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Biochemistry of Sexual Morphogenesis in *Schizophyllum commune*: Effect of Mutations Affecting the Incompatibility System on Cell-Wall Metabolism

JOSEPH G. H. WESSELS

Botanical Laboratory, University of Leiden, Leiden, The Netherlands

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A number of homokaryons of *Schizophyllum commune*, which carry various mutations affecting the incompatibility system, and a wild-type homokaryon were grown and examined for differences in the net synthesis of the cell-wall polysaccharides S-glucan, R-glucan, and chitin and in the activity of an enzyme hydrolyzing R-glucan (R-glucanase) in mycelial extracts and culture media. Only slight differences were observed for the accumulation of S-glucan and chitin. In comparison with the wild-type homokaryon, a very high S-glucan/R-glucan ratio was found in a primary *B*-factor mutant strain. Essentially, wild-type S-glucan/R-glucan ratios were restored in two strains in which additional mutations restored normal morphology: a strain carrying a secondary *B*-factor mutation and a strain carrying a modifier mutation in addition to the primary *B*-factor mutation. The S-glucan/R-glucan ratios in three *A*-factor mutants were intermediate between those of the wild-type homokaryon and the primary *B*-factor mutant. In young, growing cultures of the various homokaryons, except for the *A*-factor mutants, a correlation was found between the S-glucan/R-glucan ratios in the cell wall and the activities of R-glucanase in mycelial extracts. A certain specificity of the effect of the studied mutations on enzyme activities was indicated by the fact that, in young cultures, changes in R-glucanase activities were not paralleled by similar changes in the activities of laminarinase and maltase. The results can be correlated with particular morphological features of the homokaryons and, together with earlier results obtained with heterokaryons, indicate the activity of R-glucanase as an integral component of sexual morphogenesis regulated by the incompatibility factors.

Sexual interaction and the subsequent formation of a dikaryotic mycelium in *Basidiomycetes* exemplifies a relatively simple case of cellular differentiation and morphogenesis in a eukaryotic organism. In *Schizophyllum commune* and in many other *Basidiomycetes*, sexual morphogenesis encompasses the formation of a dikaryon which is typified by clamp connections and binucleate cells and which normally bears fruiting bodies. *S. commune* is heterothallic, and the formation of a dikaryon only ensues from the interaction of two morphologically similar self-sterile homokaryons which carry different alleles in at least one of the loci of both the *A* and *B* incompatibility factors (13). The role of each factor can be inferred from the sequences of events that occur in hemicompatible matings which result in the formation of so-called common-*A* ($A = B \neq$) and common-*B* ($A \neq B =$) heterokaryons (8, 19). The *B*-morphogenetic sequence, operative when the *B* factors are different in a mating, involves nuclear migration from one mycelium into the opposite mycelium. The *A*-morphogenetic sequence, operative when the *A* factors are different, includes nuclear pairing, conjugate nuclear division, hook-cell initiation, and hook-cell septation. The last event in the formation of the clamp, i.e., the fusion of the hook cell with the subterminal cell, only ensues if in addition to the *A* factors the *B* factors are also different.

Many hypotheses have been forwarded to explain the morphogenetic action of the incompatibility factors at the molecular level (10). A very attractive model has been advanced by Prévost (11) and Raper and Raper (13, 17) based

on genetic work with *Coprinus radiatus* and *S. commune*, respectively. This model assigns regulatory roles to the incompatibility factors.

In an attempt to identify enzymatic reactions controlled by the incompatibility genes and responsible for morphological events in *S. commune*, attention was given to the activity of a cell-wall glucan-hydrolyzing enzyme. Apart from a small amount of chitin, the cell wall of *S. commune* is mainly composed of two different glucans (21). R-glucan is an alkali-insoluble glucan with β-1,3 and β-1,6 linkages. S-glucan is an alkali-soluble glucan that appears to contain only α-1,3 linkages (1). *S. commune* produces an enzyme hydrolyzing R-glucan (R-glucanase) which can be classified as a β-1,6-glucan glucanohydrolase (23). After exhaustion of the carbon supply in the medium and concomitantly with fruiting-body expansion, R-glucanase activity rises sharply in the dikaryon (22). The in vivo activity of R-glucanase was apparent by a decrease of R-glucan in the cell wall. Under these conditions, a similar rise in R-glucanase activity was not observed in homokaryons, but, in a common-*A* mating, high R-glucanase activities were found even in the presence of glucose in the medium (24). At the same time, the S-glucan/R-glucan ratio in the common-*A* mating became two to three times higher than in the parental homokaryons.

The availability of homokaryons which carry mutations at the incompatibility loci and which closely mimic heterokaryons in morphology (13) made it possible to verify some predictions on cell-wall composition and R-glucanase activity as drawn from the work with heterokaryons (24). The present report describes the results of this study and adds to the evidence that cell-wall composition is regulated by the incompatibility factors and that this is accomplished, at least in a number of cases, by variations in the activity of R-glucanase.

MATERIALS AND METHODS

Organism. Table 1 lists the strains of *S. commune* used in this study. All strains were kindly supplied by John R. Raper of The Biological Laboratories, Harvard University, Cambridge, Mass. The *Bβ2(1)* allele was isolated originally by Yair Parag of the Department of Botany, The Hebrew University, Israel.

The striking effects of mutations affecting the incompatibility system on hyphal morphology and colonial appearance of *S. commune* homokaryons are shown in Table 1 and Fig. 1, respectively. When compared to the wild-type homokaryon (699a), the primary mutation in the *B* factor (C10-46) causes a reduction in the linear growth rate and curtails the formation of aerial hyphae (flat mycelium). In the homokaryon carrying the unlinked modifier mutation *MIV-11* in addition to the *B*-factor mutation (B10-1), wild-type morphology is fully restored. A secondary mutation in the *B* factor (D2316) also restores wild-type morphology. However, in this case linear extension of the mycelium is slower than in strain 699a and the mycelial mat is much more dense. Colonial morphology and growth rate of the strain which carries only a mutation in the *A* factor (1761) bears some resemblance to that of the primary *B*-

TABLE 1. *Description of Schizophyllum commune homokaryons*

Strain	Genotype[a]		Common designation	Reference	Homokaryotic morphology in agar cultures
699a	*A41α1-β2*	*B41α3-β2*	*A41B41*		Normal
C10-46[b]	*A43α4-β6*	*Bα3-β2(1)* *α4*[c]	*A43Bmut*	9	Mimics *A*=*B*≠ heterokaryon: irregularly shaped hyphae, disrupted septa, abnormal nuclear distribution
B10-1[b]	*A41α1-β1*	*Bα1-β2(1) MIV-11* *α3*	*A43BmutMIV*	12	Normal
D2316	*A43α4-ade-β6*	*Bα3-β2(1–3)*	*A43Bmut-mut*	18	Normal
1761[b]	*Aα1-pab-β1(1)* *α4*	*B41α3-β2*	*AmutB41*	16	Mimics *A*≠*B*= heterokaryon: mycelium with pseudoclamps
1779[b]	*Aα1-β1(1)* *α4*	*Bα1-β2(1)* *α3*	*AmutBmut*	16	Mimics *A*≠*B*= heterokaryon: mycelium with pseudoclamps
1758[b]	*Aα1-pab-β1(1)* *α4*	*Bα3-β2(1)* *α1*	*AmutBmut*	16	Mimics dikaryon: mycelium with true clamps

[a] Each incompatibility factor is constituted of two linked loci, *Aα-Aβ* and *Bα-Bβ*, with multiple alleles (14, 15). A primary mutation is indicated by one number in parenthesis following the affected locus; a secondary mutation is indicated by a second number in parenthesis. *MIV-11* is a modifier mutation outside the incompatibility loci.

[b] Isogenic by ten successive generations of backcrossing to strain 699a.

[c] Incompatibility allele designated on the line immediately above is the more probable identity.

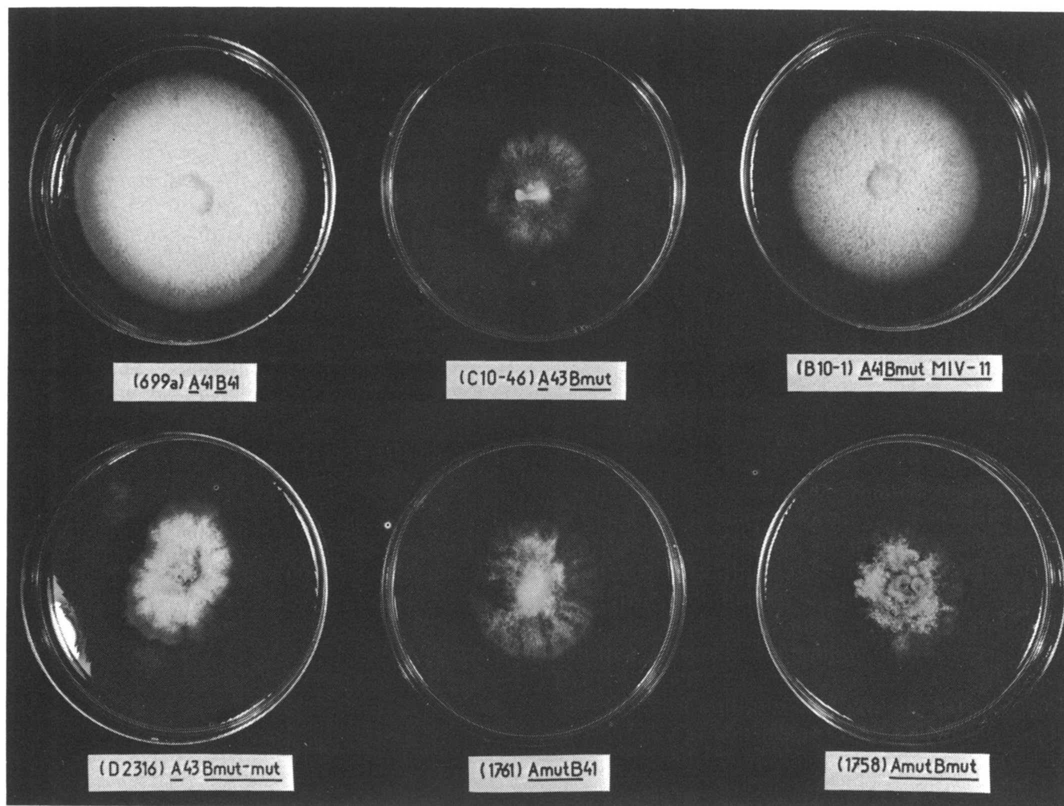

FIG. 1. *Plug-inoculated agar cultures of a wild-type homokaryon of S. commune and various strains which carry mutations at the incompatibility loci and a modifier mutation. Culture age, 7 days; temperature, 25 C.*

factor mutant. The incorporation of primary mutations of both the *A* and the *B* factor in strain 1758 entails the appearance of a restricted, densely growing mycelium (a typical characteristic of dikaryons) bearing fruiting bodies. It should be noted that strain 1779, listed in Table 1, deviates from expectations. Instead of true clamps, the hyphae of this strain bear pseudoclamps and no fruiting bodies are produced.

Culture conditions. The strains were grown on minimal medium (24). To this medium was routinely added a trace element solution (21) and, for auxotrophic strains, the medium was supplemented with the requisite growth factors (10^{-3} M, final concentration).

To follow changes in polysaccharides and protein during growth, 250-ml Erlenmeyer flasks were used containing 50 g of sand (acid-washed and glowed) and 15 ml of medium. For the purpose of determining enzyme activities during growth, 1-liter Erlenmeyer flasks containing 100 g of sand and 30 ml of medium were used. The surface of the sand-medium was inoculated uniformly with a mycelial suspension prepared by fragmenting a 5-day-old culture on minimal agar medium in a Waring Blendor (1 min at half speed). The cultures were incubated at 25 C (±0.5) with continuous fluorescent illumination (about 1,100 lux).

Chemical analyses. Prior to harvest of the mycelium, a sample of the culture broth was removed and residual glucose was determined with the Glucostat reagents (Worthington Biochemical Corp., Freehold, N.J.). Water was then added to the culture flasks and most of the sand was removed by decantation. The mycelium and some adhering sand were collected by filtration through a Jena G1 glass filter, washed with distilled water, and stored in ethyl alcohol (96%) at −18 C. The mycelia were then homogenized in a Virtis "45" homogenizer (The Virtis Co., Gardiner, N.Y.) running at half speed for 1 min, and the homogenate was made up to 50 ml with ethyl alcohol (96%).

For determination of water-insoluble polysaccharide fractions, a 10-ml sample of the alcoholic homogenate was centrifuged and the sediment was washed once with water. The sediment was then suspended in water, heated at 100 C for 10 min, and washed four times with water to remove a viscous water-soluble material of unknown composition. This slime was also present in the culture fluid and was produced in varying amounts depending on strain and culture age. Alkali-soluble cell-wall glucan (S-glucan) was then extracted from the sediment with 1 N KOH for 18 hr at 25 C. The S-glucan was precipitated by

adjusting the alkaline extract to *p*H 5 with acetic acid. The nonprecipitable polysaccharide in the alkaline extract is referred to as acid-soluble polysaccharide and probably includes glycogen. Alkali-insoluble cell-wall glucan (R-glucan) was determined as total glucan remaining after alkaline extraction. After solubilization of S-glucan in 1 N KOH and R-glucan in hot concentrated formic acid, the amount of glucan in the various fractions was determined with the anthrone reagent (4). Another 10-ml portion of the alcoholic homogenate was used for the determination of chitin (2).

Total protein in the mycelium was estimated as follows. To a 5-ml portion of the alcoholic homogenate, 1 ml of 30% trichloroacetic acid was added. The precipitated material was washed once with 5% trichloroacetic acid and then extracted with 1 N NaOH at 100 C for 10 min. Insoluble material was removed by centrifugation, and protein in the alkaline extract was determined by the colorimetric procedure of Lowry et al. (7).

Enzyme assays. Enzyme activities were determined in the 7,000 $\times$ *g* supernatant fraction of mycelial extracts and in the culture fluid, after dialysis against 0.05 M McIlvain buffer (*p*H 5.5; 23).

R-glucan (contaminated with about 15% chitin) was prepared from enzymatically cleaned cell walls of *S. commune* K35 dikaryon, as outlined elsewhere (22). Other substrates were soluble laminarin (K & K Laboratories, Jamaica, N.Y.) and maltose (British Drug Houses, England).

R-glucanase activity was determined by mixing 0.5 ml of enzyme preparation with 0.5 ml of an R-glucan suspension in McIlvain buffer (5 mg/ml). After 2 hr at 30 C, the reaction mixtures were deproteinized by addition of 1 ml of 0.3 N $Ba(OH)_2$ followed by 1 ml of 5% $ZnSO_4 \cdot 7H_2O$. After centrifugation, the supernatant fluids were filtered through glass-fiber paper (Whatman GF/A) to ascertain complete removal of insoluble glucan fragments. The amount of soluble carbohydrate in the filtrates was then determined with the anthrone reagent (4). The hydrolysis of laminarin and maltose was determined by mixing 0.1 ml of enzyme preparation with 0.4-ml solutions of these carbohydrates in McIlvain buffer (1.25 mg/ml) and incubating at 30 C for 1 hr. For laminarinase, the increase in reducing-sugar content was measured by use of the neocuproine procedure (3); the alkaline copper reagent was used to stop the enzymatic reaction. Hydrolysis of maltose was terminated by heating in boiling water for 5 min, and the amount of liberated glucose was measured with the Glucostat reagents. All enzyme activities are expressed as milligrams of glucose equivalents liberated per milligram of protein (or per milliliter of medium) during the time indicated. Protein was determined according to the method of Lowry et al. (7).

RESULTS

Changes in protein and polysaccharide fractions. Figure 2 depicts changes in the amount of various cell constituents during growth of the wild-type homokaryon and three *B*-factor mutant strains. The decrease of glucose in the medium is also given. It is important to stress that the sand-liquid cultures exhibited the cytological features given in Table 1 and also were similar to agar-plate cultures with respect to the development of aerial hyphae and apparent density of the mycelial mat (Fig. 1). Notwithstanding these morphological differences, there exist only small differences between the strains concerning the total amount of protein made. In all cases, 46 to 56% of the nitrogen added as asparagine is converted into protein. However, there may be differences in the rate of net protein synthesis. A most conspicuous difference between the strains concerns the net synthesis of the cell-wall component R-glucan. The primary mutation in the *B* factor (C10-46) causes a severe reduction in the rate of accumulation of this glucan. A partial restoration of R-glucan accumulation to the rate observed in the wild-type strain (699a) occurs when, in addition to the primary *B*-factor mutation, the modifier mutation *MIV-11* is also present (B10-1) and by a secondary mutation in the *B* factor (D2316). Such wide variations cannot be observed in the accumulation of the other two cell-wall components, S-glucan and chitin. All mutant strains made less acid-soluble polysaccharide (possibly including glycogen) than did strain 699a; the amount formed in strain D2316 comes closest to that of the wild-type strain.

Comparison of cell-wall glucan synthesis and protein synthesis (Fig. 2) shows that glucan accumulation continues after net protein synthesis ceases. Surprisingly, glucan accumulation may continue even after the glucose in the medium is exhausted. This phenomenon is absent in the previously studied *S. commune* dikaryons K8 and K35, which do not produce extracellular slime. Therefore, the possibility exists that the insoluble glucan synthesized after exhaustion of glucose in the medium derives from the breakdown of water-soluble polysaccharides previously excreted into the medium. Although changes in the amount of slime were not determined in the present experiments, Wang and Miles (20) have reported the breakdown of this material after depletion of the glucose supply.

Since the morphology of the hyphae is probably more related to the relative amounts of cell-wall components than to the absolute amounts accumulated in the cultures, the ratios of S-glucan and R-glucan were calculated. Figure 3 shows that strain C10-46 has an exceptionally high S-glucan/R-glucan ratio throughout the period measured. Obviously, the additional presence of the modifier mutation (B10-1) or the presence of a secondary mutation in the *B*

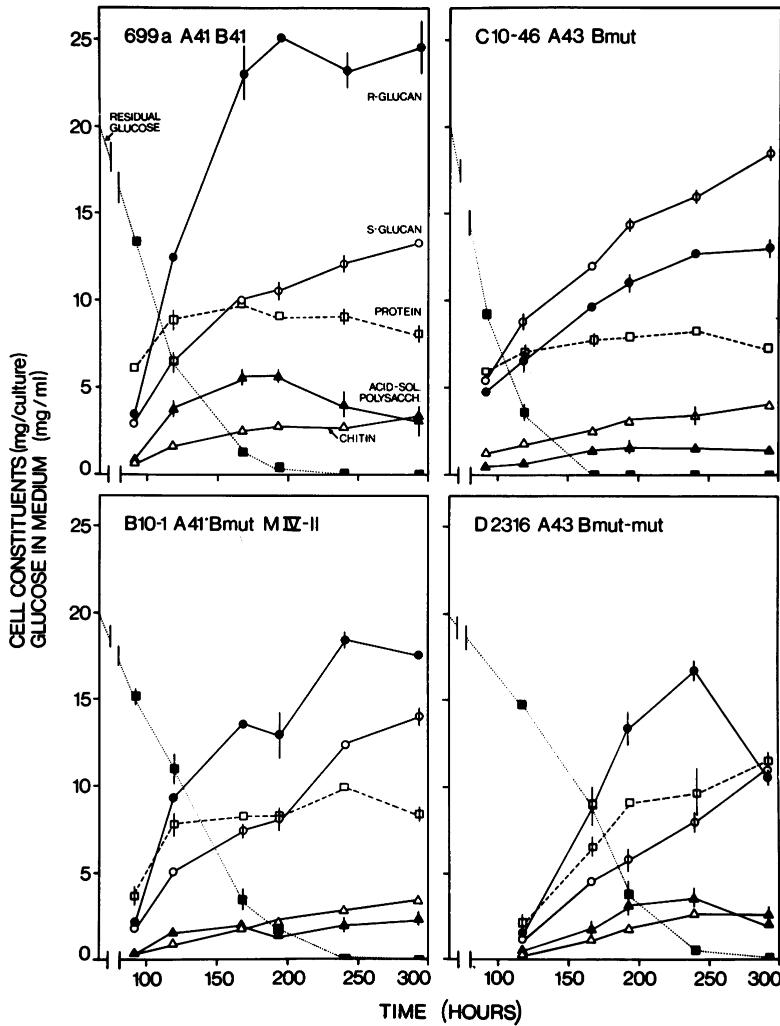

FIG. 2. *Glucose consumption and changes in polysaccharide fractions and total protein during growth of a wild-type homokaryon and B-factor mutant strains of S. commune. The cultures contained 15 ml of medium. Each point represents the mean value for duplicate cultures; the vertical lines indicate the difference between these two cultures.*

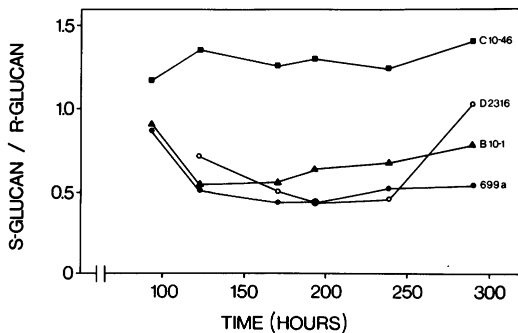

FIG. 3. *S-glucan/R-glucan ratios in a wild-type homokaryon and in B-factor mutant strains of S. commune.*

factor (D2316) restores this ratio to a value very close to that of the wild-type strain (699a).

The high S-glucan/R-glucan ratio for strain C10-46 is not only due to a decreased accumulation of R-glucan but is also influenced by a slightly increased rate of net S-glucan synthesis in this strain. Repetition of the whole experiment gave results generally in agreement except for the decrease of R-glucan in old cultures of strain D2316.

Changes in glucanase activities. Since in a common-*A* mating a high S-glucan/R-glucan ratio is correlated with a high R-glucanase activity (24), the possibility was considered that a similar relationship would hold for strain C10-46, which mimics a common-*A* hetero-

karyon. Figure 4A shows that this indeed seems to be the case; in young growing cultures of the *B* factor mutant (C10-46), high intracellular R-glucanase activities are found which are similar to those previously reported for common-*A* heterokaryons (24). The incorporation of the modifier mutation *MIV-11* in the *B*-factor mutant (B10-1) restores the low wild-type R-glucanase activity during the growth period but does not prevent a subsequent rise during late development. In the presence of a secondary mutation in the *B* factor (D2316), the R-glucanase activity in young cultures is somewhat higher than in the wild-type strain but then drops to a low level which is maintained throughout the culture period.

Since R-glucanase activity is also present in the culture fluids, the possibility exists that some of the changes depicted in Fig. 4A are due to differences in excretion of R-glucanase into the medium. Figure 4B shows that the activities of R-glucanase in the medium of strain C10-46 are generally higher than those in the media of the other strains. This clearly indicates that the high intracellular R-glucanase activities in strain C10-46 are not brought about by a decreased secretion of R-glucanase into the medium.

An important question concerns the specificity of the effect of the studied mutations on the activity of R-glucanase; therefore, the activity of other hydrolytic enzymes was also followed.

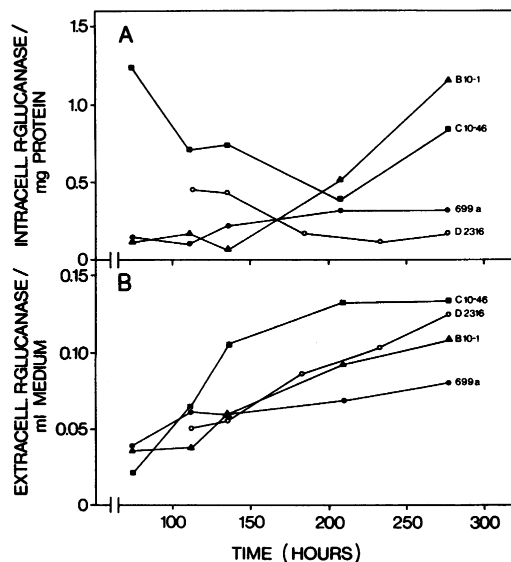

FIG. 4. *Intracellular (A) and extracellular (B) R-glucanase activities during growth of a wild-type homokaryon and B-factor mutant strains of S. commune.*

Figure 5 shows that, in older cultures, the primary *B*-factor mutants C10-46 and B10-1 exhibit a more pronounced increase in laminarinase and maltase activities than do the wild-type homokaryon (699a) and the secondary *B*-factor mutant (D2316). This is similar to the pattern observed for R-glucanase activities (Fig. 4A). However, in young growing cultures of the various homokaryons, differences in laminarinase and maltase activities do not parallel those in R-glucanase activities. Although other enzymes may be influenced similar to R-glucanase, these results suggest that, in young cultures, the examined mutations exert a more or less specific effect on the activity of R-glucanase.

It should be recalled that, with the exception of strain D2316, all cultures attain their maximal protein content at a culture age of 125 to 150 hr (Fig. 2). Consequently, only those differences in enzyme activities recorded before this culture age may be crucial to hyphal morphogenesis.

Effect of A-factor mutations. Although strain 1758 (*AmutBmut*) exhibited dikaryotic morphology in agar cultures (Table 1, Fig. 1), true clamps were rarely seen in sand-liquid cultures but pseudoclamps were abundantly present. Similarly, no fruiting bodies were produced in sand-liquid cultures. Whether this is due to the change in cultural

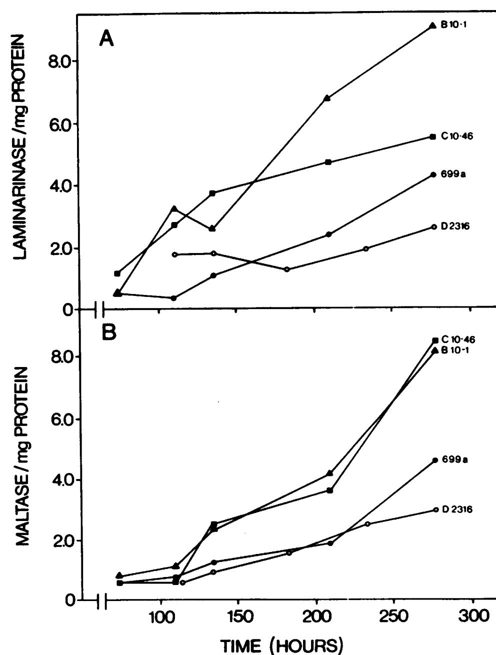

FIG. 5. *Intracellular laminarinase (A) and maltase (B) activities during growth of a wild-type homokaryon and B-factor mutant strains of S. commune.*

conditions or to the noted genetic instability of *A*-factor mutants (Raper, *personal communication*) is not known. Thus, under the present conditions, the three *A*-factor mutants, 1761, 1758, and 1779, all mimic common-*B* heterokaryons notwithstanding the additional presence of a mutated *B* factor in two of these strains.

Figure 6A shows that the S-glucan/R-glucan ratios of the *A*-factor mutants are somewhat higher than those of the wild-type homokaryon (669a). Figure 6B gives the changes in R-glucanase activities during growth, and it is clear that the elevated S-glucan/R-glucan ratios in the *A*-factor mutant strains cannot be related to increased R-glucanase activities. Analyses of the culture fluids also gave low extracellular R-glucanase activities for the mutant strains.

DISCUSSION

It seems obvious that many enzymes participate in the progression of sexual morphogenesis in *S. commune*. For some parts of the *A*-morphogenetic sequence, e.g., nuclear pairing and conjugate nuclear division, there is no suggestion as to the proteins involved. On the other hand, the events of the *B*-morphogenetic sequence, i.e., dissolution of complex septa and possibly hook-cell fusion, can reasonably be attributed to the activity of lytic enzymes affecting cell membranes and cell-wall substances. During dikaryotization, dissolution of septa only occurs during nuclear migration (5); one apparent function of the *A* factor is to switch off this part of the *B*-morphogenetic sequence. When the *B*-morphogenetic events are not curbed by the *A*-morphogenetic sequence, they act continuously. This is the case in the common-*A* heterokaryon and in homokaryons with a primary mutation in the *B* factor. These mycelia grow with little aerial hyphae; they have abundant simple septa that allow free passage of nuclei, the hyphae are irregularly shaped, and extrusion of protoplasm can sometimes be observed (6, 8, 9, 19). The latter phenomena, too, may be caused by high activities of a cell-wall material-hydrolyzing enzyme that creates weak spots in the cell wall that cannot resist the turgor pressure.

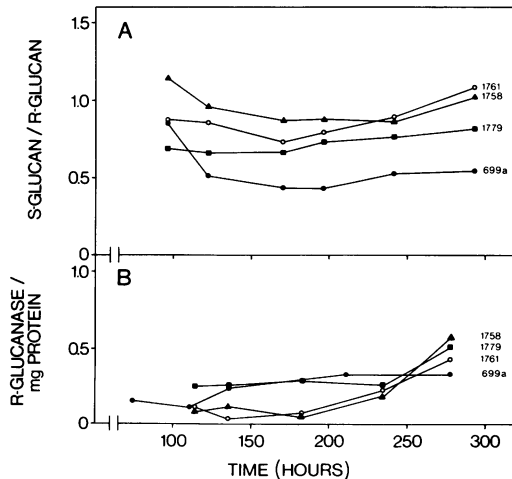

FIG. 6. *S-glucan/R-glucan ratios (A) and intracellular R-glucananase activities (B) during growth of a wild-type homokaryon and A-factor mutants of S. commune.*

In a previous study (24), a much higher S-glucan/R-glucan ratio (1.37) was found in a common-*A* heterokaryon than in the parental homokaryons (S-glucan/R-glucan ratios of 0.62 and 0.41, respectively). In young cultures, this was correlated with at least a 10–fold difference in the intracellular R-glucanase activity. Comparison of the primary *B*-factor mutant and the isogenic wild-type homokaryon in the current study demonstrates a similar relationship; the averaged S-glucan/R-glucan ratios for the two strains are 1.29 and 0.54, respectively, and this can be correlated with a nearly 10–fold difference in the activity of R-glucanase in mycelial extracts from young cultures. These results can be provisionally interpreted as repression of R-glucanase in normal homokaryons and derepression of this enzyme in the presence of a single *A* factor and either two nonallelic *B* factors or one mutated *B* factor. Although differences in the R-glucan synthesizing system cannot be ruled out, the enhanced R-glucanase activity may be responsible for the observed decrease in the net synthesis of R-glucan and, hence, for the aberrant morphology of the common-*A* heterokaryon and the primary *B*-factor mutant strain.

Modifier mutations of type IV restore wild-type homokaryotic morphology in a *B*-factor mutant (12). The results show that this is correlated with a reversal to a wild-type S-glucan/R-glucan ratio and R-glucanase activity. However, this low R-glucanase activity is only maintained during the major growth period and it increases again after exhaustion of the glucose in the medium. Apparently, the *MIV* mutation prevents expression of the *B*-factor mutation only as long as glucose is present. In terms of a repressor model, this means a transition from derepression to catabolite repression of R-glucanase. The suppression of R-glucanase activity during growth could explain the restoration of a normal S-glucan/R-glucan ratio in the cell wall and, hence, a return to wild-type morphology in a *B*-factor mutant.

A similar transition from derepression to catabolite repression of R-glucanase was also suggested

to explain the difference between common-*A* heterokaryons and dikaryons (24). In dikaryons, too, the activity of R-glucanase only increases after exhaustion of the carbon supply in the medium, and this has been related to fruiting-body expansion (22). This is consistent with the observation that *MIV* mutations are not expressed in established dikaryons, not even in double dose (12).

The secondary mutant of the *B* factor which was examined resembles wild-type strains with respect to morphology, S-glucan/R-glucan ratio, and R-glucanase activity. However, in young cultures higher levels of R-glucanase are found than in the wild-type homokaryon. This suggests that the secondary mutation is not completely effective in restoring R-glucanase repression. In this context, it may be significant that mating reactions have shown that the secondary mutation is not completely equivalent to the wild-type allele (18).

In comparison with the wild-type homokaryon, strains with a mutated *A* factor do not exhibit significantly altered R-glucanase activities. Nevertheless, the S-glucan/R-glucan ratio is slightly elevated in these strains. This is similar to results previously obtained with a common-*B* heterokaryon (24). Obviously, in these cases the effect on cell-wall glucan synthesis is brought about by a mechanism different from that suggested for the *B*-morphogenetic pathway.

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