THE BIOCHEMISTRY OF THE ACTINOMYCETALES. STUDIES ON THE CELL WALL OF *STREPTOMYCES FRADIAE*

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In order to gain some information on the comparative biochemistry of the Streptomyces, an investigation of the nature of the cell wall has been undertaken. There are only few data relating to the chemical composition of the actinomycetes. Thus, Avery and Blank (1954) found that representative species of Actinomyces, Nocardia, Streptomyces, and Micromonospora were all devoid of chitin and cellulose, which led them to the conclusion that the actinomycetales are related to bacteria rather than fungi. The present paper presents more decisive data which support this conclusion.

Early in our studies, it was observed that cells of *Streptomyces fradiae* were lyzed by lysozyme. This provided some indirect evidence concerning the chemical nature of the cell wall since Epstein and Chain (1940) as well as Meyer and Hahnel (1946) were able to extract from cells of *Micrococcus lysodeikticus* a mucopolysaccharide which was depolymerized by lysozyme. This depolymerization was accompanied by the liberation of hexosamine and other reducing sugars. Furthermore, Salton (1952*a*, *b*, 1953) has shown that purified cell walls of various susceptible bacteria can be lysed by lysozyme, and he has supplied chemical data which indicated that these cell walls were of a mucoid nature. It thus appears that lysozyme is a mucopolysaccharase, and that sensitivity to this enzyme constitutes indirect evidence for the presence of mucopolysaccharide. Sensitivity of a purified cell wall preparation of the vegetative mycelium of *Streptomyces fradiae* to lysozyme now has been demonstrated and is reported here with supporting chemical evidence for the mucoid nature of these cell walls.

MATERIALS AND METHODS

Organism. The organism used in this study was a neomycin-producing strain of *Streptomyces fradiae*, which was isolated by Waksman and Lechevalier (1949) and numbered 3535 in the Institute of Microbiology collection.

Growth conditions and harvesting. The organism was grown in a synthetic medium of the following composition: glucose, 10.0 g; monosodium glutamate, 10.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.200 g; $CaCl_2$, 0.025 g; $FeSO_4$, 0.025 g; $ZnSO_4$, 0.025 g; distilled water, 1000 g.

The cultures were incubated for 72 hr at 28 C on a shaker rotating at 250 rpm. The vegetative mycelium obtained was separated by filtration through Reeve Angel No. 802 filter paper, washed three times with distilled water, and resuspended in distilled water to give a suspension containing approximately 10 mg dry weight of cellular material per ml.

The lysozyme used in this study was crystalline egg white lysozyme obtained from Armour and Company.

Reducing power (expressed as glucose) was determined by the method of Folin and Malmros (1929). *Hexosamine* was determined by a modification of the Elson and Morgan method (1933) made by Blix (1948), and by the method of Dische and Borenfreund (1950). *Hexose* was determined by a modification by Chung and Nickerson (1954) of the anthrone method of Morris (1948).

Chromatography. Amino acids in cell wall hydrolyzates were determined by two-dimensional paper chromatography using Whatman No. 1 filter paper. The chromatograms were irrigated for 24 hr with phenol-ammonia in one direction and 18 hr with *n*-butanol-acetic acid-water (250–60–250 v/v) in the other direction. Amino acids were detected by spraying with 0.25 per cent ninhydrin in water-saturated butanol and heating at 100 C for 5 min.

Preparation of cell walls. The procedure employed to prepare the cell walls was essentially similar to that described by Salton and Horne (1951) for the preparation of bacterial cell walls by the use of the Mickle disintegration apparatus. Seven ml of the cell suspension were placed in the disintegrator cup with 2.0 g grade 12 Ballotini

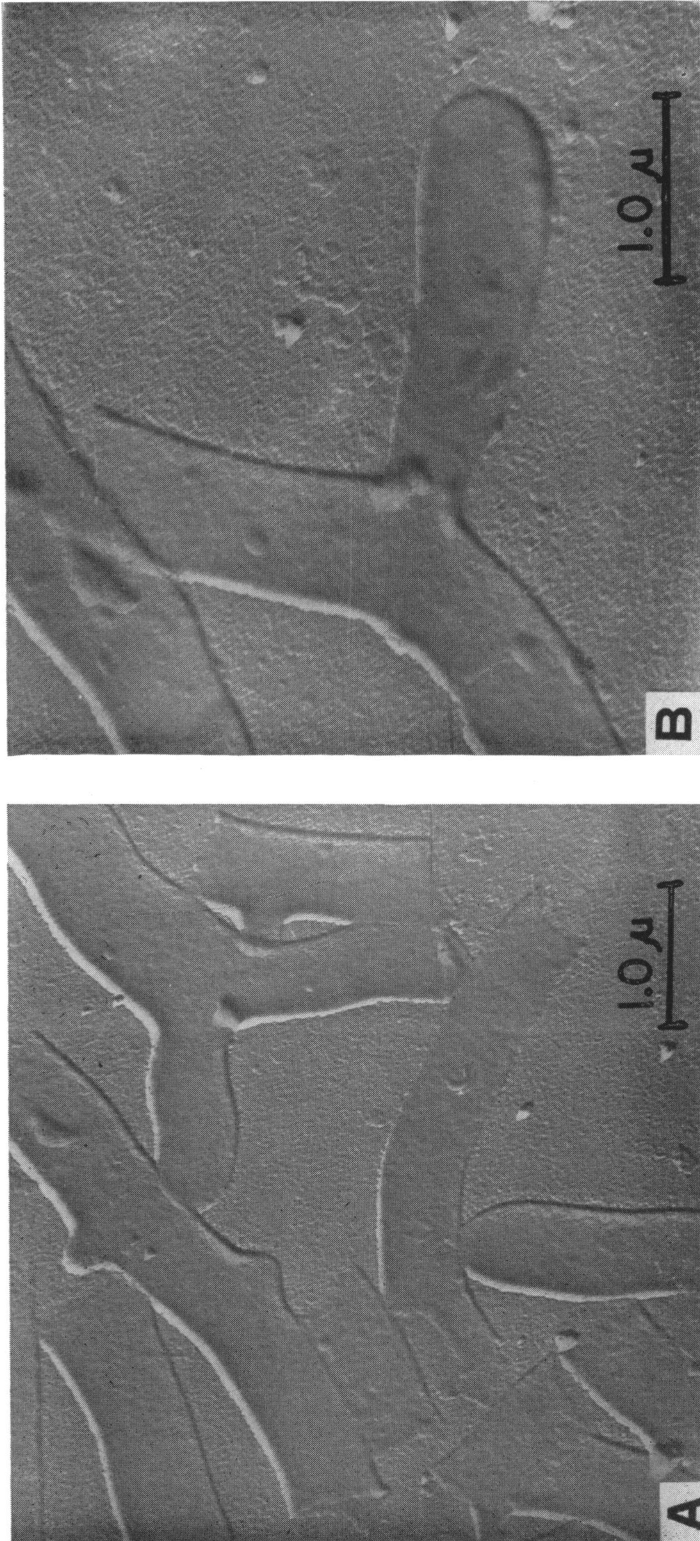

Figure 1. Cell walls of *Streptomyces fradiae*, shadowed with chromium at an angle of 25 degrees. *A*, 16,600×; *B*, 25,000×.

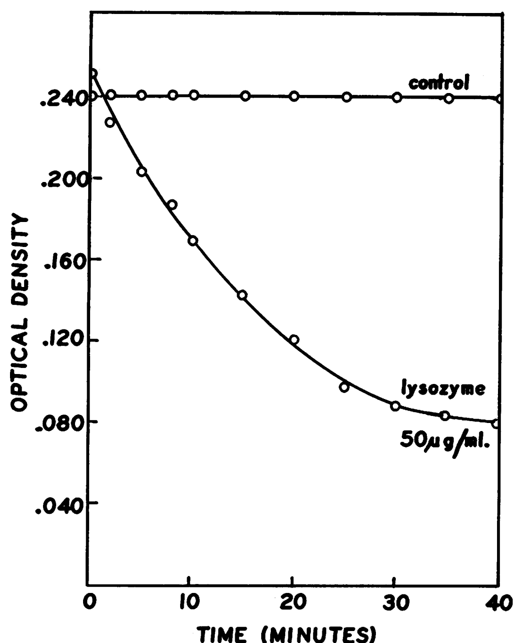

Figure 2. Lysis of cell walls by lysozyme.

beads. This was subjected to vibration at maximum amplitude for 30 min. The suspension was then centrifuged at 2500 rpm for 15 min, whereupon the unbroken cells were deposited on the bottom of the tube and the ruptured cell walls remained in the supernatant. The cell wall fragments were in turn separated by centrifugation at 10,000 rpm in an Aminco high-speed angle centrifuge for 15 min, washed twice with water, 5 times with M/15 phosphate buffer, pH 7.5, and finally 7 times with distilled water. The cell walls were then lyophilized from an aqueous suspension. The purity of the cell wall preparations was checked by electron microscopy. Photomicrographs were made using an RCA model EMU-2 electron microscope.

EXPERIMENTAL RESULTS

Properties of the cell wall: lysis by lysozyme. Electron photomicrographs of preparations made as described above showed that the cell-wall preparations so obtained were essentially free from intracellular material (figure 1).

A suspension of the cell walls in buffer showed a decrease in optical density when incubated in the presence of lysozyme, indicating a destruction of the fabric of the cell wall and its subsequent solution. A typical lysis curve is shown in figure 2. In this experiment, the cell walls were suspended in M/15 phosphate buffer, pH 6.6, in the presence and absence of lysozyme. The suspensions were incubated at 37 C and optical density readings were made at measured time intervals with a Klett-Summerson photoelectric colorimeter, employing filter No. 54. The presence of a substance susceptible to depolymerization by lysozyme in these cell walls is clearly indicated.

Carbohydrate composition. Acid hydrolysis of the cell walls resulted in a rapid clearing of the suspension accompanied by a liberation of reducing substances. Samples of 25.0 mg of the cell wall preparation were hydrolyzed in 12 ml 2 N HCl at 100 C for 2 hr. Samples were removed after 30 min, 1 hr, and 2 hr for purposes of analysis. The results are shown in figure 3. It can be seen that reducing substances were liberated rapidly. The great majority of the liberated reducing substances is accounted for by hexosamine. The remainder is very closely accounted for by hexose, as determined by the anthrone analysis. The hexose portion has not yet been positively identified. However, this demonstration of an easily hydrolyzable polysaccharide, the major portion of which is hexosamine, taken together with the suscepti-

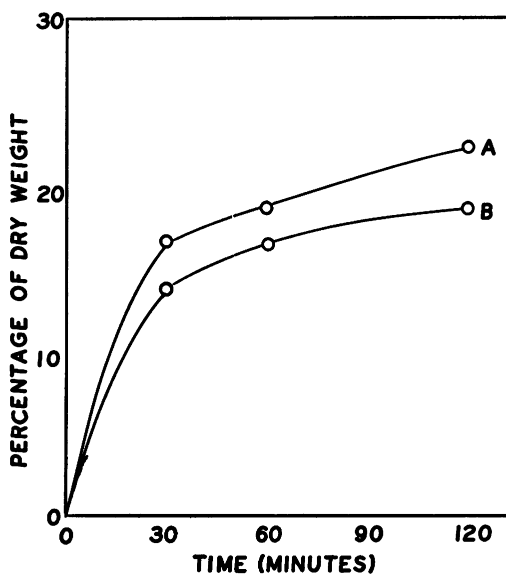

Figure 3. Rate of hydrolysis of cell walls by 2N HCl at 100 C and liberation of reducing substances. Curve *A*: Total reducing substances (expressed as glucose). Curve *B*: Hexosamine.

TABLE 1

Reducing substances liberated by hydrolysis with 2 N HCl at 100 C for 2 hr

	Per Cent of Dry Weight
Total reducing power (expressed as glucose)	22.2
Hexosamine	18.4
Hexose (anthrone analysis, glucose standard)	3.4

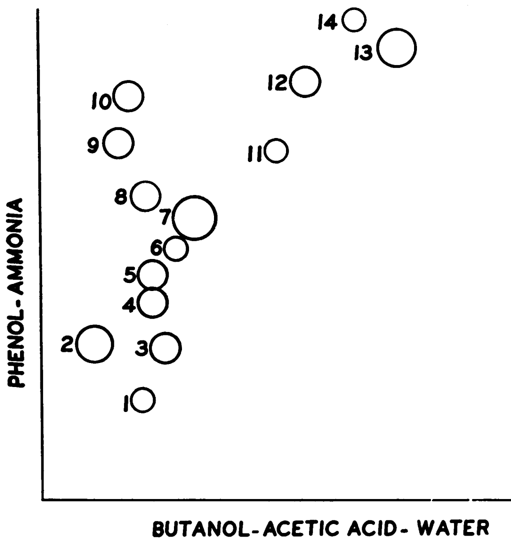

Figure 4. Amino acid chromatogram of cell wall hydrolyzate. *1*, aspartic acid; *2*, diaminopimelic acid; *3*, glutamic acid; *4*, serine; *5*, glycine; *6*, threonine; *7*, alanine; *8*, hexosamine; *9*, lysine; *10*, arginine; *11*, tyrosine; *12*, valine; *13*, leucine or isoleucine; *14*, phenylalanine.

bility to lysozyme shown above, would appear to establish the mucoid nature of the cell wall of this organism.

Amino acid composition. The amino acids present in acid hydrolyzates of the cell wall preparations were identified by paper chromatography. Samples of 15 to 20 mg of the cell-wall preparation were hydrolyzed with 5 ml 6 N HCl at 100 C for 24 hours in sealed ampoules. The hydrolyzates were dried *in vacuo* over $CaCl_2$ and NaOH pellets, redissolved in water, and concentrated to dryness again. This was repeated twice. Finally, the hydrolyzates were taken up in 0.5 ml distilled water, and subjected to chromatography.

The results obtained by two-dimensional chromatography are shown in figure 4. It is interesting to note the appearance of a spot, the position of which on the chromatogram corresponds to that of diaminopimelic acid, first found and identified by Work (1949, 1950, 1951) in *Cornybacterium diphtheriae*, and later found by Salton (1953) in *Bacillus subtilis*, *Escherichia coli*, and *Salmonella pullorum*. The chromatograms indicated the presence of the following additional amino acids: aspartic acid, glutamic acid, serine, glycine, threonine, alanine, lysine, arginine, valine or methionine, leucine or isoleucine, phenylalanine, and tyrosine. The strongest spots were produced by spots corresponding to alanine, glutamic acid, and what appears to be diaminopimelic acid. The aromatic amino acids gave extremely weak spots, indicating the presence of relatively small amounts.

DISCUSSION

The cell walls of the vegetative mycelium of *Streptomyces fradiae* have been shown to be of a mucoid nature, containing mucopolysaccharide and amino acid components.

When these data are compared with those obtained by Salton (1952*a*, *b*, 1953) on the cell walls of various bacteria, it is seen that the cell wall of *Streptomyces fradiae* is essentially similar to the cell walls of various gram positive bacteria, although certain differences do exist. In the case of *Streptomyces fradiae*, hexosamine accounted for the major part of the reducing sugar liberated on acid hydrolysis, while in the case of all the gram positive bacteria hexosamine accounted for less than half. Salton found that the cell walls of gram positive bacteria possessed a limited complement of amino acids, while cell walls of gram negative bacteria possessed a more complete range. The cell walls of *Streptomyces fradiae* appear to contain more amino acids than the gram positive bacteria, but fewer than the gram negative bacteria.

Studies are presently under way to characterize these cell walls more completely and to make comparisons among other species of Streptomyces and among other genera of the actinomycetales. It is felt that a comparative biochemical study of the cell walls may contribute much to our knowledge of this group of organisms, and may provide a sounder basis to taxonomic considerations.

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SUMMARY

Preparations of cell walls of *Streptomyces fradiae* were prepared by mechanical disintegration. They were solubilized by lysozyme indicating the presence of a mucopolysaccharide. This has been substantiated by the observation that hydrolysis with 2 N HCl at 100 C rapidly liberates reducing substances, the major portion of which was accounted for by hexosamine. A protein component appears to be associated with this mucopolysaccharide. The amino acid composition of this protein has been determined by paper chromatography.

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