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Aspergillus nidulans Mutant Lacking α-(1,3)-Glucan, Melanin, and Cleistothecia

I. POLACHECK AND R. F. ROSENBERGER*

Department of Microbiological Chemistry, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

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A mutation in *Aspergillus nidulans* led to a loss of both melanin and α-(1,3)-glucan, a major wall polysaccharide. In addition, the mutation prevented the formation of cleistothecia. Mutant walls contained increased amounts of β-(1,3)-glucan and galactose polymers, and electron micrographs indicated that they had lost the outermost wall layer. Such walls were more readily digested by lytic enzymes, and this increased susceptibility to hydrolysis was due to the absence of α-(1,3)-glucan and not of melanin. The pleiotropic effects of the mutation are discussed, with particular reference to the hypothesis that α-(1,3)-glucan acts as the endogenous carbon source for biosynthetic processes in the stationary phase of growth. In this view, glucan synthesis would be the primary target of the mutation, and the absence of glucan would result in the lack of melanin and cleistothecia, formed after nutrients are exhausted. Two other mutations that lowered the mycelial α-(1,3)-glucan content also inhibited melanin and cleistothecia production.

Hyphal walls of *Aspergillus nidulans* have been shown to contain chitin, α-(1,3)-glucan, β-(1,3)-glucan, mannose- and galactose-containing polysaccharides, proteins, and melanin (3, 17, 19, 22). As yet, however, relatively little is known about the functions of the individual wall components or about the mechanisms coordinating wall assembly. One approach to clarifying such questions would be the study of mutants unable to form particular wall polymers. In the present paper we describe the effects of a mutation, originally isolated by Martinelli and Bainbridge (15), that markedly alters the wall composition of *A. nidulans* hyphae.

The mutation *melB*°$_2$ was selected for, and described as leading to, a loss of melanin synthesis (15). We found that, in addition to lacking melanin, strains carrying *melB*°$_2$ failed to produce α-(1,3)-glucan and contained markedly increased amounts of β-(1,3)-glucan and a galactose polymer in their walls. Furthermore, such strains formed no cleistothecia, and their walls were more susceptible to autolytic enzymes. The pleiotropic changes induced by *melB*°$_2$ are discussed in relation to the suggestion that α-(1,3)-glucan acts as an endogenous carbon source for metabolic processes in the stationary phase of growth (23-25).

MATERIALS AND METHODS

Strains, media, and growth conditions. *A. nidulans* strains SM16 and BWB152 (15) were the kind gifts of S. Martinelli and B. W. Bainbridge. 13.1.OL (3) was a gift from A. Bull, and a strain carrying the mutation *acl*1 (25) was a gift from B. J. M. Zonneveld. Liquid cultures of these strains and of R21 (7) were grown in a 2% (wt/vol) glucose-mineral salts medium (17) supplemented with biotin and *p*-aminobenzoic acid under the conditions described previously (17). Production of melanin and cleistothecia was followed on Czapek-Dox agar and on the above glucose-salts medium (but with twice the salts and half the phosphate concentration) solidified with agar.

To radiolabel hyphae with [*U*-^{14}C]glucose or [1-^{3}H]glucose, the media and schedules described previously (17) were used, with the following minor modifications. In determining wall turnover, labeling was carried out in medium containing 2% (wt/vol) glucose and 0.1 μCi of [^{14}C]glucose per ml. When differential labeling of lateral and tip walls was the aim, the medium contained 1% (wt/vol) glucose and 0.25 μCi of [^{3}H]glucose per ml for the lateral wall and 0.5% (wt/vol) glucose and 1 μCi of [^{14}C]glucose per ml for the apical wall.

Preparation of walls and autolysins. Hyphal walls were isolated after sonic treatment and purified by differential centrifugation (17). When walls free from autolysins were required, the walls were inactivated by heating with sodium dodecyl sulfate (17). To obtain free autolysins, the walls were allowed to autolyze in 0.05 M phosphate buffer, pH 7 (17), and the mixture was filtered through a membrane filter (average pore size, 0.45 μm). The filtrate, containing the autolysins, was dialyzed and lyophilized.

In the experiments measuring wall turnover, replicate flasks were inoculated with equal numbers

of conidia. After growth with [^{14}C]glucose, the mycelia from both flasks were harvested (17). The total mycelial contents of one flask were resuspended in fresh medium containing unlabeled glucose and incubated further (mycelium A); the mycelial contents of the second flask were washed, dried, and weighed (mycelium B). After growth with unlabeled glucose, mycelium A was harvested, washed, dried, and weighed, and walls were purified from both mycelium A and mycelium B as described by Gooday (9). We added an extra step and further treated the purified walls with 2.5 mg of Pronase per ml for 60 min at 37°C. The cytoplasmic contamination, as measured by ribonucleic acid content, was less than 3%. The purified walls were then hydrolyzed, and the ^{14}C content was determined. If it occurs, wall turnover will result in a loss of ^{14}C during incubation with unlabeled glucose.

Fractionation of walls with KOH. α-(1,3)-Glucan was extracted from walls by pretreatment with acetic acid and incubation with 5% KOH as described by Zonneveld (23). We introduced the minor modifications of carrying out the alkali treatment under an atmosphere of N_2. The KOH extracts were neutralized with acetic acid to precipitate α-(1,3)-glucan, and this was collected by centrifugation and washed with water. The precipitate, the supernatant fluid, and the washings were all assayed for sugars, amino sugars, and amino acids after hydrolysis in 3 N HCl for 3 h at 100°C and in 6 N HCl for 18 h at 120°C.

Lysis of hyphae by Helicase. Cultures were grown for 17 h with [^{14}C]glucose, and the mycelium was collected by filtering through fine-mesh nylon netting. The washed, labeled mycelium was suspended in 0.05 M phosphate buffer, pH 7, containing 10 mg of Helicase (Industrie Biologique Francaise, S.A. Glenvilliers, France) per ml to give a final mycelium concentration of 1.5 mg (dry weight)/ml and incubated at 37°C. Samples, taken at intervals, were filtered through glass-fiber papers (GF/C, Whatman, Maidstone, England), and the radioactivity in the filtrate and on the filter was determined.

Wall hydrolysis, chemical assays, and radioisotope determinations. Walls and wall fractions were hydrolyzed with 3 or 6 N HCl (17). Sugar, amino sugar, and amino acid assays were carried out as described previously (17) except for galactose, which was determined with galactose dehydrogenase (Sigma Chemical Co., St. Louis, Mo.). Sugars and amino sugars were identified chromatographically as described previously (17). Radioisotopes were assayed by liquid scintillation counting in a Tri-Carb spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Electron microscopy. Walls were fixed and sectioned as previously described (17). Sections were stained with 1% (wt/vol) uranyl acetate in absolute ethyl alcohol and with Reynold's lead citrate (18).

RESULTS

Wall composition of *melB*$^{c}_{2}$ and wild-type strains. Walls purified from the *melB*$^{c}_{2}$ strain, SM16, contained approximately three times as much galactose and 20% less glucose than those from the parent strain, BWB152, or from R21, another derivative of the Glasgow wild type (Table 1). Fractionation of the walls (22) to determine α-(1,3)-glucan (S-glucan, alkali soluble) and β-(1,3)-glucan (alkali insoluble) showed that the *melB*$^{c}_{2}$ mutation induced striking differences in the content of these polymers (Table 2). α-(1,3)-Glucan was absent in SM16, and the amounts of glucose-containing polymer

TABLE 1. *Monomers found in acid hydrolysates of purified walls*

Strain	Percent of wall dry wt				
	Glucose	Galactose	Total sugar[a]	Glucosamine	Amino acid
R21	32	4.2	47.0	16.8	8.9
BWB152	28.4	3.5	40.1	17.3	8.4
SM16	23.8	14.1	46.3	16.5	8.3

[a] Determined with anthrone and glucose as standards.

TABLE 2. *Monomers found in acid hydrolysates of the alkali-soluble and alkali-insoluble fractions of purified walls*

Strain	Fraction[a]	Percent of wall dry wt				
		Glucose	Galactose	Total sugar[b]	Glucosamine	Amino acids
R21	S-glucan	12.1	0	14.2	0	0.6
BWB152	S-glucan	12.0	0	13.2	0	0.7
SM16	S-glucan	0.14	0	0.14	0	0.5
R21	Alkali insoluble	11.4	2.3	16.8	15.9	8.5
BWB152	Alkali insoluble	8.4	1.4	11.0	13.9	5.9
SM16	Alkali insoluble	21.0	5.0	29.0	19.2	8.3

[a] S-glucan is alkali-soluble polysaccharide, which precipitates at neutral pH; alkali-insoluble material is the remainder of the wall. For details see text.

[b] Determined with anthrone and glucose as standards.

not extracted by alkali had increased to restore the glucose content to almost its normal level. Whole walls and alkali-soluble and -insoluble fractions from strains BWB152 and SM16 were hydrolyzed by acid and chromatographed to determine the monomer composition. The S-glucan fraction contained only glucose; in whole walls and in the alkali-insoluble fractions, glucose, galactose, mannose, glucosamine, and amino acids were found. The walls of both strains contained very little galactosamine (0.12% of the wall dry weight) and SM16 thus differs from another melaninless mutant that was reported to have a high galactosamine content (3). In addition to the S-glucan, KOH extracted glucose- and galactose-containing polymers, which did not precipitate at neutral pH, from walls of strain R21 and BWB152. These, together with the S-glucan and alkali-insoluble fractions, accounted for more than 90% of the sugars detected in whole walls. Alkaline extracts of strain SM16 walls contained neither S-glucan nor glucose that remained in solution at neutral pH. Such extracts contained galactose that was not precipitated by neutralization and accounted for 64% of the galactose in whole walls.

Morphology of walls in thin sections. Under the electron microscope, walls of wild-type hyphae appeared to contain an inner electron-dense layer, a more translucent layer exterior to this, and an irregular, electron-opaque layer at the outside surface (Fig. 1-2). Walls from the *melB*$^{c}_{2}$ mutant were thinner and appeared

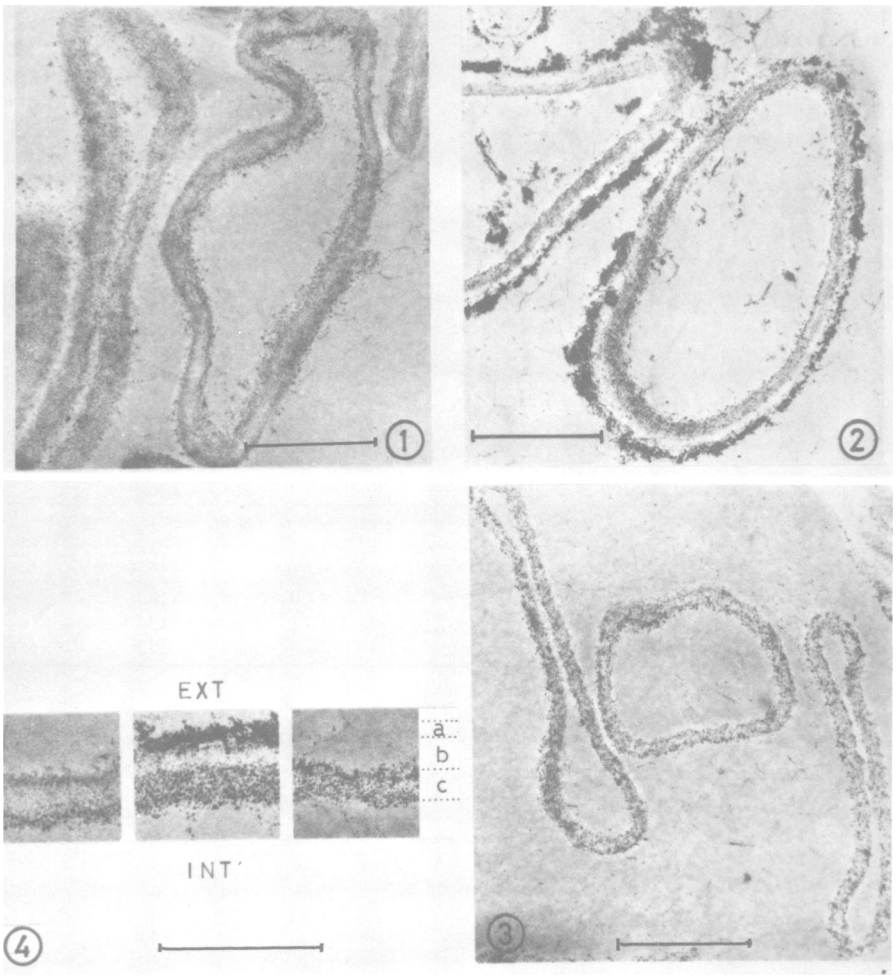

FIG. 1. *Sections through purified A. nidulans walls after staining with uranyl acetate and lead citrate. (1) SM16 walls (bar = 1 μm). (2) R21 walls. Note the three layers in wall (bar = 1 μm). (3) R21 walls, alkali-extracted to remove α-(1,3)-glucan. The two outer layers have been lost (bar = 1 μm). (4) From left to right: Composite of SM16, R21, and R21 alkali-extracted walls to illustrate the layers present in (2) and absent in (1) and (3). a, b, and c represent the three layers (bar = 0.5 μm).*

to have lost the two outer layers (Fig. 1-1). To determine whether loss of α-(1,3)-glucan can affect the banded appearance of walls, wild-type hyphae were extracted with alkali by the same procedure used to remove α-(1,3)-glucan (22). After this treatment, the walls contained only the inner electron-dense layer and were thinner, but similar in appearance to those of the *melB*°$_2$ strain (Fig. 1-3, 1-4).

Susceptibility of walls to digestion by hydrolytic enzymes. The action of autolysins on walls of R21 and SM16 was compared in two ways. These were (i) rates of hydrolysis by the enzymes that remain attached during the wall purification (17) and (ii) hydrolysis by added autolysin after the attached enzymes had been inactivated with sodium dodecyl sulfate (17). In both instances, SM16 walls were markedly more susceptible to digestion than were those of R21 (Fig. 2A, B). The much greater lysis of intact hyphae of SM16 by Helicase (Fig. 3) confirmed the difference between the two strains in resistance to enzyme hydrolysis.

The relatively rapid rate at which isolated SM16 walls were digested by bound, endogenous enzymes prompted us to determine the rate of wall turnover in growing hyphae. Both in strain SM16 and in R21, wall turnover was either absent or so low that it could not be detected by our method (Table 3).

Action of hydrolytic enzymes on apical and lateral walls. The apical walls of wild-type *A. nidulans* hyphae are better substrates for hydrolytic enzymes than the older, lateral walls (17). We therefore investigated whether apical walls of SM16 were also more easily digested or whether the whole wall had acquired the susceptibility normally characteristic of the tip. After differential labeling of the lateral and tip walls, the rates at which enzymes released monomers containing the two isotopes were followed (Table 4). Enzymes liberated the label from apical wall several times faster than would have been predicted from the isotope ratios after complete acid hydrolysis, and the apical wall was thus more susceptible to enzyme action.

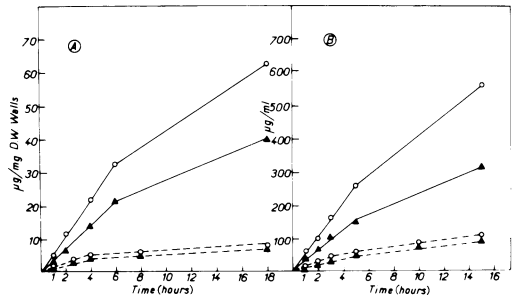

FIG. 2. *Hydrolysis of SM16 and R21 walls by autolysins. (A) Solubilization of wall components by endogenous autolysins remaining attached during purification. Symbols: (——) SM16; (- - -) R21; (○) glucose; (▲) N-acetylglucosamine. (B) Liberation of wall components by antolysins from sodium dodecyl sulfate-treated walls (10 mg/ml). Symbols as in (A).*

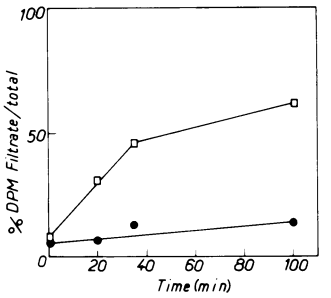

FIG. 3. *Lysis of whole hyphae by Helicase. Labeled hyphae (see text) were suspended in a solution of Helicase, and the radioactivity solubilized from hyphae is presented as a fraction of the total. Symbols: (□) SM16; (○) R21.*

TABLE 3. *Turnover of wall polymers in strains R21 and SM16*

Strain	Growth schedule[a]	Wall dry wt at harvest (mg/flask)	Total dpm in wall hydrolysates[b]	
			3 N HCl	6 N HCl
R21	14.5 h, ^{14}C	11.6	21,878	8,510
	14.5 h, ^{14}C 3.5 h, ^{12}C	25.4	23,575	8,282
SM16	14.5 h, ^{14}C	15.9	130,848	30,560
	14.5 h, ^{14}C 3.5 h, ^{12}C	41.4	165,360	29,064

[a] After growth with [^{14}C]glucose, the whole contents of replicate flasks were taken for analysis or resuspension in medium with [^{12}C]glucose. After 3.5 h, the resuspended cultures were harvested.

[b] Walls purified from the whole contents of replicate flasks were hydrolyzed in 3 N HCl for 3 h at 100°C (sugars), and the insoluble residue was hydrolyzed for a further 18 h at 120°C in 6 N HCl (glucosamine and amino acids). dpm represents disintegrations per minute, i.e., counts per minute corrected for counting efficiency and quenching.

Cleistothecia formation by SM16 and α-(1,3)-glucan content of other strains not forming cleistothecia or melanin. SM16 produced no cleistothecia under conditions where BWB152 and R21 formed large numbers. A possible relationship between α-(1,3)-glucan content, melanin synthesis, and cleistothecia production was thus examined in two additional strains; 13.1.OL, altered in melanin synthesis (3), and *acl*1, altered in ability to form cleistothecia (25). Cleistothecia and melanin are formed by stationary-phase cultures on solid media, and although their presence or absence can be detected with reasonable cer-

tainty, quantitation presents very considerable difficulties. Within the limits of the methods, however, a positive correlation between the α-(1,3)-glucan content of hyphae and the production of melanin and cleistothecia was found (Table 5).

DISCUSSION

The multiple changes found in strain SM16 raise the question of whether the *melB*$^{c}_{2}$ mutation is, in fact, a single-point mutation. The genetic evidence available does not provide a clear answer. One phenotypic change, the loss of melanin, can be readily scored in recombinants and behaves as a single mutation located on chromosome VII (15). The other changes that we have described cannot, however, be easily scored in a large number of recombinants. The recombination data are, therefore, very limited. They indicate that the determinants of the *melB*$^{c}_{2}$ phenotype are all located on the same chromosome and have so far shown no segregation between melanin loss and other markers. In our most extensive meiotic analysis, complete linkage was found between sensitivity to lytic enzymes and lack of melanin in 30 recombinants from a cross to wild type. The findings are thus in keeping with *melB*$^{c}_{2}$ being a single mutation with pleiotropic effects. In addition, ultraviolet light, the mutagen used (15), is not known to induce closely linked, multiple mutations. However, because of the small number of recombinants and since none of the phenotypic changes lend themselves to the selection of revertants, the possibility that *melB*$^{c}_{2}$ represents several linked mutations cannot be ruled out.

If the MelB$^{c}_{2}$ phenotype is indeed due to a single mutation, this could be a control mutation affecting several functions. It could also be a mutation blocking the synthesis of a single wall component required for the formation of other constituents and of cleistothecia. These speculative possibilities are raised because the latter one fits closely with recent studies on the function of α-(1,3)-glucan (23–25).

α-(1,3)-Glucan has been shown to be a stable constituent of vegetative hyphal walls and to be degraded when vegetative growth ceases (23, 25). By a variety of approaches, Zonneveld has further demonstrated a correlation between α-(1,3)-glucan content and the numbers of cleistothecia formed on starvation (23–25). On this basis, he suggested that α-(1,3)-glucan serves as the endogenous carbon source for sexual differentiation. If the *melB*$^{c}_{2}$ mutation directly affects only α-(1,3)-glucan synthesis, the above hypothesis would predict simultaneous loss of cleistothecia.

Further melanin, like cleistothecia, is not synthesized during exponential vegetative growth (3, 11, 20) but only when the nutrient supply is very low or exhausted. In wild-type *A. nidulans*, melanin can account for 20% of the wall dry weight (3), and considerable amounts of carbon and energy would be required for its synthesis. If α-(1,3)-glucan provides carbon and energy not only for cleistothecia formation but also for other synthetic processes, its absence would lead to a loss of melanin. Strains carrying *melB*$^{c}_{2}$ should in this case form the enzymes required for melanin synthesis, and indeed they contain phenol oxidases (5, 10, 11, 15). A direct test of the possibility that the mutation primarily affects only α-(1,3)-glucan synthesis must await clarification of the steps in glucan and melanin (1, 2, 10, 11, 14) biosynthesis and assays of the enzymes in these pathways. That two other, independently isolated, mutants (*acl*1 and 13.1.OL) with low levels of α-(1,3)-glucan also formed small amounts of or no melanin and cleistothecia does lend support to this view. It may be pointed out that *melB*$^{c}_{2}$ hyphae form heterokaryons and are thus capable of hyphal fusions and that *A. nidulans* mycelium is self-fertile. Lack

TABLE 4. *Susceptibility of apical and lateral walls of SM16 to enzyme hydrolysis*

Hydrolysis	dpm/ml of hydrolysate[a]		
	^{14}C	^{3}H	$^{14}C/^{3}H$
Enzymatic			
30 min, 37°C	25,037	3,307	7.58
60 min, 37°C	34,575	5,433	6.36
HCl, 6 N, 18 h	93,737	35,178	2.66

[a] Hyphae were grown for 18 h with [^{3}H]glucose (lateral wall) and then for 0.5 h with [^{14}C]glucose (apical wall). After purification of the wall fraction, one portion was hydrolyzed with *A. nidulans* autolysins (see text), and another was hydrolyzed with acid.

TABLE 5. *α-(1,3)-Glucan content, cleistothecia formation, and melanin synthesis by the wild-type and mutants*

Strain	α-(1,3)-Glucan (mg/100 mg of wall)	Melanin formation[a]	Cleistothecia formation[a]
R21	12	+++	+++
*acl*1	3.5	+	+
13.1.OL	1.8	−	−
SM16	0.15	−	−

[a] Symbols: +++, Strong pigmentation or large number of cleistothecia; +, melanin or cleistothecia formed, but weak pigmentation or small numbers; −, pigmentation absent or cleistothecia not seen.

of cleistothecia is thus unlikely to be due to blocks in cell fusion or nuclear migration.

Irrespective of its genetic nature, the *melB*°$_2$ mutation can be used to clarify some aspects of the location and function of α-(1,3)-glucan in the wall. This is possible because melanin is always absent from vegetative hyphae during exponential growth (20), and the qualitative difference between wild-type hyphae and those carrying *melB*°$_2$ will be a lack of α-(1,3)-glucan. Thus, the changes in the wall morphology of strain SM16 show that the glucan is a major component of the outermost wall layer. A similar location for this polymer has also been established in walls of *Schizophyllum commune* (12, 21) and *Agaricus bisporus* (16).

Furthermore, α-(1,3)-glucan appeared to protect young, nonmelanized hyphae from the action of hydrolytic enzymes. This protection is presumably due to the glucan's ability to hinder β-glucanases and chitinase from reaching their substrates (6, 7) and to the absence of α-(1,3)-glucanase in extracts of *A. nidulans* (25) and snail gut (6, 7). The rapid wall dissolution caused by exogenous enzymes in *melB*°$_2$ strains has, in our hands, greatly facilitated the isolation of *A. nidulans* protoplasts and may have value in such research. It also illustrates the efficiency of the in vivo controls on autolytic enzymes. Although its walls are much better enzyme substrates, growing hyphae of SM16 showed no more wall turnover than did those of the wild type. It is of interest that melanin fulfills a protective function, similar to that of α-(1,3)-glucan, in old or differentiated hyphae which, in *A. nidulans* at least, no longer contain α-(1,3)-glucan (25). The widespread occurrence of melanin in differentiated fungal structures is well documented (1, 3, 8, 11, 14, 20), as is its inhibition of enzymatic wall hydrolysis (4, 13).

We have previously shown that the young, apical walls in wild-type hyphae are a better substrate for autolytic enzymes than are older, lateral walls (17). The question thus arose of whether the protective effect shown by α-(1,3)-glucan could account for this difference in sensitivity. If the apical walls of wild-type hyphae are better enzyme substrates because they do not contain α-(1,3)-glucan whereas lateral walls do, then such differences should disappear in strains carrying *melB*°$_2$. In addition, apical walls from SM16 and from the wild type should be equally susceptible to enzyme action. In fact, apical walls of SM16 proved better substrates than either older walls of the same strain (Table 4) or apical walls of the wild type (results not shown). We take this to indicate that the glucan is inserted along the whole hypha, including the newly formed wall at the apex. This conclusion is based on the assumption that the presence of α-(1,3)-glucan is only one of several factors that affect the sensitivity of walls to autolytic enzymes.

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