and the $A\neq B\neq$ mating. The total amount of cell wall glucan in the hemi-compatible matings was also considerably lower, especially in the $A=B\neq$ mating.

Preliminary experiments revealed that, during growth on glucose, extracts of homokaryons showed low R-glucanase activities. However, extracts from a $A=B\neq$ heterokaryon displayed significantly higher activities against R-glucan. This could be responsible for the high S-glucan/R-glucan ratio in at least this heterokaryon. Changes in R-glucanase activities were therefore measured after transfer of the mycelia to the sand-liquid medium.

Figure 2 shows that glucose was present in the culture media during the entire experiment. The specific activity of R-glucanase activity in extracts (intracellular activity) of the individual parental homokaryons was low and did not change significantly during growth. In the $A=B\neq$ interaction, however, the intracellular R-glucanase activity increased sharply after 24 hr, at which time the flat growth characteristics also became apparent. Subsequently, the total protein content of the extracts from the $A=B\neq$ interaction decreased considerably (up to 70%), possibly due to an increased proteolytic activity. However, even if the total R-glucanase activity is calculated rather than the specific activity, there still remains a conspicuous difference. In the $A\neq B=$ interaction, the R-glucanase activity remained low though it increased somewhat over the homokaryotic mycelium level. In the interaction leading to the dikaryon, there was an indication of a transient small increase after which the enzyme activity fell to the low parental mycelial levels.

An R-glucanase was also excreted into the culture medium in all cases; the conspicuous increase in intracellular activity of the $A=B\neq$ interaction was not, however, accompanied by a similar increase in the medium.

An important question arose concerning the specificity of the observed change in R-glucanase activity in the $A=B\neq$ interaction as compared with other enzymes. The activity of a number of other hydrolytic enzymes involved in the metabolism of carbohydrates was therefore determined. Laminarinase and pustulanase were chosen be-

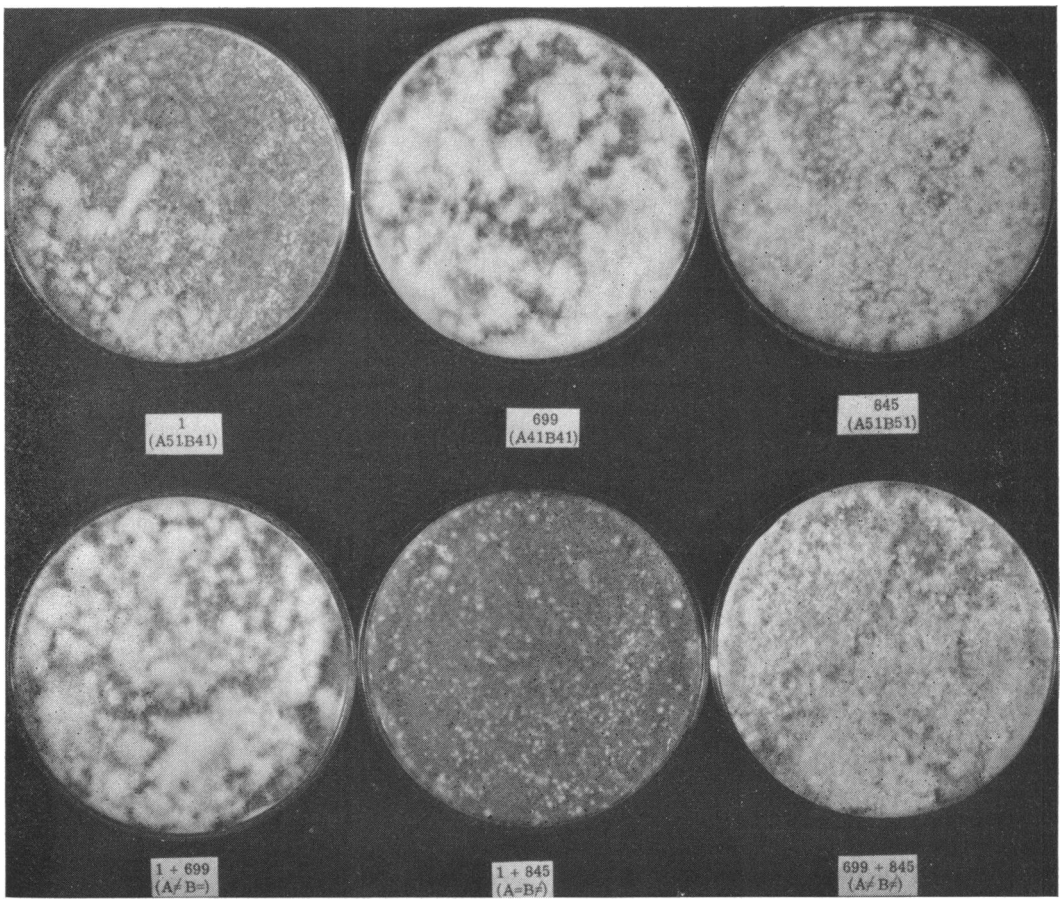

FIG. 1. *Sand-liquid plate cultures of individual homokaryons and mixtures of mycelia of Schizophyllum commune. Culture age, 110 hr; temperature, 25 C (±0.5).*

cause these enzymes hydrolyze β-(1→3) and β-(1→6) glucosidic linkages, respectively. These linkage types are also present in the cell-wall glucans of *S. commune* (18). Cellobiase and *p*-nitrophenyl-β-D-glucosidase have been studied as inducible enzymes in *S. commune* (Wilson and Niederpruem, *in press*), and were therefore included in this survey. The hydrolysis of glycogen and maltose was determined because it was observed that enzyme blanks of the $A=B\neq$ interaction became progressively low in reducing sugar liberated during enzyme incubation, suggesting the presence of a glycogen-splitting enzyme activity. All extracts were also tested against S-glucan, but none of these was active in the hydrolysis of this cell wall glucan of *S. commune*.

Figures 3 and 4 give the results of the enzyme analyses at two culture ages, 12 and 65 hr, in the aforementioned experiment. In the parental homokaryons (see Fig. 3), both the specific activities and changes thereof during the 53-hr period varied, depending on the particular strain. This is not surprising in view of the striking differences in gross morphological appearance between these homokaryons. Within these variations, no significant deviations from the homokaryons could be noted when compared with the activities of the various enzymes in the matings leading to the $A\neq B=$ and $A\neq B\neq$ mycelia, except for laminarinase (see Fig. 4). However, the changes in laminarinase occurred in all three mating reactions, and therefore could not be related specifically to just one particular cross. In the $A=B\neq$ mating, on the other hand, the increase in R-glucanase was similar to changes in enzyme activities towards glycogen and maltose. Other enzyme activities increased much less, although the 65-hr values were all somewhat higher than in the other cases. This may reflect the loss of protein during

TABLE 1. *Amounts of S-glucan and R-glucan of individual homokaryons and various matings of Schizophyllum commune*

Strain[a]	S-glucan + R-glucan (mg)	S-glucan/R-glucan
1 (homokaryon)	47.7	0.62
699 (homokaryon)	49.5	0.57
845 (homokaryon)	36.1	0.41
1 + 699 (common-B heterokaryon)	25.5	1.13
1 + 845 (common-A heterokaryon)	17.8	1.37
699 + 845 (dikaryon)	45.1	0.63

[a] Values measured at 110 hr of cultivation and mating, respectively. Mating types as in Fig. 1.

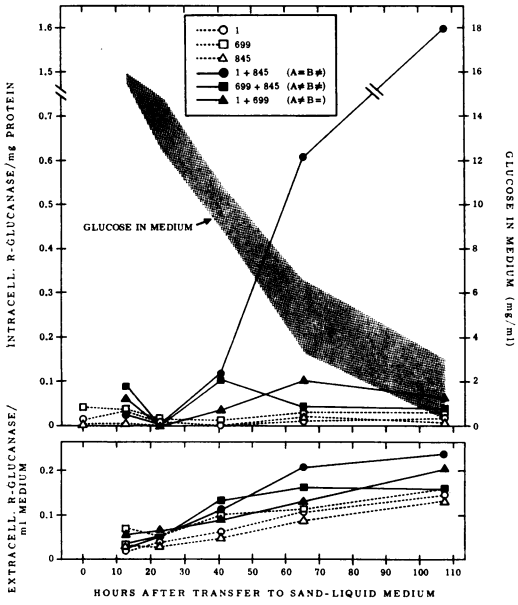

FIG. 2. *Specific activities of R-glucanase in cell extracts and culture filtrates of individual homokaryons and mixtures of Schizophyllum commune mycelia. All values found for residual glucose in the medium fall within the shaded area.*

this period, resulting in enhanced specific activities.

It is important to stress that the increase in R-glucanase was not accompanied by similar increases in laminarinase, pustulanase, cellobiase, and *p*-nitrophenyl-β-D-glucosidase, all of which cleave β-glucosidic linkages. Furthermore, in this experiment, the liberation of hydrolysis products from R-glucan and laminarin was estimated with both the reducing sugar reagent and the glucose oxidase method. It was found that, in the case of R-glucan breakdown, with these crude extracts,

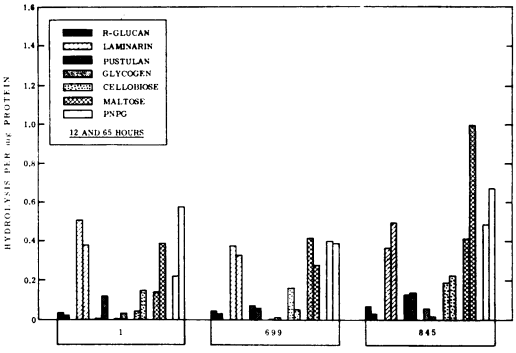

FIG. 3. *Specific activities of various hydrolytic enzymes in cell extracts of homokaryons of Schizophyllum commune during growth on glucose. Hydrolysis of specific substrates designated by key-insert. Specific activity for hydrolysis of R-glucan, laminarin, pustulan, glycogen, cellobiose, and maltose is on the basis of milligrams of glucose equivalents liberated per hour × milligrams of protein.*

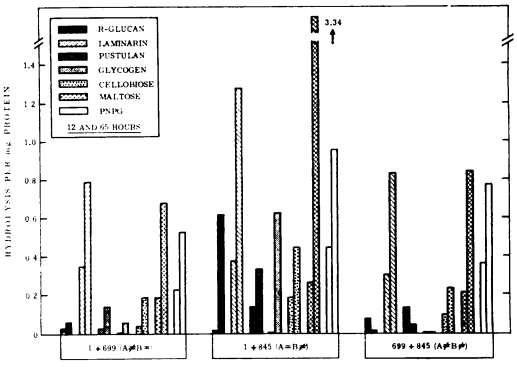

FIG. 4. *Specific activities of various hydrolytic enzymes in cell extracts during formation of heterokaryons of Schizophyllum commune in the presence of glucose in the medium. Activity expressed as in Fig. 3.*

virtually all the liberated reducing sugar could be accounted for by glucose, whereas this was only partially so in the case of laminarin hydrolysis.

Changes in enzyme activities during carbon starvation. In dikaryotic mycelium stock cultures of *S. commune* K.8 and K.35 derived from the "Kniep" stock, it was previously found that depletion of glucose from cultures led to an increase in R-glucanase activity both in extracts and in the medium (19). Since segregation of incompatibility factors was abnormal in this latter material, ambiguous results would probably be obtained by studying homokaryotic progeny of this stock. It was therefore of interest to examine the comparative effect of carbon starvation in homokaryons like strain 699 and 845 and the dikaryon derived from these strains.

Enzyme levels in these homokaryons and the

established dikaryon were determined after growth for 168 hr, at which time the glucose had virtually disappeared from the medium in all cultures and the surface of the dikaryon was covered with fruit body primordia. A number of these primordia then proceeded to produce pilei which were well established 4 days later. A second analysis was therefore performed on 260-hr cultures. Homokaryotic fruit body primordia in small numbers were also apparent in strain 845, but none of these produced pilei within the period studied here.

Figure 5 gives the changes in various enzyme activities during carbon starvation in the dikaryon 699 × 845 and the parental homokaryons. The most important result of this experiment is that the conspicuous increase in R-glucanase activity in the dikaryon was not observed in the homokaryons during nutritional deprivation. Changes in certain enzyme activities in the homokaryons were apparently strain-dependent; cellobiase and maltase activities increased in strain 699 but decreased in strain 845. Again, as in the $A=B\neq$ interaction, the increase in R-glucanase activity in the dikaryon was not paralleled by a similar increase in laminarinase, pustulanase, cellobiase, and *p*-nitrophenyl-β-D-glucosidase, but comparable changes were found in the hydrolytic activities against glycogen and maltose. In fact, the whole pattern of enzyme changes in the dikaryon during carbon starvation bore a clear resemblance to the pattern of changes encountered in the $A = B \neq$ interaction in the presence of glucose (see Fig. 4).

R-glucanase in other homokaryons and common-A mycelia. Various normal or nutritionally forced common-*A* mycelia were established by mating wild-type or auxotrophic strains of *S. commune* on minimal medium. Homokaryons and common-*A* mycelia were then grown on sand-liquid medium. At various times, remaining glucose in the medium and specific R-glucanase activity of extracts were determined. Samples of presumed common-*A* mycelia were taken from the sand plates and the mycelium was mated with a tester strain fully compatible with one of the nuclear types in the common-*A* mycelium. In all cases, a unilateral dikaryotization of the tester strain occurred, indicating that the established common-*A* heterokaryons were stable and not disrupted during transfer to the sand plates.

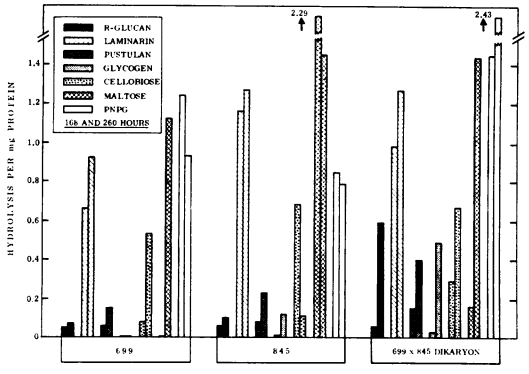

FIG. 5. *Specific activities of various hydrolytic enzymes in cell extracts of parental homokaryons and the established dikaryon of Schizophyllum commune during glucose deprivation. Activity expressed as in Fig. 3.*

Table 2 gives the mating types of the strains used and the results obtained. The table also includes the results for two morphological mutants which have been isolated from aged common-*A* mycelia (17), namely R593, a mutant called "*puff*," and R144, a mutant called "*streak*." However, many strains showed great dissimilarities in their macroscopic appearance. The extremes were a completely flat mycelium with no aerial hyphae (R367 and R144), an aerial system of compact dry-looking mycelium (strain 14) and mycelium with abundant wooly aerial hyphae (R667). In addition, some homokaryotic strains (strains 1, 845, and 5h) produced homokaryotic fruit body primordia. The production of extracellular slime also varied greatly. The common-*A* mycelia grew completely flat or with much less aerial hyphae than their homokaryotic components and produced little or no slime.

To appreciate the values given for glucose in the medium, it should be noted that all homokaryons exhausted their glucose within a period of 120 to 210 hr. Notwithstanding the fact that very low amounts of glucose could still be detected in the medium of old cultures, this indicates that homokaryons harvested at 306 hr were deprived of glucose for at least 96 hr.

Table 2 shows that the R-glucanase activity was low in extracts of all homokaryons growing on glucose and that exhaustion of glucose generally did not change this activity significantly. However, an increase was noted for strains 1, 845, and especially R367 and R144. It might be significant that the former two strains showed homokaryotic fruiting while strain 1 even produced a few fully expanded fruiting bodies within the time limits of the experiment, and that the two latter strains were completely flat. In addition, it was shown that R367 was a unilateral matter, as was R144. The morphological mutant R593 (*puff*) grew mainly submerged as little beads and did not show increased R-glucanase activities.

Glucose consumption in the common-*A* mycelia was slower than in their homokaryotic components, but in all cases the R-glucanase activity

TABLE 2. *Glucose utilization and R-glucanaose of various individual homokaryons and established heterokaryons of Schizophyllum commune*

Strain	Mating type[a]	Culture age (hr)	Residual glucose[b] (mg/ml)	R-glucanase/ mg of protein
Homokaryons				
1	A51B41	160	5.3	0.12
		306	0.4	0.51
845	A51B51	160	8.8	0.09
		306	0.5	0.31
13	A51B2	160	7.6	0.25
		306	0.6	0.37
20	A51B47	160	1.9	0.20
		306	0.0	0.22
5h	A41B51	160	8.2	0.32
		306	0.0	0.15
14	A2B51	160	7.3	0.35
		306	0.2	0.18
R667	A2B2	160	4.6	0.13
		306	0.6	0.17
R70	A2B1	160	1.8	0.02
		306	0.2	0.11
R367	A41B4	160	0.3	0.09
		306	0.0	0.73
S43	A41B2	160	5.6	0.03
		306	0.1	0.16
R593	A2B1	160	0.5	0.03
		306	0.3	0.16
R144	A1B1	160	0.3	0.10
		306	0.1	1.06
Common-A heterokaryons				
1 × 845	A51B41 × A51B51	160	13.6	1.38
		257	11.2	1.43
13 × 20	A51B2 × A51B47	160	10.1	0.56
		257	1.8	0.99
		306	0.4	1.17
R667 × R70	A2B2 × A2B1	205	18.5	1.74
R367 × S43	A41B4 × A41B2	205	16.4	0.23
		277	7.7	0.56
		306	4.5	0.30
Aberrant common-B heterokaryon				
5h × 14	A41B51 × A2B51	160	10.2	1.23
		257	2.2	0.69
		306	0.2	0.88

[a] Nutritionally-forced heterokaryons were 667A2B2 arg$^-$ × 70A2B1 ura$^-$ and 367A41B4 nic$^-$ × S43-A41B2 ura$^-$.

[b] Initial glucose concentration 20.4 mg/ml.

of extracts was higher, at least in the presence of glucose. It is also noteworthy that the common-*A* mycelia with the less elevated R-glucanase levels, 13 × 20 and R367 × S43, grew much more vigorously than 1 × 845 and R667 × R70.

Table 2 also includes the results from a cross (5h × 14) which is essentially a $A \neq B =$ mating, but the 5h strain carries an apparent suppressor mutation for the *B* factor. It is believed that extensive nuclear migration actually occurred in this mating (Mishkin and Niederpruem, *unpublished data*), and it was of interest to find that in this case, too, the presumed heterokaryon produced elevated R-glucanase levels. Strain 5h also carries a mutation promoting homokaryotic fruiting, but this character was not strongly expressed under our conditions. In the mating, however, a large number of open fruit bodies was eventually produced.

DISCUSSION

The enzymatic hydrolysis of the *S. commune* R-glucan cell wall component is still an ill-defined system; hence, the term "R-glucanase" is retained

for the enzyme instrumental in the breakdown of this polysaccharide. Results of the current study already indicate that this enzymatic activity cannot be evaluated with laminarin or pustulan as substrates because changes in enzymatic activities against these polysaccharides do not parallel those against R-glucan. The latter has an X-ray diffraction diagram identical to yeast glucan (18) but different from that of laminarin (3). Consequently, in yeasts, also, the use of laminarin or pustulan to assay enzymes active in the self-digestion of the cell wall (1, 2) may lead to ambiguous results. It is possible, however, that laminarinase and pustulanase play other roles in the complete hydrolysis of fungal cell wall glucans.

Although previous evidence suggested that changes in R-glucanase in *S. commune* reflect differences in the rate of synthesis of the enzyme (19), more critical experiments are necessary to validate this point. However, in view of a recently proposed model which assigns regulatory functions to the incompatibility factors in sexual morphogenesis of this mushroom (12), it is of interest to consider the possibility that these genes control the formation of R-glucanase.

The findings that R-glucanase activities can increase in extracts of common-*A* heterokaryons and dikaryons, but generally not in parental homokaryons, suggest that heteroallelic conditions in the *B* factors are required for derepression of this enzyme. Thus, the presence of a single *B*-factor product in the homokaryon might be implicated in a permanent repression of R-glucanase. In the common-*A* heterokaryons, R-glucanase seems to be derepressed even in the presence of glucose in the medium; in the dikaryon, derepression occurs only in the absence of glucose (19) or other readily utilizable carbon sources (Duindam and Wessels, *unpublished data*). This suggests that R-glucanase, under permanent repression in homokaryons, becomes subject to catabolite repression when two different B-factor products are present in a common cytoplasm. It then becomes necessary to postulate that in the common-*A* heterokaryon, owing to the absence of different *A*-factors, the level of the active catabolite is so low that the aporepressor is ineffective. This notion can be supported further by the fact that the growth rate and the rate of glucose consumption in the common-*A* heterokaryon are much slower than in homokaryons and dikaryons (17, see also Table 2). Alternate explanations are possible, however, For instance, the products of the *A* factors in the dikaryon could play a more direct role in catabolite repression of R-glucanase if heteroallelic conditions in the *B* factor render the *B*-factor repressor completely inactive.

The low R-glucanase activities encountered in extracts during the formation of a partially common-*B* heterokaryon generally fit the hypothesis given above. However, the R-glucanase activities seem to be somewhat higher than in the extracts of homokaryons in the presence of glucose. Also, the elevated S-glucan/R-glucan in the cell wall of this mycelium is difficult to explain. Possibly this is brought about by some change affecting the synthesis of cell-wall glucans.

Whatever the exact role of the incompatibility factors in the regulation of R-glucanase may be, the study of this enzyme, which is intimately involved in cell-wall metabolism and hence cell morphology, may offer a direct approach to elucidate some of the biochemical mechanisms underlying sexual morphogenesis in *S. commune*. For instance, the high levels of R-glucanase evoked during growth of the common-*A* heterokaryon might well be related to the high S-glucan/R-glucan in the cell wall and also to aberrant morphological features such as apparently weakened cell walls, dissolution or incomplete formation of complex septa, increase in the incidence of hyphal anastomoses, and flat growth. The increase in R-glucanase in dikaryons as a result of glucose exhaustion has been related previously to the formation of pilei in *S. commune* (19). The assignment of regulatory roles, either direct or indirect, to the incompatibility factors in the control of R-glucanase also permits speculation as to the role of this enzyme during dikaryotization. For instance, the rate of release of *A* and *B* factor products by the invading nucleus would determined repression or depression of R-glucanase. More rapid production of the *B* factor product could temporarily relieve R-glucanase repression which might be a factor in dissolution of septa and thus facilitate nuclear migration. Eventually, repression would be restored again in the dikaryotic cell when the product of the *A* factor accumulates. The occurrence of apparent complex septum disorganization under conditions where nuclear migration was known to have occurred has been found in *Coprinus lagopus* (6).

It would be of considerable interest to study the regulation of R-glucanase in those homokaryons carrying mutations in the *B* factor (8, 11), in the *A* factor (13), or in both factors, as well as in disomics (15). Examination of these mimics of heterokaryons in *S. commune* and of certain other mutations modifying sexual morphogenesis (16) could lead to support or modification of the hypotheses given above.

ACKNOWLEDGMENTS

This investigation was supported by the Netherlands Organization for the Advancement of Pure

Research (Z. W. O.) and by research grant GB-5902 from the National Science Foundation.

LITERATURE CITED

1. ABD-EL AL, A., AND H. J. PHAFF. 1966. The presence of an exo-β-glucanase in yeast. Yeast News Letter **15**:8–9.
2. BROCK, T. D. 1965. Biochemical and cellular changes occurring during conjugation in *Hansenula wingei*. J. Bacteriol. **90**:1019–1025.
3. BULL, A. T., AND C. G. C. CHESTERS. 1966. The biochemistry of laminarin and the nature of laminarinase. Advan. Enzymol. **28**:325–364.
4. DICK, S. 1965. Incompatibility in fungi, p. 72–80. Springer-Verlag, Heidelberg, Germany.
5. DYGERTS, S., L. H. LI, D. FLORIDA, AND J. A. THOMA. 1965. Determination of reducing sugar with improved precision. Anal. Biochem. **13**: 367–374.
6. GIESY, R. M., AND P. R. DAY. 1965. The septal pores of *Coprinus lagopus* in relation to nuclear migration. Am. J. Botany **52**:287–293.
7. JERSILD, R., S. MISHKIN, AND D. J. NIEDERPRUEM. 1967. Origin and ultrastructure of complex septa in *Schizophyllum commune* development. Arch. Mikrobiol. **57**:20–32.
8. KOLTIN, Y., AND J. R. RAPER. 1966. *Schizophyllum commune:* new mutations in the B incompatibility factor. Science **154**:510–511.
9. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
10. PAPAZIAN, H. P. 1950. Physiology of the incompatibility factors in *Schizophyllum commune*. Botan. Gaz. **112**:143–163.
11. PARAG, Y. 1962. Mutations in the B incompatibility factor in *Schizophyllum commune*. Proc. Natl. Acad. Sci. U.S. **48**:743–750.
12. RAPER, J. R., 1966. Genetics of sexuality in higher fungi. The Ronald Press Co., New York.
13. RAPER, J. R., D. H. BOYD, AND C. A. RAPER. 1965. Primary and secondary mutations at the incompatibility loci in *Schizophyllum*. Proc. Natl. Acad. Sci. U.S. **53**:1324–1332.
14. RAPER, J. R., AND K. ESSER. 1961. Antigenic differences due to the incompatibility factors in *Schizophyllum commune*. Z. Vererbungslehre **92**:439–444.
15. RAPER, J. R., AND M. T. OETTINGER. 1962. Anomalous segregation of incompatibility factors in *Schizophyllum commune*. Rev. Biol. (Lisbon) **3**:205–221.
16. RAPER, C. A., AND J. R. RAPER. 1966. Mutations modifying sexual morphogenesis in *Schizophyllum*. Genetics **54**:1151–1168.
17. RAPER, J. R. AND J. P. SAN ANTONIO. 1954. Heterokaryotic mutagenesis in Hymenomycetes. I. Heterokaryosis in *Schizophyllum commune*. Am. J. Botany **41**:69–86.
18. WESSELS, J. G. H. 1965. Morphogenesis and biochemical processes in *Schizophyllum commune* Fr. Wentia **13**:1–113.
19. WESSELS, J. G. H. 1966. Control of cell-wall glucan degradation during development in *Schizophyllum commune*. Antonie-van Leeuwenhoek J. Microbiol. Serol. **32**:341–355.