JOURNAL OF BACTERIOLOGY, Jan. 1975, p. 332-337

Vol. 121, No. 1
Printed in U.S.A.

Autolytic Enzymes in Hyphae of *Aspergillus nidulans*: Their Action on Old and Newly Formed Walls

Y. POLACHECK AND R. F. ROSENBERGER*

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

Received for publication 15 July 1974

Walls, purified from hyphae of the ascomycete *Aspergillus nidulans*, autolyzed on incubation and liberated glucose, mannose, galactose, *N*-acetylglucosamine, and soluble oligosaccharides. Digestion proceeded at linear rates until approximately 3% of the wall polymers had been hydrolyzed and then slowed markedly. The change in the rate of autolysis was not due to loss of enzyme activity but was caused by the disappearance of a fraction of the wall which was highly susceptible to digestion. Radioactive labeling showed that this fraction was the newly formed wall. The new wall was highly susceptible to enzyme action both when it was deposited at the apex in growing hyphae or when deposited laterally in hyphae treated with cycloheximide. The relations between wall modification and apical growth are discussed.

Microorganisms commonly produce enzymes which can hydrolyze polymers in their own walls (for review, see 3, 6), and these are considered to play important roles in cell growth. Functions in which autolysin action has been implicated include daughter cell separation (2), wall extension (5, 6), morphogenesis (22-24), and cell fusion (12).

To participate in any of these processes, autolytic enzymes must act on particular sites in the wall and in a controlled manner. There is, however, very limited information on the distribution of autolysins in the cell envelope (18), on the factors that affect this distribution (13), and on how the extent of their activity is regulated (17). This is particularly true of fungi, where studies have mainly aimed at demonstrating the presence of autolytic enzymes (11, 23, 24).

The vegetative hyphae of filamentous fungi are an extreme example of wall metabolism restricted to a specific site, since new wall is deposited only at the apex, whereas protoplasm extending back for hundreds of microns supplies precursors for wall synthesis (8, 21). They would thus appear to be well suited for studying site-specific activities of wall hydrolases. In the present work, we have investigated if wall autolysins act at the sites of wall formation in hyphae of *Aspergillus nidulans* and how their activity is controlled. Our results, based on kinetics of autolysis and differential labeling of lateral and apical walls, show that newly formed wall was indeed digested preferentially. However, this did not appear to be due to a specific location of autolysins at the sites of wall synthesis, as in *Streptococcus faecalis* (18), but to an increased resistance of older wall.

MATERIALS AND METHODS

Strain, media, and growth conditions. *Aspergillus nidulans* R21 (yellow conidia and requiring *p*-aminobenzoic acid) was grown on a mineral salts (19) medium containing 3 g of $(NH_4)_2SO_4$, 1 mg of *p*-aminobenzoic acid, and either 1 or 5 g of glucose per liter. Conidial suspensions for inocula were prepared as previously described (19), and cultures were incubated on a rotary shaker at 37 C.

Continuous radioactive labeling of mycelium was carried out by inoculating conidia into medium containing 5 g of glucose per liter and 0.05 μCi of [U-^{14}C]glucose (specific activity, 3 mCi/mmol; Radiochemical Centre, Amersham) per ml and incubating for 15 to 20 h. To label for short periods, a 15- to 20-h culture in medium containing 5 g of glucose per liter was rapidly filtered through fine-mesh nylon netting and the mycelium on the filter was washed with prewarmed medium and resuspended in medium containing 1 g of glucose per liter. [U-^{14}C]glucose or [1-^{3}H]glucose (specific activity, 5.3 Ci/mmol; Radiochemical Centre, Amersham) was then added to give final concentrations of 0.5 and 5 Ci/ml, respectively, and incubation was continued for 60 or 90 min.

Preparation of purified hyphal walls. Batches of walls were routinely prepared from 400 ml of a 15- to 20-h culture. The mycelium was collected on nylon nets by filtration, washed, and suspended in cold 0.5 M phosphate buffer (pH 7). The mycelium, suspended in 50 ml of buffer, was homogenized in an Omnimix (Sorvall Corporation, Newtown, Conn.) at 16,000 rpm for 5 min with cooling. The hyphae were then broken in a Sonifier, (model S110, Branson

Corp., Danbury, Conn.) fitted with a cooling vessel. Sonic treatment was carried on at full power output for periods of 15 min, followed by a 5-min cooling interval, until only broken hyphae were seen under the phase microscope. This required a minimum of 45 min of sonic treatment.

The wall fraction was purified by repeated centrifugations at 12,000 × *g* for 10 min and at 120 × *g* for 10 min in the cold and the precipitate was washed with 0.5 M phosphate buffer (pH 7). Finally, the walls were washed three times with distilled water by centrifuging at 12,000 × *g* for 10 min.

To obtain walls free of autolytic enzymes, the wall fraction prepared as above was heated for 5 min at 100 C in 4% (wt/vol) sodium dodecyl sulphate (SDS) and then washed by centrifugation to remove the detergent.

Autolysis of isolated walls. Walls were suspended in 0.02 M phosphate buffer, (pH 7) containing 100 μg of merthiolate per ml and incubated with shaking at 37 C. The concentration of walls in the suspension was determined by drying and weighing and was usually approximately 10 mg/ml. Samples taken for analysis were cooled and centrifuged at 12,000 × *g* for 10 min in the cold, the supernatant was filtered through a membrane filter (average pore size 5 μm; Millipore Corporation, Bedford, Mass.), and the filtrate was used for further analysis.

Isolation of the products of autolysis and of chemical hydrolysis. The supernatants from autolyzing walls were shaken with Dowex 1 and Dowex 50 ion-exchange resins to separate neutral from charged compounds. The resin was removed by centrifugation and washed three times with water, and the supernatants containing the neutral sugars were combined. Charged compounds were extracted from the resins with 3 N HCl.

Sugars were separated by thin-layer chromatography on cellulose plates (0.16 mm thickness; Eastman Kodak Co., Rochester, N.Y.) with ethyl acetate–pyridine–water (8:2:1) as solvent. Each plate was developed five times, and spots were located with alkaline silver nitrate. When determining specific activities, the sugars were first heated for 60 min at 100 C in 3 N HCl or 2 N H_2SO_4 to hydrolyze any oligosaccharides present, the acids were removed by drying or the addition of barium carbonate, and the monomers were chromatographed. Sugars were eluted from the chromatograms by scraping off the appropriate regions of cellulose and extracting with hot methanol. Radioactivity in the eluates was determined by liquid scintillation counting, and sugar concentration was determined by the colorimetric assays described below. The charged compounds eluted from the ion-exchange resins consisted solely of amino acids, and these were separated and estimated on an automatic amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.).

Walls were hydrolyzed chemically by heating in 3 N HCl or 2 N H_2SO_4 at 100 C for 3 h to obtain free sugars and at 120 C for 18 h to hydrolyze chitin and proteins. The acids were removed either by drying or by the addition of barium carbonate. The specific activities of monomers in acid hydrolysates were determined as described above for autolysates with the following exception. During autolysis, chitin was degraded to *N*-acetylglucosamine and the acetylated amino sugar was analyzed together with the neutral sugars. Acid hydrolysis, however, produced free glucosamine which was bound by the ion-exchange resins and then eluted together with the amino acids. Glucosamine was therefore assayed in the amino acid analyzer, and its radioactivity was determined by passing the column effluent from the amino acid analyzer through a flow cell fitted in a Tri-Carb spectrometer (Packard Instruments Co., Inc., Downers Grove, Ill.).

Chemical assays. In the supernatants of autolyzing walls, sugars were assayed with anthrone (20) with glucose as standard and free glucose with glucose oxidase (Glucostat, Worthington Biochemical Corporation, Freehold, N.Y.). Acetylated amino sugars were determined as described by Reissig (14), free amino sugars by the method of Levvy and McAllen (10), and amino acids with ninhydrin (1).

After elution from chromatograms, sugars were assayed with anthrone (20) using the appropriate sugar as standard and amino sugars as described above.

Electron microscopy. Walls were fixed and stained with 1.5% (wt/vol) aqueous $KMnO_4$ for 30 min at 4 C, washed with distilled water, and dehydrated through ethanol. They were then embedded in Epon 812 as described previously (19). Ultrathin sections were cut with a diamond knife on an LKB Ultratome III and examined in a Phillips EM 300 electron microscope.

RESULTS

Autolysis of isolated walls. Purified walls, incubated in buffer containing merthiolate to prevent bacterial contamination, released soluble sugars, amino sugars, and amino acids (Fig. 1). These compounds were liberated at linear rates for the first 3 to 5 h, and then the rate of

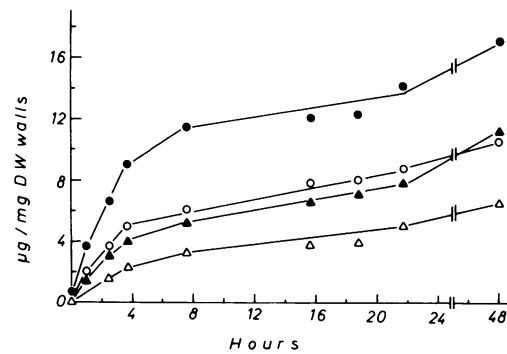

FIG. 1. *Autolysis of purified A. nidulans walls. Walls, at a concentration of 41.6 mg/ml, were incubated at pH 7, and sugars and amino acids in the supernatant were estimated. Symbols:* ●, *neutral sugars, anthrone with glucose standard;* ○, *glucose oxidase;* ▲, *amino sugars;* △, *amino acids, ninhydrin with arginine standard.*

digestion slowed markedly. In the pH range of 5.5 to 8, autolysis was most rapid at pH 7, and after 48 h at this pH, approximately 3% of the wall (dry weight) had been solubilized (Fig. 1). No soluble compounds were liberated from walls heated with SDS (results not shown).

The great reduction in the rate of autolysis after only a fraction of the wall had been hydrolyzed suggested that either the enzymes had lost activity or that easily accessible substrate had been utilized. The addition of fresh walls, which had been heated with SDS, showed that enzyme activity had not been lost and that disappearance of substrate was responsible for the change in rate (Fig. 2). The liberation of amino acids did not increase in rate when fresh SDS-treated walls were added (results not shown), but this is not surprising, since SDS would be expected to remove the protein from the wall preparation.

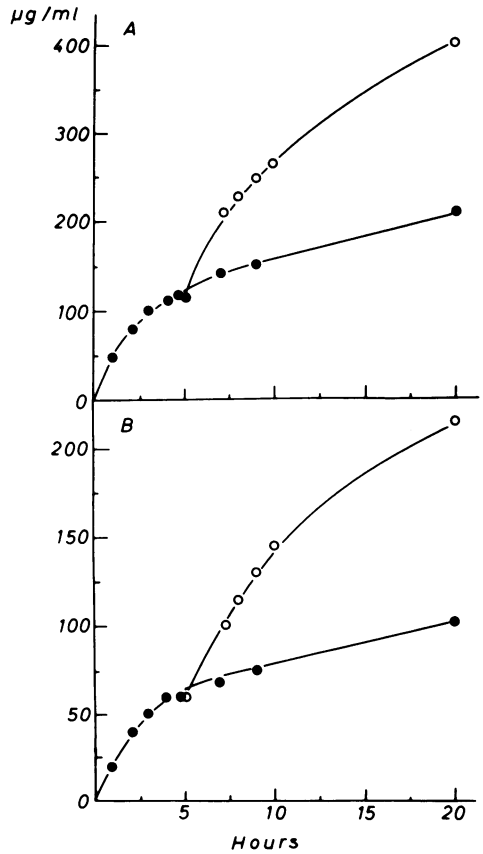

FIG. 2. *Effect of adding SDS-treated walls to autolyzing walls. Walls, at a concentration of 8.2 mg/ml, were incubated, and after 5 h SDS-treated walls were added to 1 portion (final concentration of SDS-treated walls, 10 mg/ml). (A) Neutral sugars (anthrone) in supernatant. Symbols: ●, untreated walls; ○, untreated plus SDS-treated walls. (B) Amino sugars in supernatant. Symbols are the same as in (A).*

Walls isolated from hyphae treated with cycloheximide were used in some labeling experiments, and these showed autolysis kinetics quite similar to the ones described above. However, the rate of protein digestion relative to that of glucans and chitin was markedly less than in walls from normally growing hyphae (Table 1).

Nature of autolysis products. Chromatography of the supernatants from autolyzing walls showed the presence of oligosaccharides and four reducing spots which co-chromatographed with glucose, mannose, galactose, and *N*-acetylglucosamine. The structure of the oligosaccharides was not investigated further in the present work except to show that acid hydrolysis of the combined fraction yielded glucose, mannose, galactose, and glucosamine. Column chromatography on an automatic amino acid analyzer confirmed that the amino sugar formed by autolysis was glucosamine and showed the presence of 14 amino acids among the autolysis products. No D amino acids could be detected with D amino acid oxidase.

The monomers produced by autolysis were thus identical to those formed by acid hydrolysis of *A. nidulans* walls (9, 16), and all the major wall polymers must have been autolysin substrates.

Association of autolysins with the envelope fraction. Walls purified by the method de-

TABLE 1. *Autolysis of walls from hyphae with and without cycloheximide treatment*

Wall sample	Amino acids[a]/sugars in autolysate	
	Neutral sugars[b]	Amino sugars
With cycloheximide[c]		
5 h	0.2	0.29
15 h	0.16	0.24
48 h	0.16	0.23
No cycloheximide[d]		
5 h	0.33	0.66
15 h	0.35	0.6
48 h	0.4	0.59

[a] Ninhydrin with arginine as standard.

[b] Anthrone with glucose as standard.

[c] Hyphae, grown for 15 h, were shaken for 3 h with 100 μg of cycloheximide per ml, and walls were isolated. The purified walls were incubated for the stated times.

[d] As in footnote [c], but no cycloheximide addition.

scribed above or by variations of it were not completely free from cytoplasm and membrane fragments. This was apparent from electron micrographs (Fig. 3) and from the trace amounts of ribose found in acid hydrolysates of the preparations. However, little autolytic activity could be detected in the cytoplasmic fraction obtained after the sonic treatment step in wall purification. When cytoplasm (1.7 mg of protein/ml, final concentration) was added to

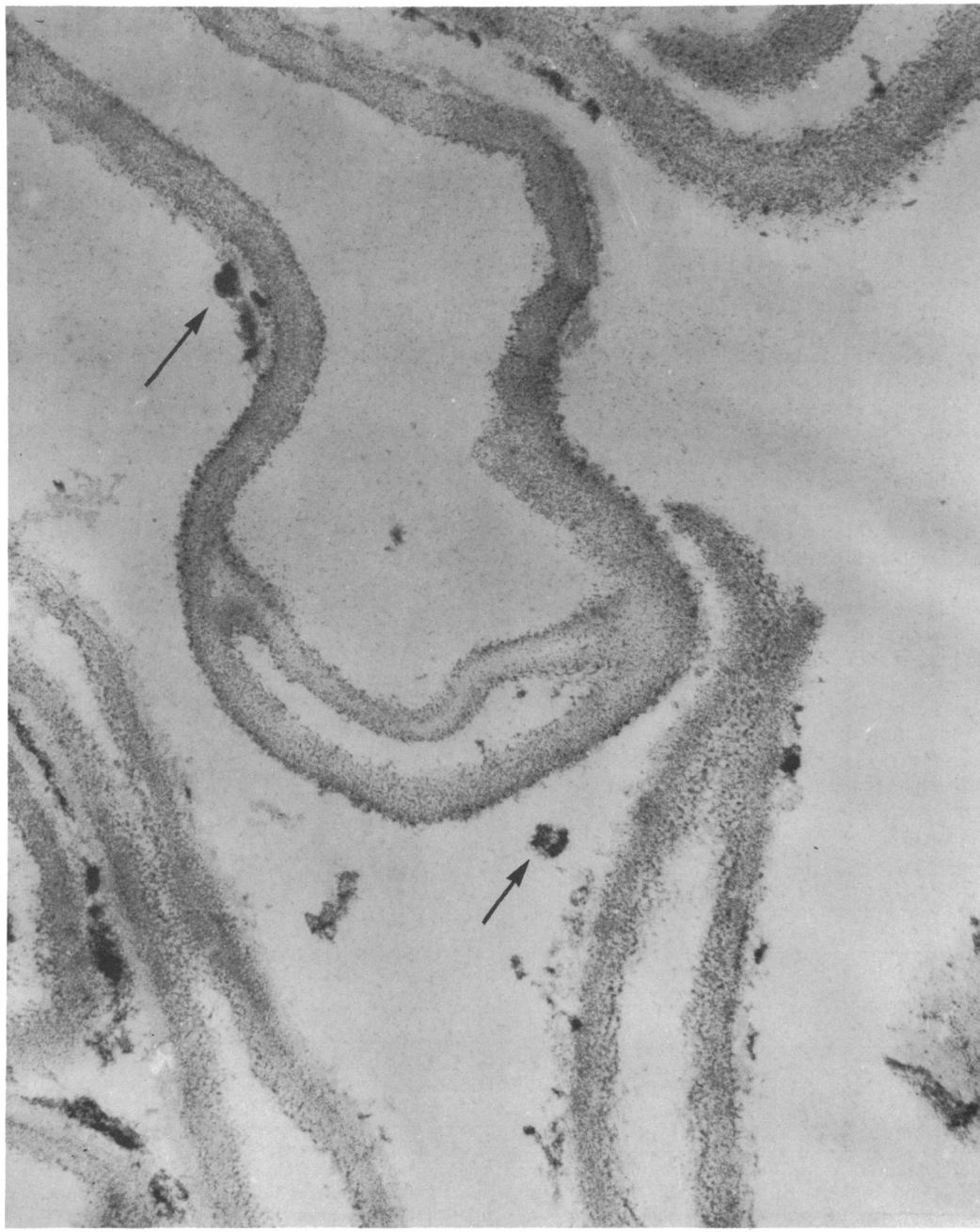

FIG. 3. *Section through purified A. nidulans walls after staining with permanganate. ×49,000. Note that no characteristic membrane profiles are associated with the walls. The arrows indicate contaminating cytoplasm.*

SDS-treated walls, the rate at which free glucose and amino sugars were liberated was 14 and 7%, respectively, of that obtained with an equal concentration of purified walls not treated with SDS. When cytoplasm (1.7 mg of protein/ml) or purified walls (0.033 mg of protein/mg wall [dry weight]) were added to SDS-treated walls labeled with ^{14}C, the radioactivity solubilized per hour was 340 counts per min/mg of cytoplasmic protein and 2,580 counts per min/mg of wall protein. Thus, the source of autolytic activity in the wall preparations was not the small amount of cytoplasmic contamination. Rather, autolysins appeared to be firmly enough attached to the cell envelope to withstand repeated washing with 0.5 M phosphate buffer.

Location of autolysis sites on hyphal walls. Hyphae lay down new wall at the apex, and selective labeling of the apical wall was therefore possible by pulse labeling cultures with a radioactive substrate. Isolation of the walls of such hyphae and determination of the specific activity of the autolysis products would then show if there was any preferential digestion of apical as opposed to older wall.

In Table 2, the specific activities of autolysis of products isolated in a typical experiment are compared with those of the monomers obtained from a portion of the same wall by chemical hydrolysis. The differences in the two sets of specific activities show that autolysis did not occur at random along the hyphal wall but preferentially in the apical portion. The differences in the specific activities of the products of autolysis and of chemical hydrolysis tended to be greatest after short periods of autolysis. For comparison with data in Table 2, the ratio of specific activities after 15 h of autolysis was 3.7 for glucose, 2.8 for glucosamine, and 3.2 for galactose.

TABLE 2. *Relative rates of autolysis in apical and lateral walls*[a]

Monomer	Sp act of purified monomer[b]	
	From acid hydrolysate	From autolysate[c]
Glucose	9	39.4
Galactose	5.8	23.8
Mannose	10.4	24.7
Glucosamine	9.5	29.8

[a] Hyphae were grown for 15 h with nonradioactive glucose and then for 1 h with [^{14}C]glucose. After purification of the wall fraction, one portion was hydrolyzed by acid and another allowed to autolyze.

[b] Expressed as 10^3 counts/minute per micromole.

[c] Monomers were isolated after 1 h of autolysis.

Location of autolysis sites on the walls of hyphae treated with cycloheximide. The apical wall is also the newly synthesized portion of the hyphal envelope and the above findings do not answer the question of if it is the age of the wall or its position on the envelope that determines susceptibility to autolysis. To investigate this, we used the finding that in the presence of cycloheximide, wall is laid down all around the hypha and not exclusively at the apex (19). Hyphae were grown first with [^{14}C]glucose, then for a short period with [^{3}H]glucose to label the tip, and finally incubated in medium containing cycloheximide and nonradioactive glucose. The wall fraction was purified, and the specific activity of sugars obtained by autolysis and acid hydrolysis was determined. The content of ^{14}C and ^{3}H in the autolysis products was several times lower than that in the sugars isolated after acid hydrolysis (Table 3). The distribution of ^{3}H, the isotope labeling the tip, may be noted in particular. It indicates that apical wall was broken down less rapidly than that deposited subapically in the presence of cycloheximide. Thus, the newly formed, unlabeled wall was digested preferentially by the autolytic enzymes, and it was the age rather than position on the envelope which determined the rate of hydrolysis.

DISCUSSION

In common with other species of microorganisms (3, 6, 11, 23), hyphae of *A. nidulans* contained enzymes which could hydrolyze their own wall polymers and which were attached to the hyphal envelope. These enzymes have not yet been isolated and characterized, but the products of their action clearly show that they

TABLE 3. *Relative rates of autolysis in lateral wall, apical wall, and wall made in the presence of cycloheximide*[a]

Monomer	Ratio of sp act of acid hydrolysis[b] to sp act of autolysis[b]	
	^{14}C	^{3}H
Glucose	2.84	2.16
Galactose	5.8	5.35
Glucosamine	6.13	4.47

[a] Hyphae were grown for 15 h with [^{14}C]glucose, then for 1 h with [^{3}H]glucose, and finally for 3 h with nonradioactive glucose and 100 μg cycloheximide per ml. After purification of the wall fraction, portions were hydrolyzed by acid or autolyzed.

[b] Counts per minute per micromole of purified monomer.

could digest all the major wall polymers (9, 16). However, only a small proportion of each of these polymers was readily hydrolyzed, whereas the remainder was digested at a much slower rate.

The labeling experiments showed that the fraction highly susceptible to autolysis was the newly formed wall. Even when newly formed wall was deposited in close vicinity to old lateral wall, as in the presence of cycloheximide, it was digested preferentially. Preferential autolysis of new wall also explains the relatively slow rate of protein hydrolysis in walls from hyphae treated with cycloheximide (Table 1). The youngest wall, formed in the presence of cycloheximide, would not contain proteins, and any amino acids liberated would have had to come from older, resistant sections.

It is of interest to compare wall autolysis in *A. nidulans* to that in *Streptococcus faecalis*, where autolysins again preferentially digest newly formed wall (18). In the coccus, the whole wall is susceptible to autolysis, but active enzyme is specifically located at sites of wall synthesis. In hyphae, there appears to be no localization of autolysin, but the wall acquires resistance shortly after its deposition.

No conclusions can be drawn at present about the biochemical nature of the change to resistance and how general a phenomenon this may be in filamentous fungi. However, Wessels and Koltin (23) have implicated a change in the susceptibility of R glucan to autolysin action as a factor in the morphogenesis of *Schizophyllum commune*. Several lines of evidence have demonstrated that, in a variety of species, new wall undergoes modification after its deposition. Microscope observations have shown that older wall is thicker than apical wall (7), some fluorescent dyes stain the tip but not older sections of hyphae (4), and abrupt changes in external osmotic pressure induce swelling primarily in the hyphal apex (15). It is tempting to speculate that such modification is responsible for changes in the rate of autolysis, and we are attempting to investigate this process and its possible role in determining sites of wall synthesis.

LITERATURE CITED

1. Cocking, E. C., and E. W. Gemm. 1954. Estimation of amino acids by ninhydrin. Biochem. J. **58**:xii.
2. Forsberg, C., and H. J. Rogers. 1971. Autolytic enzymes in growth of bacteria. Nature (London) **229**:272-273.
3. Ghuysen, J. M. 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. Bacteriol. Rev. **32**:425-464.
4. Gull, K., and A. P. J. Trinci. 1974. Detection of areas of wall differentiation in fungi using fluorescent staining. Arch. Mikrobiol. **96**:53-57.
5. Higgins, M. L., and G. D. Shockman. 1970. Model for cell wall growth of *Streptococcus faecalis*. J. Bacteriol. **101**:643-648.
6. Higgins, M. L., and G. D. Shockman. 1971. Procaryotic cell division with respect to walls and membranes. CRC Crit. Rev. Microbiol. **1**:29-72.
7. Hunsley, D., and J. H. Burnett. 1970. The ultrastructural architecture of the walls of some hyphal fungi. J. Gen. Microbiol. **62**:203-218.
8. Katz, D., D. Goldstein, and R. F. Rosenberger. 1972. Model for branch initiation in *Aspergillus nidulans* based on measurements of growth parameters. J. Bacteriol. **109**:1097-1100.
9. Katz, D. and R. F. Rosenberger. 1970. A mutation in *Aspergillus nidulans* producing hyphal walls which lack chitin. Biochim. Biophys. Acta **208**:452-460.
10. Levvy, G. A., and A. McAllan. 1959. The N-acetylation and estimation of hexosamines. Biochem. J. **73**:127-132.
11. Mahadevan, P. R., and V. R. Mahadkar. 1970. Role of enzymes in growth and morphology of *Neurospora crassa*: cell-wall-bound enzymes and their possible role in branching. J. Bacteriol. **101**:941-947.
12. Niederpruem, D. J., and J. G. H. Wessels. 1969. Cytodifferentiation and morphogenesis in *Schizophyllum commune*. Bacteriol. Rev. **33**:505-535.
13. Pooley, H. M., and G. D. Schockman. 1969. Relationship between the latent form and the active form of the autolytic enzymes of *Streptococcus faecalis*. J. Bacteriol. **103**:457-466.
14. Reissig, J. L., J. L. Strominger, and L. F. Leloir. 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem. **217**:959-966.
15. Robertson, N. F. 1959. Experimental control of hyphal branching and branch form in hyphomycetous fungi. J. Linn. Soc. London Bot. **56**:207-211.
16. Ruiz-Herrera, J. 1967. Chemical components of the cell wall of *Aspergillus* species. Arch. Biochem. Biophys. **122**:118-125.
17. Sayare, M., L. Daneo-Moore, and G. D. Schockman. 1972. Influence of macromolecular biosynthesis on cellular autolysis in *Streptococcus faecalis*. J. Bacteriol. **112**:337-344.
18. Shockman, G. D., H. M. Pooley, and J. S. Thompson. 1967. Autolytic enzyme system of *Streptococcus faecalis*. III. Localization of the autolysin at the sites of cell wall synthesis. J. Bacteriol. **94**:1525-1530.
19. Sternlicht, E., D. Katz, and R. F. Rosenberger. 1973. Subapical wall synthesis and wall thickening induced by cycloheximide in hyphae of *Aspergillus nidulans*. J. Bacteriol. **114**:819-823.
20. Trevelyan, W. E., and J. S. Harrison. 1952. Studies on yeast metabolism. 1. Fractionation and microdetermination of cell carbohydrates. Biochem. J. **50**:298-303.
21. Trinci, A. P. J. 1971. Influence of the width of the peripheral growth zone on the radial growth rate of fungal colonies on solid media. J. Gen. Microbiol. **67**:325-344.
22. Wessels, J. G. H. 1965. Morphogenesis and biochemical processes in *Schizophyllum*. Wentia **13**:1-113.
23. Wessels, J. G. H., and Y. Koltin. 1972. R-glucanase activity and susceptibility of hyphal walls to degradation in mutants of *Schizophyllum* with disrupted nuclear migration. J. Gen. Microbiol. **71**:471-475.
24. Zonneveld, B. J. M. 1972. A new type of enzyme, an exo-splitting $\alpha(1,3)$ glucanase from non-induced cultures of *Aspergillus nidulans*. Biochim. Biophys. Acta. **258**:541-547.