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THE PARTICULATE AND CHITIN NATURE OF THE RODLET MOSAIC LAYER IN STREPTOMYCES COELICOLOR A3(2) AERIAL SPORES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Richard Allen Smucker, B.S., M.S.

* * * * *

The Ohio State University

1976

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Pfister. 1975. Babesia sp.: The relationship of stage of
development to structure of intra- and extra- cellular

A comparison by electron microscopy of Plasmodium berghei
freed by ammonium chloride lysis to P. berghei freed by
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FIELDS OF STUDY

Major Field: Microbial Cytology

Studies in Methodology for Cell Fractionation. Professor R.M. Pfister

Studies in Mesosome Isolation. Professor R.M. Pfister

Studies in Cell Wall Analysis. Professor R.M. Pfister
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ................................................................. ii
VITA ................................................................................................ iii
LIST OF TABLES .......................................................................... vii
LIST OF FIGURES ....................................................................... viii
INTRODUCTION ......................................................................... 1

LITERATURE REVIEW ............................................................. 3
- General characteristics of Streptomyces .................................. 3
- Sheath characteristics and wall composition .......................... 6
- Wall composition .................................................................... 11
- Fungal spore surfaces ............................................................... 12
- Amino-sugar occurrence ............................................................ 14
- Sugar isolation and detection .................................................... 18
- Secondary metabolism .............................................................. 20
- Actinophage and secondary metabolites ................................. 22
- Culture medium considerations ............................................... 23

MATERIALS AND METHODS .................................................. 26
- Bacterial cultures .................................................................... 26
- Culture maintenance ................................................................. 26
- Fermenter growth ................................................................... 26
- Shake flask culturing ............................................................... 27
- Cells cultured for polymer extractions ..................................... 27
- Cytochemical analysis of the rodlet mosaic ............................ 28
- Polysaccharide extractions ....................................................... 29
  A. Enzymatic rodlet isolation .................................................. 29
  B. Rodlet isolation by precipitation ........................................... 30
    1. Chloroform-butanol deproteinization ................................. 31
    2. Freon 113 deproteinization ............................................... 32
<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitin purification</td>
<td>32</td>
</tr>
<tr>
<td>Polymer hydrolysis</td>
<td>33</td>
</tr>
<tr>
<td>Protein analysis</td>
<td>34</td>
</tr>
<tr>
<td>Carbohydrate analysis</td>
<td>34</td>
</tr>
<tr>
<td>Thin layer chromatography</td>
<td>36</td>
</tr>
<tr>
<td>Gas chromatography</td>
<td>37</td>
</tr>
<tr>
<td>Infrared absorption spectra</td>
<td>38</td>
</tr>
<tr>
<td>Density gradient centrifugation</td>
<td>38</td>
</tr>
<tr>
<td>Scanning electron microscopy</td>
<td>39</td>
</tr>
<tr>
<td>Transmission electron microscopy</td>
<td>39</td>
</tr>
<tr>
<td>Rodlet length distribution and rodlet diameter</td>
<td>42</td>
</tr>
<tr>
<td>RESULTS</td>
<td>43</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>99</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>113</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>115</td>
</tr>
<tr>
<td>Appendix A</td>
<td></td>
</tr>
<tr>
<td>Liquid nitrogen cryo-impacting.</td>
<td>115</td>
</tr>
<tr>
<td>Appendix B</td>
<td></td>
</tr>
<tr>
<td>Culture media</td>
<td>116</td>
</tr>
<tr>
<td>Appendix C</td>
<td></td>
</tr>
<tr>
<td>Linear regression analysis</td>
<td>118</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>119</td>
</tr>
<tr>
<td>Table No.</td>
<td>Title</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Rodlet diameters</td>
</tr>
<tr>
<td>2</td>
<td>Rodlet length statistics</td>
</tr>
<tr>
<td>3</td>
<td>Freeze-etch replication monitoring of <em>Streptomyces</em> spores.</td>
</tr>
<tr>
<td>4</td>
<td>Chemical analysis of rodlets</td>
</tr>
<tr>
<td>5</td>
<td>Chitinase effect on precipitated rodlets</td>
</tr>
<tr>
<td>6</td>
<td>Thin layer chromatography on microcrystalline cellulose</td>
</tr>
<tr>
<td>7</td>
<td>Summary of infrared spectra of crab chitin and rodlets.</td>
</tr>
<tr>
<td>8</td>
<td><em>Streptomyces</em> bacterial-fungal similarities</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Scanning Electron micrograph of <em>S. coelicolor</em> colony.</td>
<td>46</td>
</tr>
<tr>
<td>2</td>
<td>Scanning electron micrograph of ether-treated <em>S. coelicolor</em> colony</td>
<td>46</td>
</tr>
<tr>
<td>3</td>
<td>Shadowed spore of <em>S. coelicolor</em>.</td>
<td>48</td>
</tr>
<tr>
<td>4</td>
<td>Negative stained <em>S. coelicolor</em>.</td>
<td>48</td>
</tr>
<tr>
<td>5, 6, 7</td>
<td>Critical point dried <em>S. coelicolor</em>.</td>
<td>50</td>
</tr>
<tr>
<td>8, 9</td>
<td>Freeze-etch replica of <em>S. coelicolor</em>.</td>
<td>52</td>
</tr>
<tr>
<td>10, 11</td>
<td>Thin section of OsO$_4$-ruthenium red <em>S. coelicolor</em>.</td>
<td>54</td>
</tr>
<tr>
<td>12, 13</td>
<td>Freeze-etch replica of <em>Streptomyces</em> OSU #474 cells that were partially embedded</td>
<td>57</td>
</tr>
<tr>
<td>14</td>
<td>Ether-treated <em>S. coelicolor</em> rodlet length distribution</td>
<td>61</td>
</tr>
<tr>
<td>15</td>
<td>Control <em>S. coelicolor</em> rodlet length distribution</td>
<td>61</td>
</tr>
<tr>
<td>16</td>
<td>Ether-treated <em>Streptomyces</em> OSU #474 rodlet length distribution</td>
<td>63</td>
</tr>
<tr>
<td>17</td>
<td>Control <em>Streptomyces</em> OSU #474 rodlet length distribution</td>
<td>63</td>
</tr>
<tr>
<td>18, 19</td>
<td><em>S. coelicolor</em> spores from submerged culture.</td>
<td>66</td>
</tr>
<tr>
<td>20</td>
<td>Freeze-etch replica of growth from liquid media containing agar (<em>Streptomyces coelicolor</em>).</td>
<td>70</td>
</tr>
<tr>
<td>21</td>
<td>Negative stain of rodlet-like fibers from agar shake culture (<em>S. coelicolor</em>).</td>
<td>70</td>
</tr>
<tr>
<td>Figure No.</td>
<td>Title</td>
<td>Page No.</td>
</tr>
<tr>
<td>-----------</td>
<td>-------</td>
<td>----------</td>
</tr>
<tr>
<td>22</td>
<td>Freeze-etch of control <em>S. coelicolor</em> rodlet mosaic.</td>
<td>73</td>
</tr>
<tr>
<td>23</td>
<td>Freeze-etch replica of 0.1 N HCl treated <em>S. coelicolor</em> rodlet mosaic.</td>
<td>73</td>
</tr>
<tr>
<td>24</td>
<td>Freeze-etch replica of chitinase treated <em>S. coelicolor</em> rodlet mosaic.</td>
<td>73</td>
</tr>
<tr>
<td>25</td>
<td>Electron micrograph of negative stained enzymatically isolated rodlet-like fibers.</td>
<td>76</td>
</tr>
<tr>
<td>26</td>
<td>E.M. of negative stained enzymatically isolated rodlet-like fibers prepared by density gradient centrifugation.</td>
<td>76</td>
</tr>
<tr>
<td>27</td>
<td>E.M. of negative stained precipitated rodlet-like fibers.</td>
<td>79</td>
</tr>
<tr>
<td>28</td>
<td>E.M. of freeze-etch replica of precipitated rodlet-like fibers.</td>
<td>79</td>
</tr>
<tr>
<td>29</td>
<td>Standard curve for Lowry protein analysis.</td>
<td>83</td>
</tr>
<tr>
<td>30</td>
<td>Standard curve for glucose determination.</td>
<td>83</td>
</tr>
<tr>
<td>31</td>
<td>Standard curve for D-glucosamine determination.</td>
<td>83</td>
</tr>
<tr>
<td>32</td>
<td>Standard curve for NAGA determination.</td>
<td>83</td>
</tr>
<tr>
<td>33</td>
<td>Gas chromatograph of acetic acid.</td>
<td>85</td>
</tr>
<tr>
<td>34</td>
<td>Infrared spectra of precipitated rodlets and crab chitin.</td>
<td>92</td>
</tr>
<tr>
<td>35</td>
<td>E.M. of freeze-etch replica of purified crab chitin.</td>
<td>95</td>
</tr>
<tr>
<td>36</td>
<td>E.M. of negative stained rodlet-like crab chitin fibers.</td>
<td>95</td>
</tr>
<tr>
<td>37</td>
<td>E.M. of <em>S. coelicolor</em> A3(2) actinophage.</td>
<td>98</td>
</tr>
<tr>
<td>Figure No.</td>
<td>Title</td>
<td>Page No.</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>38</td>
<td>Schematic of the relationships among the spore envelope layers of <em>Streptomyces</em></td>
<td>102</td>
</tr>
<tr>
<td>39,40</td>
<td>Freeze-etch replica of mature aerial <em>S. coelicolor</em> spores</td>
<td>102</td>
</tr>
<tr>
<td>41,42</td>
<td>Freeze-etch replica of TCA treated aerial <em>Streptomyces coelicolor</em> spores</td>
<td>104</td>
</tr>
</tbody>
</table>
INTRODUCTION

*Streptomyces* are ubiquitous soil and water bacteria (36) which have been widely utilized for commercial antibiotic production. Considerable effort has been focused on *Streptomyces* species characterization (72) but relatively little is known about their cellular ultrastructure and wall composition.

The spore and sporulating hyphal wall rodlet mosaic is one ultrastructural component that has been demonstrated by transmission electron microscopy in the *Streptomyces* investigated (61, 85, 86, 87). The rodlet mosaic is one of the major components of ornamentations observable in whole mounts of mature spores examined by electron microscopy. The spore morphology is species specific and there are four basic types depending upon the degree of protrusion: smooth, warty, spiny, and hairy (80).

Freeze-etch replication and electron microscopy appear to be the best methods to examine the rodlet mosaic images and in the replicas the rodlet mosaic subunits appear to be individual fibrils (rodlets) which criss-cross over each other, creating the mosaic effect. The individual rodlets (fibrils) generally occur in closely apposed pairs (88).
A morphologically similar rodlet mosaic has also been demonstrated on *Penicillum*, *Aspergillus*, and *Trichophyton* spores (31, 32, 33), but no comparable structure has been demonstrated in the eubacteria. Bradley and Ritzi (8) suggested that *S. venequeulae* rodlet mosaic is lipid-like, but Wildermuth, Wehrli, and Horne (88) indicated that the *S. coelicolor* rodlet mosaic is not lipid-like. Wildermuth et al. (88) suggested that the nature of the rodlet mosaic would only be ascertained when its components could be isolated and purified.

Objectives of the present work will be to establish the ultrastructural spore envelope relationship of the *Streptomyces* rodlet mosaic and to isolate and chemically characterize the *S. coelicolor* rodlet mosaic.
LITERATURE REVIEW

General Characteristics of Streptomyces

The generic name "Streptomyces" divided into its constituent Greek adjective and noun means "a pliant, or bent fungus" (streptos=pliant or bent; and myces=fungus).

Various terms have been used to label the genus Streptomyces. Russian literature uses "Actinomyces" whereas most other countries use the designation "Streptomyces". Although the term "actinomycetes" technically refers to the entire group of the Actinomycetales it is frequently used to designate the commonly studied genus, Streptomyces. The former usage will be adhered to in all cases in this and following discussions.

The Actinomycetales are bacteria with fungal-like morphology; unlike many true bacteria, the actinomycetes form a variety of structures which can be used in their classification (3). Buchanan, in 1917 (9), placed the Streptomyces in one (Actinomycetales) of six ordinal groups of the class Schizomycetes. He used the following description to characterize the actinomycetes in general: "(Actinomycetales, Order III). 1) thread bacteria or ray fungi which show filaments, frequently with true branching; 2) not water forms; 3) a group intergrading with the fungi". These early observations and
placement of the *Streptomyces* and other Actinomycetales with the bacteria were superseded by later observations and conclusions.

Moore and Chapman (54) review that Bessey placed the Actinomycetes in family Moniliaceae, of the form class Deuteromyces (Division II of *Bergey’s Manual of Determinative Bacteriology*, 7th ed. 1957). Supportive evidence for the fungal classification had been: 1) observation of true nuclei; 2) myceloid colonial habit; 3) reproduction by exogenous conidiophores (closest similarity to fungi observed in *Streptomyces*, (22)); and 4) lack of sexual reproduction.

Moore and Chapman, in 1947 (54), demonstrated by electron microscopy of thin sections that the *Streptomyces* do not possess nuclear membranes and that they divide amitotically.

Tresner, Davies, and Backus (80) suggest the use of the electron microscope for describing *Streptomyces* spore surface configurations which are features stable enough to be used as taxonomic criteria (smooth, warty, spiny, or hairy surface textures).

*Streptomyces* naturally occur in neutral and slightly alkaline soils. Hyphae are infrequently found in soils (44, 52).

Kalakoutskii and Pauzharitskaja (38) described microscopically recognizable stages in the morphological life cycle of the *Streptomyces*: 1) primary (substrate) mycelium; 2) production of secondary aerial mycelium; 3) sporulation; and 4) spore germination. Erikson (22) noted that occasionally *S. coelicolor* substrate hyphae fused prior to aerial hyphae production, but generally aerial hyphae were produced by direct branching from substrate hyphae.
Wildermuth (85) used electron microscopy of thin sections to describe the cellular differentiation of *Streptomyces coelicolor* A3(2) in more detail. The substrate hyphae usually occurred as a loose network of branching filaments with homogeneous walls and cytoplasm. One-day old aerial mycelia appeared white with single or short-chained spores, and after several days appeared pale gray, consisting almost entirely of chains of spores. When the colony gains a gray-brown aerial coloration it consists entirely of spores and many of the older substrate mycelia are lysed.

Carvajal (12) indicated that *Streptomyces* in submerged culture also exhibited a morphological life cycle. Four *Streptomyces* species grown in corn steep liquor medium began spore production after 36-80 hr of culturing, and after 100 hr spores germinated and the life cycle was repeated.

These developments occur aerobically in both fermentation vessels and in shake flasks but more slowly in shake flasks. Maximum sporulation in shake flasks occurred between 4-6 d. In the *S. griseus* fermentation culture, maximum sporulation and maximum streptomycin activity occurred at 120 hr.

Germination of *Streptomyces* spores appears to occur by enzymatic degradation of the spore wall material. Evidence supporting this idea is the occurrence of well defined circular holes observed in carbon replicas of germinated spore ghosts (16). Vernon indicated that spores appear to be released by longitudinal splitting of the sheaths. Vernon made his conclusions based upon images of shadowed whole mounts of germinated spores (82).
Ebner and Frea (21) report the heat survival of *S. fradiae* is a function of morphological age of the culture. By treating cell populations at 55°C for 10 min during different stages in the life cycle they determined that heat survival was at a minimum when the conversion of spores to vegetative mycelium was complete and increased when spores were produced.

As indicated by Weinberg (84) our understanding of the relationship of secondary metabolites to bacterial cells is not well understood; however, Redshaw, McCann, Sankaran, and Pogell (63) communicated several observations concerning antibiotic production. *S. alboniger* aerial mycelium mutants still produced puromycin. Development of aerial mycelium was stimulated in these mutants (mutant with respect to aerial mycelium production when glucose was in the medium) by the addition of 5 mM adenine and may indicate some possible role the nucleosides have in secondary metabolism regulation.

Features of *Streptomyces*’ life cycle which correlate with Weinberg’s (84) concepts of secondary metabolism include: 1) antibiotic production; 2) spore production; 3) sheath appearance on spores and sporulating hyphae; and 4) development of aerial spore hydrophobicity (17).

**Sheath Characteristics and Wall Composition**

Development of hydrophobic properties of aerial spores and the appearance of the hyphal-spore sheath components (88) may be due to the same metabolic product or they may be due to totally separate
products. Details of these considerations will be examined in a later section of this review.

Sheath appearance is readily demonstrated by freeze-etch replication and transmission electron microscopy of aerial hyphae and spores of *Streptomyces* (88). Sheath presence is usually inferred by the visualization of the rodlet mosaic or by the assumption that the loosely attached material around spores, as seen in thin section, contains the rodlet mosaic. Rodlets have not been observed in thin section, in the surveyed literature.

*S. coelicolor* walls form two layers while progressing from aerial mycelial to spore stages. Only the inner component grows inward to form the cross walls which therefore delimit the spores. The outer component remains intact for some time and acts as a sheath around the developing spores (30).

Beaded structures appear on the outer and inner components of the aerial wall (thin section studies). The inner layer of the spore wall is about 30 nm thick. The central layer, 12 nm thick, may be from the outer component of the original substrate mycelial wall, and is separated from the outer aerial wall layer by what appears (in thin section) to be a space (30).

There does not appear to be any morphological evidence for the existence of capsule or slime layer in the *Streptomyces* (83).

As early as 1961, Glauert and Hopwood (30) indicated that a superficial fibrous layer was present on the aerial hyphae and mature spore walls. They describe the outer aerial wall component as acting
"like a sheath" around the developing spores which are subsequently liberated from the "enveloping sheath". Use of the term "sheath" in the context the above authors use it is not necessarily synonymous with its use in later literature.

Wildermuth, Wehrli, and Horne (88) coined the term "surface membrane" to describe the fibrous layer and its associated matrix observed in negative staining. Variations in the sheath (surface membrane) characteristics (looseness or tenacious association with mature spores) within the same culture of S. griseus was indicated by Williams and Sharples (89). Williams and Sharples (89) and Rancourt and Lechevalier (61) conclude that spores, if they have spines or hairs, have a persistent sheath. In contrast, if the spores are smooth they possess a less tightly bound sheath. It is important to note that this tenacious character was deduced from thin sections and from negatively stained whole mounts.

A number of investigators have indicated a species specific modulation of the outer sheath which permits grouping the genus Streptomyces with respect to degree of sheath modification. These characteristics, resolvable with the electron microscope, are grouped as smooth, warty, spiny, or hairy (8, 61, 86, 88, 89).

Bradley and Ritz (8) used carbon replicas of S. venezuelae to observed that the spores' surfaces were covered by an ordered array of tiny rods (10 x 100 nm). The 10 nm wide structures called "rods" by Bradley and Ritz (8) are likely equivalent to the two parallel rodlets observed in freeze-etch replicas by Wildermuth, et al. (88). Wildermuth (87) indicated that these irregular rodlet
patterns essentially lie in a single plane initially, and develop, producing spines surrounding *S. viridochromogenes* spores. Williams and Sharples (89) portray the ornaments of *S. finlayi* and *S. viridochromogenes* as having thick persistent sheaths and the smoothly spored strains, *S. venezuelae* and *A. griseus*, maintaining loosely attached sheaths.

In *S. coelicolor* A3(2), individual rodlets are the subunits of the sheath mosaic. The rodlets appear to have longitudinal furrows when observed in negatively stained preparations. These rodlet images, when negatively stained, are 40-450 nm long, whereas freeze-etch images of the same spore populations have rodlets imaged as 40-250 nm long units. This change in length indicates that the negative staining procedures may disrupt the original rodlet mosaic configuration and therefore the apparent rodlet lengths (88).

Rancourt and Lechevalier (61) interpret the persistent fibrous sheath's presence around *S. viridochromogenes* as indicative that the fibrous material is more than simply an inert membrane.

Wildermuth, Wehrli, and Horne (88) found that attempts to isolate the material from *S. coelicolor* A3(2) resulted in rodlets always being intact with a "membranous" component (data taken by negative stainings of centrifugation pellets). Negatively stained preparations of whole mounts of aerial spores showed an extremely thin sheet covering the rodlet mosaic. Wildermuth and coworkers conclude that since the rodlets can not be isolated as particulates by their differential centrifugation techniques that the rodlet images may well be the
result of invaginations of a continuous sheet of material. At the present the rodlet mosaic components have not been isolated and chemically identified in any of the reviewed literature.

Elucidation of the chemical nature of the sheath of *Streptomyces* has been approached by a number of indirect methods. Erickson (22) stained for lipids by using Sudan IV and light microscopy to examine the various morphological stages of *Actinomyces (Streptomyces)* coelicolor. She found that substrate mycelia did not stain; day old branches moderately stained; and that mature spores stained evenly around the colonial aerial conidial mats. Individual filaments of mature colonies tended to stick together and when stained produced a blurred image. Vernon (82) used the Erikson Sudan IV staining technique to attempt to stain cytoplasm-free sheaths but the stain did not associate with their preparations.

Using negatively stained preparations for electron microscopy Wildermuth, Wehrli, and Horne (88); and Bradley and Ritzi (8) investigated the effects of various organic solvents on the rodlet sheath mosaic. Using solvents such as chloroform, ether, xylene, acetone, and benzene, Wildermuth et al. (88) found no modifications of the *S. coelicolor* rodlet images. In contrast, Bradley and Ritzi (8) found that some of these solvents removed the image of carbon replicated rodlets of *S. venezuelae*, a conclusion which led them to interpret the rodlet character as lipid-like.

Whether the rodlets are lipid-like or not will only be determined when the rodlets can be purified and chemically characterized (88).
Microelectrophoretic studies have indicated Streptomyces spores to have a net negative charge at most pH levels and have an isoelectric point of pH 2.5. Micromonospora and Streptomyces examined by titrations and electrophoresis, display apparent carboxyl and amino groups. Thermoaotinomyces and Nocardia have only the carboxyl function (Douglas, 17). It is of interest to note that Thermoaotinomyces and Nocardia apparently do not possess the fibrous portion of the sheath.

Streptomyces, Micromonospora, Nocardia and Thermoaotinomyces treated with lysozyme exhibit a reduced net negative charge at pH 7. Interpretation of these results has not been discussed (17).

On the basis of Sudan IV staining there is some suggestion that spores of Streptomyces have considerably higher content of lipid than do the mycelia (22).

Wall Composition

A number of reviews have indicated that the bacterial cell wall composition is constant with respect to cell age and nutrient conditions and it is assumed that wall composition data is a valid basis for taxonomic characterizations (71).

In addition to differentiating bacterial genera, wall analyses are used to differentiate the Streptomyces, and in general, the actinomycetes, from the fungi (14). Avery and Blank (2) were unable to detect chitin or cellulose in aerial growth of Streptomyces, Nocardia, Micromonospora, or Thermoaotinomyces. Avery and Blank
concluded that the Actinomycetes have nothing in common, chemically, with the true fungi.

Chromatographic preparations for *Streptomyces* wall component analysis seem to be adequately prepared by hydrolysis at 100°C for 2 hr with 2 N HCl. Becker, Lechevalier, and Lechevalier (3) indicated that the *Streptomyces* cell walls have only glucosamine, muramic acid, alanine, glutamic acid, and LL-diaminopimelic acid as major components. These findings were confirmed using *Streptomyces* sp NCTC 7807 (Maxted Strain) with no apparent chromatographic difference between aerial spores and substrate mycelium.

Many of the *Streptomyces* sp and other actinomycetes, are sensitive to lysozyme action and in some cases N-acetylglucosamine monomers are freed from mycelial walls (74).

Teichoic acids have not been found in vegetative or spore walls of *Streptomyces* (16).

**Fungal Spore Surfaces**

Classification of the *Streptomyces* and the fungi in the *Bergey’s Manual* has indicated the borderline position of the *Streptomyces* with respect to eubacteria and the fungi. One would expect that in addition to morphological cycle similarities that there may be some ultrastructural similarities in the cell walls and perhaps the cytoplasmic elements.

Hess, Sassen and Remsen (32) and Hess and Stocks (33) have shown that in outer wall images of conidia from frozen-etched *Penicillium* and *Aspergillus* there is a striking resemblance to the rodlet mosaic
of the *Streptomyces* aerial conidia. In the fungi examined, the rodlet diameter was 5 nm with 10 nm spacings between the rodlet units. Rodlet mosaics were somewhat species specific, but also varied even within a particular culture (perhaps a reflection of individual spore maturation?). There was always an obvious mosaic triangular arrangement formed by criss-crossing of the rodlet units (32, 33).

Ethanolic and aqueous potassium hydroxide solutions each prohibited visualization of the rodlet mosaic layers on *Penicillium* spores. It was not known if the treatments dissolved the material or merely facilitated the rodlet mosaic removal. Conjecture by the authors indicated that the rodlet mosaic might be cutin (waxy layer, typical of vascular plant surfaces) (32).

Both the *Aspergillus* and *Penicillium* freeze-etch replicas promote the suggestion that there is a superficial layer which covers the rodlet layers. Two lines of evidence suggest this external layer: 1) fuzziness of rodlet images in carbon replicas and 2) the occasional observance of additional material around the fracture faces which show up best when the etching (sublimation) times are extended to remove additional water and reveal more deeply embedded material.

*Trichophyton rubrum* and *T. mentagrophytes* spores examined by freeze-etch replication had rodlet mosaic patterns (triangulation) (31) nearly identical to those of the *S. coelicolor* (88). Average lengths of the *Trichophyton* rodlets were 120-130 nm (31).

*Linderina penispora* and *L. macrospora* have an outer amorphous layer covering an inner fibrillar layer composed of fibrils
organized at random (92). The nature of this fibrillar layer was examined only with whole mounts and electron microscopy, but had some ultrastructural similarity to the rodlet mosaics of Streptomyces.

**Amino Sugar Occurrence**

Since the first isolation of glucosamine from crustacean chitin (41), there has been elucidated a wide variety of amino sugars with various backbone structures. It appears that most of the naturally occurring amino sugars (glycosamines) are all members of the 2-desoxy-2-aminoaldoses.

The two most predominant 2-desoxy-2-aminoaldoses are glucosamine (chitosamine) and galactosamine (chondrosamine). The second, parenthetical, names indicate common biological sources of the material (28). Glucosamine and galactosamine occur in soils probably as the respective end products of chitin and galactosminan and as cell wall decomposition hydrolysates (15).

Of the many naturally occurring amino sugars, the most abundant amino sugar, glucosamine, is commonly found in the polymeric form of chitin and in bacterial peptidoglycan. Glucosamine also occurs in some forms of nucleotide derivatives but is only rarely found in the free form. In naturally occurring products it appears that amino groups of glycosides are usually protected by acetyl and occasionally by formyl or methyl groups (71).

The difficulties involved in unequivocal identification of amino sugars should be considered when analyzing the distribution of amino
sugars in nature. This is especially true in the case of glucosamine. Its presence has often been inferred from color reactions and from paper chromatograms, and only rarely is it isolated (crystallized) and identified unequivocally (71).

With the exception of *S. octosporus*, 29 other species of yeast, including Baker's yeast, *S. cerevisiae*, contain chitin as determined by the chitosan sulfate test (68). *S. commune* shows an innermost layer of chitin microfibrils, 60 nm thick. This material was the residue remaining after sequential treatments with boiling water, hot ethanol, 5% KOH, 5% acetic acid, and 2% KMnO₄, and oxalic acid, 0.5 N HCl and water washes (37).

The relative amount of chitin in fungal cell walls varies from species to species but culture age positively correlates with the percentage of chitin present (71). Normal cells in *S. cerevisiae* culture are essentially devoid of chitin. Chitin synthesis is only essential during septum formation. Chitin synthetase is inhibited by polyoxin D, which also blocks septum formation (10).

Arthropod chitin isolation procedures have generally included boiling in aqueous acid and then in alkali. The results indicate that even much less drastic treatment such as boiling in pure water induce extensive changes. Chitin isolated by conventional methods yields acetic acid after complete hydrolysis, but at levels significantly lower than theoretically expected amounts (23). Improvements on chitin isolation and purification must involve procedures where the carbohydrate moiety is not disturbed (23).
Foster and Hackman (24) proposed that HCl and NaOH cleaning steps in preparing chitin should be replaced with EDTA (ethylene diaminetri-chloracetic acid) washes at pH 9 then pH 3. The EDTA treatments removed no carbohydrate but only decalcified the material. Subsequent ethanol washing to remove pigments followed by lipid removal with ether completed the chitin purification. The conclusion following this type of isolation was that protein was bound to the native chitin and was not even completely removable by hot alkali.

Using conventionally purified chitin Dweltz and Anand (20) hydrolyzed alpha and beta chitin with concentrated HCl by constant stirring over a boiling water bath for 2-3 hr then immediately cooling and diluting the hydrolysate with water followed by decolorization with charcoal, filtration and then vacuum drying. Using ninhydrin detection on chromatograms developed in benzidine/n-butanol/pyridine/H2O (1:5:3:3, v/v) they detected only glucosamine and no galactosamine, indicating that glucosamine is the backbone of chitin.

A number of organisms produce chitinolytic activities. Streptomyces are commonly implicated in the degradation of chitin. The end products of the chitinase system vary with respect to the method of isolation (64).

It was through the hydrolytic activities of chitinase enzymes that the N-acetyl-D-glucosamine monomeric nature of chitin was established.

The organization of the chains of polymers has not been settled (11, 19, 23, 60).
Using infrared spectroscopy and X-ray diffraction Dweltz (19) concluded that beta chitin is linked in the side-to-side fashion with a molecule of water and that this water linkage is not true with alpha chitin. Therefore the dry state of beta chitin can be considered as a monohydrate of alpha chitin; that is, beta chitin contains one molecule of water for each molecule of N-acetyl-D-glucosamine.

Carlstrom (11) on the other hand concludes a helical structure for beta chitin rather than the straight chain structure promoted by Dweltz (19). The disagreement comes from conflicting data interpretation and calculations.

Infrared analysis is dependent upon the preparation methods used for the samples. Spedding (75) indicates that mulling polysaccharides does not usually destroy the crystallinity of the samples but the preparation of a pressed disc (e.g., KBr window) may render the sample amorphous. The change towards amorphic state is signaled by the broadening of absorption bands of the infrared spectrum.

Falk and coworkers (23) studied the extracellular fibers of the diatom *Thalassiosira fluviatilis* Hustedt with X-ray analysis, infrared analysis, and proton magnetic resonance and found a crystalline polymer that they identified as chitin (poly beta-1,4-2-acetamido 2-deoxy-D-glucosamine). This structure is the same as that proposed for chitin. Infrared spectra of chitin indicates a much less highly ordered macrostructure than does the very sharply defined spectra of the chitan polymer.
Acetate recovery from fully hydrolyzed chitin and the chitan indicates that chitan has very little if any deacetylated N-acetylglucosamine and that the conventionally produced crab chitin has numerous regions containing only the glucosamine moiety. Falk and coworkers conclude that the name chitin should be reserved as a group name for these polysaccharides which contain glucosamine in addition to N-acetylglucosamine (23).

Sugar Isolation and Detection

Determination of sugar residues from natural products is usually preceded by hydrolytic release from the parent polymers of simple carbohydrates, lipopolysaccharides, or mucopolysaccharides. Hydrolysis can be achieved enzymatically or with mineral acids and in either case presence of labile sugars must be considered.

The low recovery of hexosamines from polymers as described in the literature is probably due to incomplete hydrolysis, or to destruction of the monomers by over-hydrolysis (27).

Some of the carbohydrate polymers are readily hydrolyzed into individual sugars whereas others are very resistant to degradation. Chitin requires rather rigorous hydrolysis, with 4 N-6 N HCl for 12-16 hr, to be completely depolymerized. However, during this procedure the acetyl- moiety is completely cleaved from the D-glucosamine backbone (23).

Of the wide variety of colorimetric reactions used to determine sugar levels in polymers and sugar containing substances a few are
commonly employed. The standard methods are Dubois et al. (18) phenol-sulfuric acid for unsubstituted sugars; Lee and Montgomery (42) deamination followed by phenol-sulfuric for amino sugars; various modifications of the anthrone system and the Morgan and Elson (55) method for determining N-acetyl glucosamine. Even though these assays are relatively specific it is usually accepted that where interfering substances such as some of the amino acids are present, color development variances must be considered in data interpretation.

In some cases the specific structural features of the sugar moiety required for color development have been described. For example, the Morgan-Elson reaction for N-acetyl glucosamine requires: 1) 2-acyl amido group; 2) a free reducing group; and 3) a free hydroxyl group at carbon-4. Since alkali labile substituents on carbon-1 and -4 such as O-acetyl groups are eliminated in the first stage of the Morgan-Elson determination it would be easy to confuse such ensuing positive results with the simpler NAGA. Supportive evidence should be used such as thin layer chromatography (34).

Muramic acid, a common constituent in bacterial cell walls, can be readily distinguished from glucosamine and galactosamine and other sugars by isolating these sugars on paper chromatograms (77). In the past several decades thin layer chromatography has frequently surpassed conventional paper chromatography for separations of sugars as well as other natural components. Some of the absorptive materials used include silica gel G, Kieselguhr, and more recently, microcrystalline cellulose (43, 76, 90, 95).
Microcrystalline cellulose was first suggested as a chromatographic medium by Wolfrom et al. (90) and has a number of features which make it very convenient for rapid separations of sugars and amino acids: 1) $R_f$ values and separation characteristics of the microcrystalline matrix is quite closely related to those of conventional paper chromatography; 2) development times of the chromatogram reduced to 1.5-3 hr; 3) thin layer plates of microcrystalline cellulose does not need to be heat activated but the silica gel and kieselguhr plates do; and 4) microcrystalline cellulose adheres tightly enough to the physical carrier (commonly, glass) so that a stream of air may be used to improve drying rates during sample applications and mild pencil lead pressure will not disturb the dry matrix.

For many of the 4, 5, 6, and 7 carbon sugars, whether they are substituted or fully hydroxylated, the ammoniacal solution of silver nitrate is one of the most common indicators (35) although this reagent is not specific for any class of sugar. Boric acid, anthrone, and anisidine are also quite useful as general detection systems and have some keto-sugar differentiation sensitivities. Amino containing sugars are indicated by complexing with ninhydrin to give reddish-purple spots. Only the amino sugars complex with the ninhydrin (95).

**Secondary Metabolism**

Secondary metabolites of bacteria and other microorganisms have stimulated industrial and research interest since the discoveries of penicillin and streptomycin.
Weinberg reviewed concepts of secondary metabolism, indicating that there is no evidence that antibiotics and other secondary metabolites are produced by actively growing cells. Secondary metabolites include those metabolites unique to non-replicating cells and are without known function to the producer cells. In general, the overall process of secondary metabolism includes: 1) derepression of the appropriate portion of the genome to obtain transcription and translation of the appropriate synthetases; 2) subsequent activity of the latter for a finite time. In batch culture, the derepression occurs just prior to the beginning of the stationary phase. Even though much industrial effort has been involved with the production of antibiotics since the Waksman discovery of Streptomycin (83) the molecular events that initiate as well as those that terminate secondary metabolisms are poorly understood (84).

One of the most commonly studied Streptomyces, *Streptomyces coelicolor*, also produces antibiotic secondary metabolites. There are a number of pigmented antibiotics produced by *S. coelicolor*, one of which is water soluble, 'coelicolorin' (81). Even though this antibiotic is only weakly affective against Gram-positive bacteria, this species of *Streptomyces* is the only species of the genus which has been well studied with respect to its genetic characteristics (13).

Although the control of the antibiotic production and other secondary metabolic products is not understood there are at least
several morphological features usually correlated with the time of (cell age) antibiotic production. These features include aerial spore production and the appearance of a spore sheath which envelops the spore producing aerial hyphae and the mature spores (88).

In the case of *S. fradiae* the implication of UDP-N-acetylglucosamine in neomycin synthesis implies some common intermediates with cell wall synthesis (67).

There is some experimental evidence that the secondary metabolites have feedback controls on early life cycle metabolism. *S. griseocarneus* spores fail to germinate when greater than 5 μg/ml hydroxystreptomycin or streptomycin are in the growth medium. If the spores are permitted to germinate and grow for 24 hr first, then the presence of the antibiotics do not appear to affect growth (5).

As one of the features associated with branch pathways (secondary metabolism) the rodlet mosaic component of the aerial growth sheath has been demonstrated in all the *Streptomyces* investigated (8, 87, 88).

Easily demonstrated by electron microscopy, the rodlet mosaic physical and chemical nature has not been elucidated. The major obstacle in characterizing the rodlet mosaic has been its isolation and purification.

**Actinophage and Secondary Metabolites**

Direct correlation exists between the phage genome of the *Streptomyces* and the secondary metabolites such as the antibiotics
produced. Most of the industrial cultures used for the production of antibiotics from *Streptomyces* are lysogenic. In *Streptomyces coelicolor* A3(2) the antibiotic pigment is controlled by the prophage genome integrated into the cell genome (45).

*S. coelicolor* A3(2) is the only *Streptomyces* strain well examined genetically. Its øC31 phage genome is mapped and partially characterized (13).

There is some indication that the nutrient conditions of the growth medium can affect the spontaneous expression of free actinophage in some *Streptomyces* (62).

Phagolysis of the *Streptomyces* can occur in hyphae, ungerminated spores, and germinated spores. All phases can undergo such lysis in *Actinomyces* (*Streptomyces* streptomycini strain 1072 and *A. candida* *candida* strain 36 (39). Extracellular filaments (of unknown nature) of different lengths and diameters were present outside the phagolyzed cells.

**Culture Medium Considerations**

*Streptomyces* are generally capable of utilizing a wide range of substrates for their energy and carbon sources with varying growth rates and end products depending upon the culturing circumstances.

The *Streptomyces* represent one group of organisms which are able to degrade the ubiquitous chitin found in soils and water supplies (36). Many members of this group will grow on 'simple' washed agar or complex media such as beef heart infusion and yeast extract, growing best at neutral pH levels (72).
Since many of the traditional methods for taxonomic placement have been based upon the variable secondary metabolites such as colony color, mycelial color, and diffusable pigment colors as well as gross morphological features such as colony appearance and spore arrangement, widely variable reports from different laboratories are the consequences of inconsistent use of media.

One of the goals of the International *Streptomyces* Project was to coordinate systematic methods of preparing media and sources of media constituents to obtain valid descriptions of basic metabolic characteristics of the various *Streptomyces*. The various media used and methods of preparation were summarized by Shirling and Gottlieb (72).

In addition to the chemical considerations of *Streptomyces* and other microorganisms growth, it is important to consider the surface-to-volume ratio of liquid-to-solid surface area. Interactions within media (e.g., concentration affect at particle surfaces) modifies product recovery and growth rates in bacterial cultures. Dissolved chemicals (nutrients) tend to accumulate at interfaces and therefore the medium concentrations effectively increase at particulate interfaces (7, 59, 66, 91, 93, 94).

Therefore not only the type of material that is used in the medium should be recorded but also the particle size of any suspended material must be considered.

Since a number of the *Streptomyces* have the ability to hydrolyze agar under certain conditions it is important to note the composition
of agar. It is composed of linearly linked, alternating units of D-galactose (linked β-D-(1→4)) and of L-galactose (linked α-L-(1→3)) containing a half-ester sulfate on about 1 out of 10 D-galactose units and pyruvic acid as a 4,6-O(l'carboxyethylidene) group on about one out of 51 D-galactose units (4).
MATERIALS AND METHODS

Bacterial Cultures

*Streptomyces coelicolor* A3(2) (OSU #686 from ISP #5049) was supplied by E.B. Shirling (Ohio Wesleyan College, Delaware, Ohio); and *Streptomyces* OSU #474, which was isolated from the Western Basin of Lake Erie, were used for cytochemical studies. *S. coelicolor* A3(2) (described in the literature review) was used for the polysaccharide extractions. *Streptomyces OSU* #474 was melanin positive and had smooth aerial spores arranged in flexuous chains.

Culture Maintenance

Substrate mycelia were grown for 5 d in tryptone-yeast extract broth (72). Mycelia and media aliquots (0.5 ml) were sealed in 1 ml ampoules and frozen directly in liquid nitrogen (78).

Inocula for the various studies were maintained on glycerol-asparagine agar containing trace salts (Appendix B). Sporulated cultures were maintained on slants of the glycerol-asparagine medium for periods up to 2 mo at 4 C without loss of culture viability.

Fermenter Growth

*S. coelicolor* A3(2) spore formation was monitored by phase microscopy of growth in a 5-£ fermenter (Virtis Minifermenter) which
contained 3-£ of tryptone-yeast medium (Appendix B). The fermenter was aerated at 500 ml/min, maintained at 25 C, and stirred at 75 rpm.

**Shake Flask Culturing**

The glycerol-asparagine medium, without agar, was used as a control, and 0.5% agar-agar and 1% acid-washed 60/80 mesh Chrom W (diatomaceous earth) (Applied Sciences) were added to experimental flasks. The inoculum was prepared by suspending spores obtained from glycerol-asparagine agar slants. Equivalent inocula were quantitatively added to each of 6-25 ml flasks, which initially contained only the glycerol-asparagine medium. The flasks were shaken at 125 rpm for 1 wk. After 1 wk, 2 cultures were left unmodified, 2 cultures received 1 g sterile Chrom W each, and 2 cultures each received 1 g gamma irradiated, sterilized agar. The experimental and control flasks were incubated at 25 C for an additional 7 d.

Two additional flasks contained steam sterilized agar (0.5% in glycerol-asparagine medium). *S. coelicolor A3(2)* spores were inoculated into these flasks and incubated for 2 wk at room temperature.

**Cells Cultured for Polymer Extractions**

Gram quantities of aerial growth were required for chemical extractions. Dialysis membrane (Union Carbide) was soaked in distilled water, autoclaved, and laid over glycerol-asparagine agar medium in petri dishes. Spore inocula obtained from 5 d glycerol-asparagine agar slants were spread over the dialysis membrane. Growth (8-14 d) was easily stripped from the agar surfaces. Intact
colonial growth was separated from the dialysis membrane by gentle stirring of the membranes in 0.01% Triton-X-100. Spores and aerial hyphae (cells) were Tri-R-Stirrer homogenized and then washed free of the Triton-X-100 by four centrifugation washes in distilled water at 10K(x)g for 20 min ea. Cells were lyophilized for 10-20 hr and stored at -20 C. The lyophilized preparation normally had a deep purple color.

Cytochemical Analysis of the Rodelt Mosaic

Mature aerial growth of *S. coelicolor* A3(2) and *Streptomyces* (OSU #474) was collected from cultures grown for 8-14 d on glycerol-asparagine agar (Appendix B) by scraping cells from agar surfaces with a microscope slide, homogenized and pelleted by centrifugation. Separate samples were treated with solvent or enzyme mixtures and cells were pelleted for freeze-etch replication.

The following solvents and solutions were used one at a time to treat different cell pellets: chloroform; chloroform/methanol (1/1); diethyl ether; hog pancreatic phospholipase (Worthington Biochem) (1% in unbuffered 0.005 M CaCl₂); pepsin (Worthington Biochem) (0.5% in 0.01 N HCl); trysin (Worthington); 5% (W/V) trichloroacetic acid (TCA) in H₂O; 0.1 N NaOH (100 C); H₂O (100 C); H₂O (121 C); egg white lysozyme (Worthington) (10-25 mg/ml in 0.1 M sodium borate buffer, pH 9.2); 0.1 N HCl (100 C); and *Streptomyces griseus* chitinase (lot #15C-0194, Sigma) (0.5% in 0.1 N phosphate buffer, pH 7.2).

Each of the organic solvents caused the sporulated growth to aggregate.
Polysaccharide Extractions

Two different procedures were used to isolate the rodlet-like polymers: A) enzymatic treatments; and B) isolation by ethanol precipitation. Precipitated polymers (procedure B) were deproteinized with: 1) chloroform-butanol; and 2) Freon 113.

A. Enzymatic rodlet isolation: The rodlet mosaic was considered to be a carbohydrate polymer because it was affected by HCl (Table 3). The mosaic was not affected by lysozyme or trypsin so these two enzymes were utilized, in sequence, to attempt isolation of the rodlet fibers. The following protocol was followed:

(a) Cells were lysozyme treated for 8 hr at 42 C.

Lysozyme 50 mg/ml
in NaB03 buffer
pH 9.2, containing 0.1% NaCl

(b) Cells were washed by centrifugation, 3x's, at 8700(x)g.

(c) Cells were disrupted at 20K psi by two passages through a French pressure cell.

(d) The cell paste was resuspended in trypsin and treated for 9 hr at 25 C.

Trypsin 1 mg/ml
5% CaCl 0.44 ml/10 ml Tris buffer
pH 8.0

The enzyme-broken cell mixture was Tri-R-Stirrer homogenized for 30 sec, incubated, and rehomogenized.
(e) The sample was washed 3x's with distilled water by centrifugation at 8700(x)g.

(f) The supernates were pooled and washed over a Diaflo type XM300 ultrafilter (Amicon) (MW cutoff at 300K Daltons) with 500 ml of pre-filtered 'clean' water (see below). Purified nitrogen was used at 20 psi for the washing procedure, in a 250 ml Amicon ultrafiltration cell.

(g) The material retained by the Amicon XM300 ultrafilter was lyophilized and stored at -20 C.

The pellet and supernate from step (e) were examined by electron microscopy for presence of rodlet-like fibers.

B. Rodlet Isolation by Precipitation: The following procedure is essentially that of Freeman (26). All water used for the Freeman (26) procedure and the Sevag (70) polymer deproteinization was double distilled and 'cleaned' by 0.45 µm membrane filtration (Millipore) and steam sterilization. The isolation steps were as follows:

(a) Centrifuged washed S. coelicolor A3(2) cells were disrupted by the liquid nitrogen cryo-impacting (LNIC) process (73) (Appendix A) and stored in liquid nitrogen until used.

(b) Proteins and nuclear material were removed from the cells by refluxing the disrupted cells at 100 C in 1 M acetic acid for 12 hr. The acetic acid solution became yellow and the precipitated clumps retained the purple coloration characteristic of washed whole cells, from aerial growth.

(c) The entire refluxed mixture was centrifuged (10 K(x)g) for 30 min at 5 C in 150 ml Sorvall Pyrex centrifuge bottles.
(d) The supernatant was dialyzed against 5°C distilled water for 48 hr with water changes at 12 hr intervals. Just prior to use, the dialysis tubing (Union Carbide) was prewashed in three changes of distilled water.

(e) The dialyzed material, which had formed aggregates, was again clarified by centrifugation (10 K(x)g) at 5°C for 30 min.

(f) The supernatant was reduced to 30-50 ml by flash evaporation in a side-by-side flash evaporator (Bucheler). The material was heated to 60°C and the evaporated water was trapped on a dry-ice/acetone cooled rotating glass trap.

(g) The condensed product was treated with -40°C ethanol (EtOH). The alcohol was used at the ratio of 780 ml EtOH per 100 ml sample and kept at -20°C until precipitation was evident (12-24 hr).

(h) The ethanol-induced precipitate was collected by centrifugation at 10 K(x)g at 5°C for 30 min. The pelleted product was deproteinized by one of the two methods described below:

1. Chloroform-butanol Deproteinization (70): Protein and lipids were removed from the ethanol precipitate by the following sequence:

   (a) The ethanol precipitate from the Freeman procedure was resuspended in 100 ml 'clean' water. Sodium acetate (10 g) and glacial acetic acid (1 ml) were dissolved in the mixture.

   (b) A mixture of 20 ml chloroform and 4 ml n-butanol was added. The entire mixture was shaken on a horizontal shaker for 3 hr.

   (c) The emulsion was centrifuged for 20 min at 650(x)g and a dense white emulsion was visible at the organic solvent-water interface.
(d) Aqueous phases were pooled and repeatedly treated (up to 10 times) with the chloroform-butanol mixture until there was no visible precipitate at the solvent-aqueous interface after centrifugation.

(e) Ethanol was added to the aqueous phase (780 ml EtOH/100 ml) aqueous sample. The ethanol was cooled to -40 C before adding it to the aqueous sample. The mixture was placed at -20 C overnight.

(f) Precipitated product was recovered by centrifugation (5 C) at 5K(x)g, lyophilized, and stored at -20 C.

2. Freon 113 Deproteinization (51): The Markowitz and Lange procedure was used as follows:

(a) The aqueous fraction was extracted 6x's with equal volumes of Freon 113 at 4 C. Each of the six steps were composed of eight 2 min low speed homogenizations with a Waring blender. Between each 2 min period, the blender jar was packed in ice for several minutes, to retard Freon 113 boiling (bp = 56 C). The Freon 113 fractions were extracted twice with water.

(b) The pooled aqueous fractions were treated with ethanol (780 parts to 100 parts H2O), initially at -40 C, then overnight at -20 C.

(c) The flocculent precipitate was recovered by centrifugation (5 C) at 10K(x)g for 30 min.

(d) The precipitate was washed 3x's with absolute ethanol, 3x's with chloroform, and resuspended in 'clean' distilled water. The aqueous suspension was lyophilized to a light tan fibrous powder.

Chitin Purification

Crude crab chitin (Calbiochem) was reduced from the 5 mm flake form to nearly colloidal size by the LNCI method (73). Ten grams of the pulverized chitin were treated for 2 hr with 2 N HCl, washed 15x
with water, treated with 2 N-NaOH at 80 °C for 8 hr with constant stirring, washed 10 times with water, decolorized with 5% of 60°C NaOCl (Chlorox) for 3-2 hr periods, and washed with 10 vol of distilled water. The resultant material which settled out of suspension was treated first with 3 changes of acetone, then 3 volumes of methanol, partially dried with a stream of air then dessicated in a hot air oven at 80°C. The resultant tan colored cake was homogenized in distilled water. Rehydrated chitin appeared colloidal and remained in suspension. The chitin was lyophilized and stored at -20°C.

**Polymer hydrolysis**

Isolated polysaccharide (rodlet-like) samples were prepared for thin layer chromatography and gas chromatography by hydrolysis with various concentrations of HCl from 0.5 N to 6 N HCl for periods of 2-18 hr at 95°C or 115°C. Acid was removed from the hydrolyzed samples by drying samples in the presence of NaOH pellets and silica gel (Matheson, Coleman and Bell). N-propanol was added to the samples so they would not lose more than half of their liquid volumes until the pH level was 6.5-7.

'Clean' distilled water was used to resuspend samples to specified volumes, and hydrolysates were stored at -20°C except when used for analysis.

A few samples were hydrolyzed with 0.5 N H$_2$SO$_4$ for 17 hr at 100°C. Hydrolysates were adjusted to pH 6 by adding BaOH. Residual BaSO$_4$ precipitate was removed by centrifugation at 10,000(x)g for 20 min.
Protein Analysis

Unhydrolyzed protein was determined by the Lowry method (46). Bovine serum albumin fraction V (Sigma) was the protein standard. Lowry colorimetric stock solutions were modified as follows: the CuSO₄ solution was made in distilled water, and the sodium tartrate (molarity compensated) was added to the buffer solution containing Na₂CO₃ (Allied Chemical). All solutions were stored at -20°C until used. Standard curves and sample absorbance were recorded at 540 nm with a Beckman DU spectrophotometer. Absorbance maxima for standards and for all samples were confirmed with a Bausch and Lomb Model 505 recording spectrophotometer.

Carbohydrate Analysis

Unsubstituted sugars in solution were determined by the Dubois, et al. (18) colorimetric procedure which utilized 1 ml of 5% aqueous phenol mixed with 1 ml of sample for 10 min. Concentrated H₂SO₄ (5 ml) was added to the phenol-carbohydrate mixture. Heat of mixing promoted color development which was complete after 30 min. Standard D-glucose (Mallinckrodt) solutions and experimental samples were quantitatively recorded as absorbance at 488 nm.

Free amino sugars were determined by the Lee and Montgomery (42) method. The colorimetric determination (42) was similar to the phenol-sulfuric acid method of Dubois, et al. (18) but was preceded by deamination of the amino sugars. The Lee and Montgomery procedure was modified by the use of 0.3 ml of 25%
aqueous phenol rather than 0.1 ml of 80% aqueous phenol. The deamination proceeded as follows: One and one half ml of sample at neutral pH; 0.2 ml 5% sodium nitrate; and 0.2 ml 40% acetic acid were mixed together and permitted to stand for 10 min. Aqueous ammonium sulfamate (0.2 ml of 15% soln) was added and the mixture was shaken vigorously for 30 sec, initially, and 5 sec every 10 min for a total of 30 min.

All of the above procedures are done at room temperature. Color reactions of deaminated sugars were determined by the phenol-sulfuric acid sequence with appropriate absorption values recorded at 488 nm.

N-acetyl-D-glucosamine (NAGA) concentrations were determined by the Morgan-Elson (55) procedure in which 0.1 ml 0.5 N Na$_2$CO$_3$ was added to 1 ml of sample. The buffered sample was immersed into a boiling water bath for 5 min (±5 sec). The water bath treated samples were immediately cooled in 25 C water. Each sample was acidified with 8 ml glacial acetic acid and 1 ml of 2% dimethylaminobenzaldehyde (Baker) (in glacial acetic acid/HCl, 95/5, v/v) was added. The final solution was mixed by inversion and permitted to develop for 40 min at ambient temperatures prior to recording absorbance maxima and quantitative values (at 540 nm).

Qualitative absorption spectra of all standards and samples were monitored to ensure the reliability of the assays for both carbohydrate and protein analysis. Standards used for protein
analysis were checked for the presence of sugars and the carbohydrate samples were evaluated for protein.

**Thin Layer Chromatography**

Silica gel G and microcrystalline cellulose (Applied Sciences) (250 μm layers on glass) were used for thin layer chromatographic separations of sugar hydrolysates.

Silica gel G plates were heat activated at 100°C for 30-60 min just prior to sample applications. The microcrystalline cellulose plates did not need heat treatment or dessication prior to sample applications.

Silica gel G plates were developed with n-propanol/NH₄OH/H₂O (144/48/24, v/v) for 2 hr. The solvent front usually ascended to 10 cm from the origin.

Microcrystalline cellulose plates were developed using two development systems: 1) pyridine/ethyl acetate/acetic acid/H₂O, (5/5/1/3, v/v) for 2-2.5 hr, producing a 13-14 cm solvent front; 2) normal-butanol/pyridine/water (3/2/1.5, v/v) (78) gave a solvent front at 14.5 cm in one hour. All separations were run at ambient temperatures.

The summarized procedures of Sweig and Sherma (95) were used for sugar indicators. All plates were spot-checked for UV fluorescence before and after indicator sprays were added. The 5% ammoniacal silver nitrate was used as a general spray for free sugars. The 2% anthrone, 3% p-anisidine, and 1% benzidine were made
up as spraying reagents in n-butanol. Amino sugars were indicated by 0.2% ninhydrin in 95% ethanol or acetone (spraying and dipping, respectively). Sprayed chromatograms were heated for 5-10 min at 100-105 C.

Para-anisidine was difficult to dissolve in n-butanol so 3 g p-anisidine was dissolved in 10 ml of methanol then diluted to 100 ml with n-butanol. The resulting solution permitted efficient detection of the location of NAGA. NAGA was not readily detected with any of the other indicator sprays used.

**Gas Chromatography**

Acetic acid was released by complete polymer hydrolysis. A 1.05 mg sample was dispersed in 1 ml of 6 N HCl and was hydrolyzed for 10 hr at 115 C in a Teflon capped 3 ml ReactiVial (Applied Science). Since acetic acid is volatile the hydrolyzed sample was put on ice and 1.1 ml of 6 N NaOH was added to bring the sample pH above neutrality. The sample was dried at 60 C. The residue was diluted with clean distilled water and adjusted to pH 3 with 6 N HCl.

A Varian gas chromatograph (Model 274010-00) in conjunction with a Linear recorder was used to record peak areas of acetic acid standards and samples. Column temp was 125 C, injector temp was 175 C, and the detector temp was 150 C. Nitrogen was the carrier gas. Samples were detected with the flame ionization detector. Acid washed Chrom W 60/80 mesh, was coated with SP1200 (Supelco) (1 g mixed with 0.1 g H₃PO₄ in acetone), and packed in a 6' x 1/8"
glass column. Glass wool was used only on the detector end of the column. The column was conditioned overnight at 200 C and water conditioned just prior to use with 3 injections of 5-7 µl of 'clean' distilled water (57). Acetic acid peak locations were identified with respect to elution time.

**Infrared Absorption Spectra**

Samples of polymer (0.75 mg) were suspended in water containing 300 mg of KBr, homogenized, and lyophilized. Samples were thoroughly dried in vacuo at 80 C over magnesium perchlorate and then macerated with a dessicated mortar and pestle. Clear windows were made with a Perkin-Elmer pellet die and a Carver Press, Model C. The windows remained clear for extended periods of storage. Infrared spectra were obtained with the Perkin-Elmer Model 237B Infrared Spectrophotometer. The normal slit width was used.

**Density Gradient Centrifugation**

Polymer isolates were analyzed by gradient centrifugation. Linear sucrose gradients were made with an Isco Gradient Maker, Model 570. Nitrocellulose tubes (5/8" x 4") were filled with gradients, overlayed with experimental samples, and placed in Beckman SW27 swinging buckets. Samples were centrifuged for 6 hr at 5 C and 21 K RPM. Blue Dextran (molecular weight 2 x 10^6) (Pharmacia) was used to calibrate gradients. Paper White BP (DuPont) was added to rodlet samples.

Centrifuged gradients were fractionated with an Isco Fraction Collector, Model 180. Polymer associated Paper White BP was
detected with long wave ultraviolet (Mineralight, SL 3660, U-V Products) induced fluorescence.

Fractionated samples which correlated with Paper White Fluorescence and the Blue Dextran were dialyzed against water and examined by electron microscopy.

**Scanning Electron Microscopy**

Colonies of *S. coelicolor* A3(2) mature (10 d) colonies were excised from glycerol/asparagine agar and frozen in liquid Freon 12 for 20 sec, transferred to liquid nitrogen, and lyophilized at $10^{-3}$ torr for 8 hr. Dry samples were stored in a dessicator until rotary shadowed with Au:Pd (60:40) at 90 and 25 degrees from a distance of 12.5 cm from the sublimation sources.

Control and 30 sec diethyl ether treated whole colonies were examined in a Mini-Sem Scanning electron microscope (Hikachi) operating at 15 KV, and specimen images were recorded on Polaroid P/N 55 film. Micrographs were made from the original negatives directly on Ektamatic SCF paper.

**Transmission Electron Microscopy**

The various samples of intact cells, particulates, and isolated polymers were negatively stained with 1% uranyl acetate or shadowed with Au:Pd (60:40) (Fullam, Schenectady, N.Y.).

Impressions of 10-12 d colonial growth were prepared for critical point drying by gently touching carbon-formvar coated 300 mesh copper grids (Ladd) to colony surfaces with subsequent dehydration in a series of ethanol. The ethanol was replaced by amyl acetate
and finally the amyl acetate was replaced with liquid CO$_2$ and critical point dried in a critical point drying apparatus (Tousimis Res. Corp., Rockville, Md).

Mature intact colonies of *S. coelicolor* A3(2) aerial growth were fixed according to the Luft (48) ruthenium red/osmium conjugation protocol. Samples were first fixed for 1 hr with a solution of 7.5% glutaraldehyde/0.1 M cacodylate buffer (pH 7.2)/0.15% ruthenium red in water (1/1/1) and washed three times with 0.1 M cacodylate buffer (pH 7.2). Samples were post fixed with a mixture of 5% aqueous OsO$_4$/0.1 M cacodylate buffer/0.15% ruthenium red (1/1/1) for 1 hr. Specimens were dehydrated in a graded series of 35-100% ethanol, treated with propylene oxide, and 1/1 mixture of propylene oxide and completed Epon 812 resin (47). Colonies were embedded in 100%, complete Epon 812 resin, and the resin was polymerized for 12 hr at each of 37 C, 45 C, and 60 C.

Thin sections were obtained with an LKB Ultratome III (LKB Instruments, Rockville, Md) using either glass knives or a diamond knife and sections were post stained with 2% Reynold's lead citrate (65).

The freeze-etch imaging of the rodlet mosaic layer appears to be the most efficient method of detecting this component for electron microscopy (88). The freeze-etch replication process, suggested by Moor (53), was used to process cell replicas in a Balzer's High Vacuum Freeze Etching Device (Balzer High Vac, Santa Ana, Calif).

Control and treated cell samples (solvents or enzymes), were centrifuge pelleted, placed on Au carrier discs, frozen for several
seconds in liquid Freon 12, then stored in liquid nitrogen until placed in the vacuum jar on the -100 C specimen carrier. Fractured specimens were etched for 3-4 min at 2 x 10^{-6} torr, shadowed with platinum, and coated with carbon. Organic residues on the replica were removed by floating the replica on concentrated H_2SO_4 for 3-5 hr after successive 5 min treatments in 25%, 50%, and 75% H_2SO_4. The reverse order of acid washes was followed by several rinses of the replica in distilled water. The final cleaning was overnight in a 5% NaOCl solution (Chlorox).

 Rodlets have not been shown in thin section in the surveyed literature and it has not been known whether the common processes involving embedment in epoxy resins and the dehydration procedures remove these structures. *Streptomyces* OSU #474 cells were fixed as indicated for the ruthenium red fixation and processed through the embedding procedures up to 7 hr in the 45 C polymerization oven. The resin at this stage was rather viscous so cells and resin (as a suspension) were diluted with propylene oxide (1/1, v/v), mixed by inversion, and centrifuged at room temperature for 30 min at 8,000(x)g. The cell pellet was transferred to the -130 C Balzer specimen stage prior to freeze fracturing. No etching period was employed and the remainder of the processing was the same as for other freeze fracture replications except for an addition to the cleaning procedures. The replica was floated from the specimen stub into propylene oxide and remained in propylene oxide for 30 min. The replica was washed in a descending methanol series (100%,
75%, 80%, 25%) and floated in distilled water before it was cleaned according to the sulfuric acid and sodium hypochlorite procedures used for all normally processed freeze-etch replicas. All materials used for transmission electron microscopy were examined and photographed in a Phillips 300 EM (Phillips Electronics, Cincinnati, Ohio). Photographs were taken on 35 mm Fine Grain Release Positive film (Kodak) and developed as negatives with Dektol developer (Kodak) stock solution diluted with water (1/3, v/v) at 23 C for 2½ min. Negatives were printed with various contrasts using Kodak contrast filter adjustments on Ektamatic SCF papers.

Rodlet Length Distribution and Rodlet Diameter

Periodic calibrations of the Phillips 300 EM at respective instrument settings were monitored with 0.234 μm latex spheres (Fullam) and with 54,800 li/in diffraction grating replicas (Fullam). A Zeiss Particle Analyzer (Model TGZ-3) was employed to aid in recording uninterrupted lengths measured on prints. Rodlet diameters of individual rodlet fibrils were measured using a Bausch and Lomb Measuring Magnifier. Statistical evaluations were based on 1200-1400 uninterrupted rodlet length measurements and 20-35 rodlet width measurements.
RESULTS

Nine to 12 d agar lawns of *Streptomyces coelicolor* A3(2) exhibited a gray aerial spore surface and purple pigmented agar which resulted from diffusion of water soluble pigmented antibiotics into the medium. The 9-12 d sporulating growth exhibited a hydrophobic characteristic indicated by water beading on the culture surface. The hydrophobic property was lost after a treatment for 30 sec with diethyl ether. The ether treatment produced a lawn devoid of the gray coloration but did not remove the purple pigment visible in the agar and also visible in centrifuged washed pelleted spores taken from the mature aerial mycelial growth.

Figure 1 and Figure 2 are scanning electron micrographs of untreated and 30 sec ether treated colonies, respectively. Loss of an ether soluble matrix (M) surrounding mats of filaments and spores in the control (Fig 1) is indicated by the clearer image of individual cells in the ether treated sample (Fig 2).

The rodlet components of the sheath mosaic could not be observed with the scanning electron microscope so it was imperative to use transmission electron microscopy to investigate the occurrence and characteristics of the rodlet mosaic. Mature (9-12 d) colonial growth of *Streptomyces coelicolor* A3(2) on solid agar was processed
for transmission electron microscopy by shadowing (Fig 3), negative staining (Fig 4), critical point drying (Fig 5, 6, 7), freeze-etch replication (Fig 8, 9), and thin sectioning of osmium-ruthenium red fixed cells (Fig 10, 11).

Processing cells by dessication for shadowing and negative staining appears to often disrupt the rodlets (R) and remove them from the parent cell (Fig 3, 4). An amorphous granular matrix (GM) (Fig 3) is also displaced and frequently masks the rodlets or is otherwise associated with the disrupted rodlets.

Examination of critical point dried spores (Fig 5, 6) revealed that the granular matrix (GM) remained adherent to the mature spore surfaces but was removed, in many cases, from the immature sporulating hyphae (Fig 7). In contrast, the rodlets (R) have not generally been displaced from the cell surface and are covered with the granular matrix (GM) in the critical point dried preparations (Fig 6, 7).

Electron microscopy of freeze-etch replicas permits visualization of the unmasked rodlet mosaic pattern (RM) which is composed of apparently criss-crossing individual rodlets which are frequently aligned in parallel groups of two rodlets (circles in Fig 8). The mosaic appearance is apparently intact in the freeze-etch preparations; at least in comparison to the images seen in the other methods of preparation where the individual rodlets are isolated from the cells.

The granular matrix (GM) visible in Fig 8, 9 has a variable thickness but usually (when observed) in freeze-etch preparations is tightly apposed to the rodlet mosaic (RM).
Figure 1 and 2. Scanning electron micrographs of lyophilized aerial *S. coelicolor* A3(2) intact colonial growth (Fig 1) and 30 sec ether treated colonial growth (Fig 2). The matrix (M) visible in the untreated aerial colony (Fig 1) is soluble in ether and its absence in Fig 2 permits clearer visualization of hyphal filaments (F) and chains of spores (S).
Figure 3. Transmission electron micrograph (TEM) of a germanium shadowed, air-dried *S. coelicolor* A3(2) spore which shows the released rodlet-like (R) structures and the rodlet associated granular matrix (GM).

Figure 4. TEM of uranyl acetate negatively stained spores showing the dissociated rodlet (R) images.
Figures 5, 6, and 7. TEM of critical-point dried *S. coelicolor* A3(2) aerial cells showing the granular matrix (GM); rodlets (R); and in Fig 5, an emergent germ tube (H).
Figures 8 and 9. TEM of freeze-etch replica of aerial *S. coelicolor* A3(2) spores. The exterior face of the rodlet mosaic (RM) is covered with the granular matrix (GM) which lies exterior to the rodlet mosaic, and exhibits a varying thickness.
Figures 10 and 11. TEM of ruthenium red/osmium fixed *S. coelicolor* A3(2) aerial spores showing the sheath (FS) which appears to separate (Fig 10) from the granular matrix (GM) (Fig 11) in mature spore chains. The arrows indicate the thin dark band of material which is interpreted as the rodlet mosaic layer.
Thin sections of the ruthenium red-osmium fixed spores often illustrate a layer of what has been often called the fibrous sheath (FS) and appears to separate from mature spores (Fig 10). There also are frequent cases in which the zone between the spores is filled with a granular matrix (GM) (Fig 11).

Examination of the freeze-etch replica of partially embedded cells in which epoxy monomer was partially removed with propylene oxide (Fig 12, 13) clearly indicates that the rodlet mosaic (RM) remains intact at least through 7 hr of the 45 C polymerization step. It would not be expected that further extraction by the hydrophobic embedding resin mixture would occur after the 7th hr of 45 C incubation since the mixture is very viscous at this stage.

The granular matrix (GM) which is present in the various methods of cell preparation is also visible in the specimens which had been partially embedded (Fig 13) and appears to be tightly apposed to the rodlet mosaic.

If the fracture plane exposing the rodlet mosaic is traced to the area (arrows) in Fig 13 where the spore coats are fractured in cross section, it can be observed that the rodlet mosaic is probably only 1 or 2 rodlets thick. If it is assumed that the mosaic is always as thin as is apparent in Fig 13 then it would be expected to be difficult to identify rodlets in thin section.

Average rodlet widths determined from freeze-etch and negatively stained preparations of *S. coelicolor* and *Streptomyces OSU #474* are summarized in Table 1. The standard deviations of each type of
Figures 12 and 13. TEM of freeze-etch replicas of *Streptomyces* OSU #474 aerial growth that had been partially embedded prior to freeze-etch replication. The rodlet mosaic (RM) and granular matrix (GM) are visible.

Occasionally the rodlet fibrils are aligned in parallel groups of more than two (circles in Fig 12).

Arrows in Fig 13 indicate the fracture plane which occurred perpendicular to the rodlet mosaic, exposing an "edgewise" view of a portion of the mosaic.
### TABLE 1. Rodlet Diameters

<table>
<thead>
<tr>
<th>Sample</th>
<th>Freeze-etch replication (nm)</th>
<th>Uranyl acetate negative stain (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. coelicolor A3(2)</strong></td>
<td>(whole cell) 5.0(31, 0.34)¹</td>
<td>(purified rodlets) 4.1(20, 0.53)</td>
</tr>
<tr>
<td>Purified crab chitin</td>
<td>4.8(18, 0.68)</td>
<td>4.3(22, 0.43)</td>
</tr>
<tr>
<td><em>Streptomyces OSU #474</em></td>
<td>(whole cell) 4.5(33, 0.64)</td>
<td></td>
</tr>
</tbody>
</table>

¹Sample size and standard deviation, respectively, in parentheses.
preparation are about the same as the differences between the respective sample means.

The rodlet mosaic was most readily identified in freeze-etch replication so this preparation method was used for subsequent in situ observations. In all the freeze-etch replicas of *Streptomyces coelicolor* A3(2) and *Streptomyces* (OSU #474) examined, the exposed rodlet mosaic patterns appeared to be made of randomly arranged rodlet clusters. The hypothesis of random rodlet arrangement was checked by plotting the frequencies of lengths of uninterrupted rodlet segments. Since the measurements were performed with a Zeiss Particle Analyzer, calibrated uninterrupted rodlet lengths were necessarily registered at intervals rather than as continuously variable measurements. Curves generated by measurement frequencies of rodlet lengths (Fig 14, 15, 16, 17) all appear to be similar to the normal curve but are skewed slightly to the right.

Table 2 summarizes the descriptive statistical data for the frequency curves generated by individual rodlet lengths. Statistical analyses indicate mean length and other sample differences.

After 30 hr of growth at 25°C in the fermenter, *S. coelicolor* A3(2) produced spores (Fig 18) and the spores continued to be present until 98 hr when the culture system was terminated. The possibility that the spore-like bodies were contaminants in the tryptone-yeast extract medium was ruled out by plating duplicate samples on both glycerol-asparagine medium and plate count agar (Difco). Freeze-etch replicas did not reveal rodlet mosaic images in these submerged spores (S) or hyphae (H) (Fig 19).
Figures 14 and 15. Frequency distributions of uninterrupted rodlet segments from freeze-etch replicas of ether treated (Fig 14) and untreated (Fig 15) *S. coelicolor* A3(2) aerial cells.
STREPTOMYCES COELICOLOR A3(2)

**ETHER TREATED**

* n = 1318

**RODLET LENGTH (nm)** 252 278

**FREQUENCY**

Median = 71 nm
Mean = 78 nm

STREPTOMYCES COELICOLOR A3(2)

**CONTROL**

* n = 1455

**RODLET LENGTH (nm)** 252 278

**FREQUENCY**

Median = 50 nm
Mean = 61 nm
Figures 16 and 17. Frequency distributions of uninterrupted rodlet segments from freeze-etch replicas of ether treated (Fig 16) and untreated (Fig 17) *Streptomyces* OSU #474 aerial cells.
STREPTOMYCES SP. (OSU #474)

**ETHER TREATED**

- Median = 68 nm
- Mean = 79 nm

$n = 1415$

**CONTROL**

- Median = 68 nm
- Mean = 82 nm

$n = 1401$
### TABLE 2. Rodlet Length Statistics.

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>$\bar{X}$ (nm)</th>
<th>Mode (nm)</th>
<th>Median (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. coelicolor A3(2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1455</td>
<td>61.00</td>
<td>41.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Ether treated</td>
<td>1318</td>
<td>78.50</td>
<td>63.0</td>
<td>71.0</td>
</tr>
<tr>
<td><strong>Streptomyces OSU #474</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1401</td>
<td>81.92</td>
<td>48.7</td>
<td>68.3</td>
</tr>
<tr>
<td>Ether treated</td>
<td>1415</td>
<td>78.69</td>
<td>60.0</td>
<td>68.3</td>
</tr>
</tbody>
</table>
Figure 18. Light micrograph of sporulating submerged *S. coelicolor* A3(2) growth. Spores (S) and hyphal filaments (F) are visible in the growth from the tryptone-yeast extract fermenter culture.

Figure 19. TEM of freeze-etch replica of *S. coelicolor* A3(2) submerged fermenter growth. Rodlets are not visible in replicas of spores (S) or hyphae (H).
Batch cultures of *S. coelicolor* A3(2) grown in 100 ml of modified glycerol-asparagine medium in 250 ml Erlenmeyer flasks also produced spores, and in addition, produced the purple antibiotic pigment similar to the sporulation-associated pigment produced by aerial growth on the glycerol-asparagine solid agar medium. Control flasks containing only the basic glycerol-asparagine medium had very little growth and produced no spores or observable changes in the medium. Flasks containing 0.5% agar and those containing 1% acid-washed Chromosorb W 60/80 mesh produced both spores and the purple pigmentation within 7 d of growth at 25 C on a 125 RPM shaker. Growth in the agar containing flask began producing spores within 4 d and pigmentation appeared about the same time. Pigmentation began occurring at 7 d in the presence of the diatomaceous earth but the degree of growth and intensity of pigmentation was not as high as in the agar containing media.

The diatomaceous earth-containing flasks were intended to serve as controls for nutrient and viscosity effects and particulate-solution interface phenomena introduced by the agar.

*S. coelicolor* A3(2) hydrolyzed the agar in solid media and in shake flasks containing agar. The initial lumpy agar texture in shake flasks was reduced to a heavy sirup consistency.

The agar containing shake culture medium supernate which remained after 10 K(x)g centrifugation and the pelleted cell masses were examined for the presence of released rodlets and for wall associated rodlets, respectively. Since spores produced in liquid
media frequently germinate soon after formation (12) it seemed likely that rodlets (which are not associated with the germ tubes (H), in Fig 5, would be released into the medium. Freeze-etch replicas of the "liquid" agar grown spores (Fig 20) indicates that rodlets (R), may be present on intact cells. Examination of the supernate by negative staining and electron microscopy (Fig 21) indicated the presence of rodlet-like material, which appears similar to the rodlet-like fiber isolated from aerial spores (Fig 26).

Two phases of the submerged growth appeared after 3 d in the agar-containing medium. A few of the spheres were purple while the others remained the usual tan color. Macerated clumps stained for light microscopy revealed spores associated with many of the unpigmented clumps but spores were not associated with the purple colored growth. The spores may have been released by the time the antibiotic pigment was produced.

Results of electron microscopic examinations of freeze-etch replicas of cells treated with various solvents and enzymes are summarized in Table 3. A particular solution was recorded as having "no effect" if the predominant freeze-etch image was similar to that found in the untreated control (Fig 22). Two of the reaction mixtures had readily observable effects on the appearance of the replicated rodlet mosaic appearance. The mild (0.1 N HCl) acid hydrolysis altered the interlacing appearance of individual rodlets and destroyed the double rodlet pair arrangement usually observed in untreated samples (Fig 23). The chitinase effect is demonstrated in Fig 24 where the lack of individual rodlet definition is characteristic.
Figure 20. TEM of freeze-etch replica of *S. coelicolor* A3(2) hyphal element grown in glycerol-asparagine shake culture, containing 0.5% agar agar. The typical parallel fiber rodlet image (R) is visible.

Figure 21. TEM of negative stained rodlet-like fibers (R) precipitated from the (agar-containing) shake culture of *S. coelicolor* A3(2).
TABLE 3. Freeze-etch Replication Monitoring of *Streptomyces* Spores.

<table>
<thead>
<tr>
<th>Rodlet Mosaic Modification</th>
<th>Solution</th>
<th>Effect</th>
<th>No Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chloroform\textsuperscript1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloroform/methanol\textsuperscript1 (1/1, v/v)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diethyl ether\textsuperscript1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phospholipase</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trichloracetic acid</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pepsin</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 N NaOH (at 100 C)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H\textsubscript{2}O (at 100 C)</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H\textsubscript{2}O (at 121 C)</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Lysozyme (at 20 or 100 mg/ml)\textsuperscript1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 N HCl (at 100 C)\textsuperscript1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chitinase</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript1*Streptomyces* OSU #474 also treated with these solutions. Results were identical with respect to the generalized "Effect"/"No effect" designations.
Figures 22, 23, and 24. TEM of freeze-etch replicas of untreated (Fig 22), 0.1 N HCl/100 C treated (Fig 23), and chitinase treated (Fig 24) S. coelicolor A3(2) aerial spores. The control rodlet mosaic (RM) (Fig 22) is in sharp contrast to the greatly altered mosaics in the HCl (Fig 23) and chitinase (Fig 24) treatments, which show vestigial rodlet elements (R) of their mosaics.
Some of the treatment conditions were repeated for *Streptomyces* OSU #474 and results were in agreement with those for *S. coelicolor*, at least with respect to gross rodlet mosaic appearance.

Rodlet mosaic images of freeze-etch replicas of the control and the ether treated cells appeared similar but frequency distributions of the individual uninterrupted rodlet lengths for *S. coelicolor* (Fig 14, 15) indicate that ether treatments induced rearrangements of the rodlet mosaics.

The cytochemical freeze-etch analysis and the particulate behavior of rodlets when treated with ether indicated that the rodlets could possibly be isolated by procedures designed to isolate carbohydrate polymers.

Rodlet polymer (R) isolated by sequential use of lysozyme and trypsin, then purification by Amicon Diaflo XM300 dialysis produced electron micrographic images showing many small spherical beaded structures (B) (Fig 25). Subsequent sucrose density gradient purification yielded contaminant-free rodlet-like images (Fig 26).

Chemical analysis of the enzymatically prepared rodlet preparation, which was not purified using the density gradient system, is summarized in Table 4. Recovery of rodlets is described in terms of glucose equivalents (13.9%) and protein equivalents (46%). The high percentage of protein recovery indicated by the color reaction in the Lowry protein analysis could be partially explained by possible amino sugar enhancement of the colorimetric reaction. Electron
Figures 25 and 26. TEM of negative stained, enzymatically isolated rodlet-like fibers from *S. coelicolor* A3(2) aerial growth. The double parallel fiber arrangement (R) is frequently visible. Amicon XM300 ultrafiltered samples contained numerous spherical particles (B) (Fig 25) which were removed from the rodlet fibers by density gradient centrifugation. Figure 26 represents a rodlet fiber sample that was purified by gradient centrifugation.
microscopy indicated that spherical particulates did contaminate the enzymatically isolated product (Fig 25) so alternate polymer isolation procedures were employed.

Rodlet-like particles were removed from lyophilized cells of *S. coelicolor* A3(2) by the combined polysaccharide purification methods suggested by Freeman (26) and Sevag (70). The sodium acetate in the Sevag method was sometimes precipitated with ethanol then removed by dialysis. Deproteinization with Freon 113 was used as an alternative to the Sevag method (51), and material isolated by the procedures were pooled for analyses.

Lyophilized polymer extracts, light tan in color, were readily suspended in water, but aggregated when in either chloroform, ether, benzene, acetone, pyridine, or ethanol. The ethanol precipitate yield varied from 0.08% to 0.1% with respect to dry weight of intact cells.

Submerged growth (in amounts equivalent to aerial growth) from both the fermenter and shake cultures in tryptone extract failed to yield a visible precipitate when extracted with the combined Freeman (26) and Sevag (70) or the combined Freeman (26) and Markowitz and Lange (51) polysaccharide isolation procedures.

Electron microscopy of uranyl acetate negatively stained precipitated rodlet extract (Fig 27) demonstrated the parallel arrangement of rodlet fibrils (R) that is characteristically observed in freeze-etch replications of intact rodlet mosaics (Fig 8). Freeze-etch replication of the precipitated rodlet polymer extract (Fig 28) also confirmed the parallel arrangement of extracted rodlet polymer.
Figures 27 and 28. TEM of precipitated rodlet fibers from *S. coelicolor* A3(2) aerial growth. The parallel rodlet fiber (R) arrangement is visible in the negative stained (Fig 27) and the freeze-etch replica (Fig 28) of precipitated rodlet fibers.
Chemical analyses of the precipitated rodlet-like polymer are summarized in Table 4. Standard curves for protein determination (Fig 29); glucose determination (Fig 30); glucosamine determination (Fig 31); and N-acetylglucosamine (NAGA) (Fig 32) show the high degree of reproducibility attainable with the assay procedures used.

Percentage recovery values listed in Table 4 were calculated on the basis of weight values determined with the particular colorimetric assay divided by the dry weight of the original sample in the reaction mixture.

Gas chromatographic determinations for acetic acid were based on the standard curve shown in Fig 33. The coefficient of correlation for the gas chromatography dilution curve ($r^2 = 0.91$) was somewhat lower than for the colorimetric sugar analyses. The expected value for acetic acid per cent recovery calculations was based upon the molar ratio of 44/203, acetic acid/unhydrated NAGA respectively and assuming the polymer consisted of NAGA repeating units.

The linear equations determined by linear regression analysis (Appendix C) are indicated in Fig 20-32. Quantitative determinations for standards and for unknowns were based on these equations.

Efficiency of the recovery of glucose from glycogen is nearly 100% but for the isolated rodlet-like polymer it is about 15%. Glucosamine analysis is also highly efficient in recovery of the glucose moiety of glycogen but very poor (2.2%) for the rodlet-like polymer. Since the glucosamine assay that was used involved a preliminary deamination (42), it was important to consider molar relationships when making comparisons. Since the % recovery values

<table>
<thead>
<tr>
<th>Sample</th>
<th>PERCENTAGE RECOVERY</th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Glucosamine</td>
<td>BSA frV$^1$</td>
<td>NAGA</td>
<td>Acetic$^2$</td>
</tr>
<tr>
<td>Enzymatically isolated rodlets, unhydrolyzed</td>
<td>13.9(4)$^3$</td>
<td></td>
<td></td>
<td>46.0(3)</td>
<td></td>
</tr>
<tr>
<td>Precipitated rodlets (PR), unhydrolyzed</td>
<td>15.0(6)</td>
<td>2.2(3)</td>
<td>19.0(3)</td>
<td>0.3(3)</td>
<td>0.0(2)</td>
</tr>
<tr>
<td>P.R. 1 N HCl, 5 hr, 95 C</td>
<td>32.0(3)</td>
<td>25.6(2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P.R. 6 N HCl, 12 hr, 95 C</td>
<td>4.0(3)</td>
<td>20.7(3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P.R. 6 N HCl, 18 hr, 95 C</td>
<td>2.0(3)</td>
<td>22.3(3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P.R. 6 N HCl, 10 hr, 115 C</td>
<td>4.8(3)</td>
<td></td>
<td></td>
<td>139(3)</td>
<td></td>
</tr>
<tr>
<td>Chitin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.8(2)</td>
</tr>
<tr>
<td>Glycogen</td>
<td>99.7(3)</td>
<td>130(3)</td>
<td>16.0(3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA frV</td>
<td>2.5(3)</td>
<td>7.4(2)</td>
<td>99.9(3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-glucose</td>
<td>103(3)</td>
<td>130(3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-galactose</td>
<td>93.7(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-mannose</td>
<td>145(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-glucosamine -HCl</td>
<td>2.5(3)</td>
<td>100(3)</td>
<td>12.0(3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAGA</td>
<td>3.6(3)</td>
<td>8.5(3)</td>
<td></td>
<td>99.8(3)</td>
<td></td>
</tr>
</tbody>
</table>

$^1$Bovine serum albumin, fraction V (BSA frV)

$^2$Calculations based on the unhydrated-NAGA/acetic acid ratio of 203/44 Daltons.

$^3$Numbers in parentheses indicate no. of replicates.
Figures 29, 30, 31, and 32. Standard curves and respective linear equations for bovine serum albumin, fraction V (BSA frV) protein standards (46) (Fig 29); D-glucose standards (18) (Fig 30); D-glucosamine-HCl standards (42) (Fig 31); and N-acetyl-D-glucosamine standards (55) (Fig 32).
**PROTEIN BSA frV STANDARDS**

\[ \tau^2 = 0.98 \]

\[
\mu g = 598.7 (\text{abs}) + 1.26
\]

---

**D-glucosamine STANDARDS**

\[ \tau^2 = 1.00 \]

\[
\mu g = 109.4 (\text{abs}) - 0.98
\]

---

**N-acetyl-D-glucosamine STANDARDS**

\[ \tau^2 = 0.98 \]

\[
\mu g = 706.2 (\text{abs}) - 7.11
\]
Figure 33. Characteristic acetic acid peak obtained by gas chromatography of aqueous acetic acid samples. The peak identified "water" is apparently some low molecular weight compound eluted from the column.

Linear regression analysis of diluted acetic acid standards generated the linear equation:

$$\mu g = (\text{peak area}) (51.56) - 0.63$$

The coefficient of determination ($r^2$) was 0.91.
were determined on a weight basis the 130% recovery value for glycogen is converted to 100.1% when the actual glucose proportion in glucosamine-HCl (0.84) is used for correction. Glucosamine-HCl per cent recovery from the unhydrolyzed rodlet-like precipitate is 2.2%. The Lowry protein reaction produced a 19% recovery value and NAGA determinations on the unhydrolyzed precipitated rodlet extract gave only a 0.3% recovery.

Completely hydrolyzed precipitated rodlet material (6 N HCl at 115 C for 10 hr) analyzed for acetic acid content yielded 139% of the expected acid values.

Hydrolysis reaction mixtures of precipitated rodlets (0.1% soln) in 1 N and in 6 N HCl were initially clear, the polymer readily going into suspension. Particulates 1 mm long appeared in suspension after several hours at 95 C and 115 C. These particulates remained visible throughout the 95 C hydrolysis but were completely disrupted after 10 hr at 115 C with 6 N HCl.

Chitinase treated purified rodlet extracts lost their rodlet appearance after 60 hr of enzyme hydrolysis (Table 5). The Sigma chitinase definition of unit of activity was the release of 1 mg glucose/24 hr/unit. Glucose was released at 42-47% recovery depending upon whether the reaction mixture was filtered with 0.45 μm Milipore filter (Table 5). If the polymer repeating unit is assumed to be NAGA, the 42% recovery at 70 hr is corrected, by the glucose proportion (0.71), to 59.2% recovery. In contrast, very little NAGA was
### TABLE 5. Chitinase Effect on Precipitated Rodlets.

<table>
<thead>
<tr>
<th>Sample</th>
<th>NAGA % recovery</th>
<th>D-glucose % recovery</th>
<th>E.M. monitoring of rodlets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitinase</td>
<td></td>
<td>6.4(3)</td>
<td>(not examined)</td>
</tr>
<tr>
<td>0.45 μm filter sterilized rodlet-enzyme</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mixture¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time 0</td>
<td>0.3(3)</td>
<td>15.0(6)</td>
<td>Rodlets appear normal</td>
</tr>
<tr>
<td>Time 65 hr</td>
<td>3.5(2)</td>
<td>43.0(2)</td>
<td>Rodlet images essentially absent</td>
</tr>
<tr>
<td>Time 70 hr</td>
<td></td>
<td>42.0(1)</td>
<td>Rodlet images absent</td>
</tr>
<tr>
<td>Rodlet-chitinase mixture prop. as above but not filtered</td>
<td>(not examined)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time 23 hr</td>
<td>3.4(2)</td>
<td>47(1)</td>
<td></td>
</tr>
<tr>
<td>Time 60 hr</td>
<td></td>
<td>44(1)</td>
<td></td>
</tr>
</tbody>
</table>

¹The rodlet-chitinase mixture was composed of rodlet precipitate (667 μg/ml, unbuffered) and the Sigma supplied *Streptomyces griseus* chitinase system (330 μg/ml). The total reaction mixture volume of 3 ml was homogenized with a Tri-R-Stirrer homogenizer, 2 ml was filtered with a 0.45 μm Milipore membrane into a sterile Reacti-Vial and permitted to incubate at 25 C. Using aseptic technique, periodic samples of 1 μl were withdrawn from the vial and processed for negative staining with uranyl acetate and electron microscopy.
free in the reaction mixtures even after 65 hr. It was anticipated that values would be higher in the unfiltered mixture since some of the unhydrolyzed purified rodlets might be trapped during filtration.

Purified rodlet molecular weight was estimated by correlation with density gradient centrifugation patterns of Blue Dextran 2000, which has an average molecular weight of $2 \times 10^6$. The purified rodlet-Paper White BP band of fluorescence, the purple pigment-associated band of particles from the supernate of the agar containing shake culture, and the center of the Blue Dextran 2000 band coincided. It appears that the precipitated rodlets have a molecular weight of about $2 \times 10^6$. Electron microscopy of negative stained dialyzed fractions, representing the purified rodlet band and the 0.5% agar culture supernate, confirmed the presence of rodlet-like fibers.

Thin layer chromatography (TLC) of HCl-hydrolyzed rodlet extracts always showed a spot which co-chromatographed with D-glucosamine-HCl in both of the developing systems which were used (Table 6). In the 0.5 M $\text{H}_2\text{SO}_4$ and the 1 N HCl hydrolyzed samples an additional spot co-chromatographed with D-glucose. Silica gel TLC confirmed the glucosamine identity.

The glucosamine-like sample spot was ninhydrin positive, and the spot co-chromatographing with glucose was detected with ammoniacal silver nitrate. Spots at $R_f$ 0.18 were faintly visible only with the 3% $p$-anisidine spraying reagent, and NAGA was poorly visible with all reagent sprays except with the $p$-anisidine.
TABLE 6. Thin Layer Chromatography on Microcrystalline Cellulose.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Developer A&lt;sup&gt;1&lt;/sup&gt; R&lt;sub&gt;f&lt;/sub&gt;</th>
<th>Developer B&lt;sup&gt;2&lt;/sup&gt; R&lt;sub&gt;f&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>.48</td>
<td>.40</td>
</tr>
<tr>
<td>D-fructose</td>
<td>.52</td>
<td></td>
</tr>
<tr>
<td>D-glucosamine-HCl</td>
<td>.36&lt;sup&gt;3&lt;/sup&gt;</td>
<td>.34&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>NAGA</td>
<td></td>
<td>.57&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Xylose</td>
<td>.55</td>
<td></td>
</tr>
<tr>
<td>Muramic acid</td>
<td>.44</td>
<td></td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td></td>
<td>.09</td>
</tr>
<tr>
<td>D-galactose</td>
<td>.43</td>
<td></td>
</tr>
<tr>
<td>Rodlet precipitate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 N H&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt; 18 hr</td>
<td>.15</td>
<td>.26</td>
</tr>
<tr>
<td>1 N HCl/5 hr 95 C</td>
<td>.47</td>
<td>.35&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>6 N HCl/12 hr 95 C</td>
<td>.35&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>6 N HCl/19 hr 95 C</td>
<td>.35&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
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</tbody>
</table>

<sup>1</sup>Developer A: pyridine/ethyl acetate/acetic acid/water (5/51/3) (Wolf from et al., 1965).

<sup>2</sup>Developer B: Butanol/pyridine/water (3/2/1.5) (Payne and Kieber, 1953).

<sup>3</sup>Ninhydrin positive.

<sup>4</sup>Readily visible only with 3% p-anisidine.
Infrared spectra of precipitated rodlet-like fibers and of chitin (Fig 34) prepared for spectral analysis in KBr windows have very similar characteristics. Figure 24 shows only the 'fingerprint' portion of the spectrum and Table 7 summarizes characteristics of the entire spectrum (samples in triplicate) obtained with the Perkin-Elmer IR Spectrophotometer.

Evidence indicates that the rodlet mosaic structure visualized in freeze-etch replicas (Fig 8) and in negative staining (Fig 4) of whole cells of Streptomyces coelicolor A3(2) is probably chitin. Chitin from any source should have similar freeze-etch and negative staining characteristics.

Electron micrographs of freeze-etched (Fig 35) and negatively stained (Fig 36) purified crab chitin look very similar to the analogous images of precipitated rodlets (Fig 8 and 4). The crab chitin freeze-etch replica (Fig 35) has the resemblance of the rodlet mosaic pattern and the parallel rodlet fibril images typical of Streptomyces spore rodlets.

Measurements of negatively stained and of freeze-etched chitin polymer fiber diameters average 4.3 nm and 4.8 nm, respectively (Table 1). These values are quite close to the 4.1 nm and 5.0 nm values for the S. coelicolor A3(2) rodlet diameters, and are less than 1 standard deviation from the respective means of spore rodlet diameters.

S. coelicolor A3(2) was cultured on plates containing nutrient agar in one experiment (Difco) and incubated at ambient temperatures
Figure 34. Infrared spectra of precipitated rodlets and of crab chitin from KBr windows.
INFRARED SPECTRA

PRECIPITATED RODLETS

CRAB CHITIN

frequency (cm\(^{-1}\))

absorbance
TABLE 7. Summary of Infrared Spectra of Crab Chitin and Rodlets.

<table>
<thead>
<tr>
<th>CHARACTERISTIC ABSORPTION FREQUENCIES</th>
<th>Precipitated Rodlets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitin</td>
<td>Precipitated Rodlets</td>
</tr>
<tr>
<td>1P 3425</td>
<td>P 3325-3380</td>
</tr>
<tr>
<td>P 3250</td>
<td></td>
</tr>
<tr>
<td>P 3095</td>
<td>2S 3095</td>
</tr>
<tr>
<td>P 2950</td>
<td>S 3055</td>
</tr>
<tr>
<td>P 2920</td>
<td>S 2950</td>
</tr>
<tr>
<td>P 2880</td>
<td>P 2905</td>
</tr>
<tr>
<td>P 1655</td>
<td>P 2880</td>
</tr>
<tr>
<td>P 1625</td>
<td>P 2849</td>
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<tr>
<td>P 1550</td>
<td>S 1675</td>
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<td></td>
<td></td>
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<tr>
<td>1P 1380</td>
<td>P 1515-1525</td>
</tr>
<tr>
<td>P 1310-1325</td>
<td>S 1505</td>
</tr>
<tr>
<td>P 1255-1265</td>
<td>S 1455</td>
</tr>
<tr>
<td>P 1199-1205</td>
<td>S 1420</td>
</tr>
<tr>
<td></td>
<td>3BP 1425</td>
</tr>
<tr>
<td></td>
<td>BP 1400</td>
</tr>
<tr>
<td></td>
<td>P 1380</td>
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<td>BP 1335</td>
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<tr>
<td>1P 1065</td>
<td>BP 1073</td>
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<tr>
<td>BP 1055</td>
<td>S 1055</td>
</tr>
<tr>
<td>S 1005-1020</td>
<td>S 1020</td>
</tr>
<tr>
<td>S 970</td>
<td>BP 1002-1010</td>
</tr>
<tr>
<td>P 950</td>
<td>S 950</td>
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<tr>
<td>P 930</td>
<td>S 930</td>
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<td>P 880</td>
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<td>S 775</td>
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<td>S 745</td>
<td>S 750</td>
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<td>S 720</td>
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<tr>
<td></td>
<td>S 710</td>
</tr>
<tr>
<td></td>
<td>S 680</td>
</tr>
<tr>
<td></td>
<td>S 655</td>
</tr>
</tbody>
</table>

1'P' = peak  
2'S' = shoulder  
3'BP' = broad peak
Figures 35 and 36. TEM of freeze-etch replica (Fig 35) and negative stained (Fig 36) purified crab chitin. The characteristic parallel fiber arrangement (R) is frequently evident.
for two weeks. Two types of colonies developed: one produced the typical grey aerial growth coloration, spores, and the diffusable purple pigment; and the other colony type was abnormal in that it appeared flat, producing no aerial growth, spores, or associated grey pigment, and failed to produce the purple diffusable pigment. Subculture of the abnormal colony type on nutrient agar and glycerol-asparagine agar produced only the normal type of pigmented colony.

Carbon coated copper grids were gently pressed onto the surface of both types of colonies, stained with uranyl acetate, and examined by electron microscopy. The variant colonies showed a predominance of lysed cellular material and numerous densely stained spherical units (H) with associated tail (T) structures. Close inspection (Fig 37) indicated that these newly appearing units have the properties of the common morphology of many bacteriophage.

Aberrant growth occurred in one batch of *S. coelicolor* A3(2) cultured on the glycerol-asparagine solid agar medium. The cells did not sporulate or produce antibiotic pigment, even after 4 wk of growth at room temperatures. From 12 ℓ of agar (20 ml/petri dish) only 0.2 g of washed and lyophilized aerial tan colored growth was recoverable. Normal agar cultures would produce about 5.5 g of lyophilized purple cells from 12 ℓ of medium. Light microscopy of the abnormal growth indicated that essentially no spores were present. The rodlet polymer was not precipitable from the abnormal growth.
Figure 37. TEM of negative stained *S. coelicolor* A3(2) cells disrupted by what was apparently actinophage lysis. The typical head (H) and tail (T) phage structures are frequently observed.
DISCUSSION

Hydrolyzed *Streptomyces* vegetative cell and spore walls do not have chromatographically distinguishable components (3).

There are very clear ultrastructural distinctions between the vegetative walls and aerial walls, and it would seem likely that some molecular rearrangements probably are responsible for the morphological modifications. One of the most pronounced distinguishing features of the *Streptomyces* aerial spore wall structure is the rodlet mosaic. The chemical composition and the cellular function of the rodlet mosaic has not previously been defined.

Wildermuth, Wehrli, and Horne (88) treated aerial spores of *S. coelicolor* A3(2) with various organic solvents and examined the treated spores by negative staining and electron microscopy. Their conclusion was that the rodlets were not lipid-like and were not removed from spores by the organic solvents. Bradley and Ritzi (8) used carbon replication procedures to evaluate the *S. venezuelae* rodlet nature and concluded that the rodlets were removed by some organic solvents and were lipid-like.

The reasons for the conflicting results indicated by Wildermuth et al. (88) and Bradley and Ritzi (8) may be due to differences in
specimen preparation. Bradley and Ritzi (8) did not define their procedures for removing rodlets with ethanol, benzene, and xylene.

Erikson (22) used Sudan IV to demonstrate the lipid nature of aerial Actinomyces (Streptomyces) coelicolor. Substrate hyphae did not retain the lipid stain. Bradley and Ritzi (8) interpreted Erikson's results and their own results by concluding that Streptomyces spore rodlet mosaics are probably responsible for the water-dispersion properties of aerial Streptomyces spores.

Erikson (22) recognized that homogenized aerial growth of Actinomyces (Streptomyces) coelicolor did not stain with the Sudan IV lipid stain. The ether soluble matrix (M) in Fig 1 is possibly the Sudan IV stainable material which could conceivably be removed from cell masses by homogenization.

It appears that the ether soluble matrix (M) (Fig 1), and not the rodlet mosaic (RM) (Fig 8), is responsible for the hydrophobic properties of aerial growth of Streptomyces coelicolor A3(2).

Differential centrifugation methods have not isolated rodlets from an associated "thin membrane" observed by negative stain (88). The "thin membrane" together with the rodlet mosaic has been labeled the spore fibrous sheath (8, 30, 61, 87). It has been stated that the fibrous sheath is released from the spore during spore maturation (8, 89) but studies did not offer evidence indicating that what was called "fibrous sheath" actually contained rodlets.

Figure 38 shows a schematic of the physical relationship of the mature Streptomyces coelicolor A3(2) spore envelope components.
Figures 38, 39, and 40. Schematic (Fig 38) illustrating the relative positions of the various mature spore envelope layers.

Components from the innermost layer to the outermost layer are: plasma membrane (PM); spore wall layer one (SW$_1$); spore wall layer two (SW$_2$); rodlet mosaic (RM); and granular matrix (GM).

These layers are identified in the Fig 39 and 40 freeze-etch replicas; except the two spore wall layers (SW$_1$ and SW$_2$) are only distinguished in freeze-etch replicas of TCA treated spores (Fig 41 and 42).
Figures 41 and 42. TEM of freeze-etch replicas of 5\% trichloroacetic acid (TCA) treated (4c) *S. coelicolor* spores. The innermost spore wall layer (SW$_1$) is exposed by the fracture plane between it and the second spore wall layer (SW$_2$). The rodlet mosaic (RM) is visible external to the two spore wall layers.
The thickened spore wall (SW) has two layers which were demonstrated in thin section (Fig 10) and freeze-etch replication of 5% TCA treated spores (Fig 41 and 42). The innermost layer (SW₁) is electron-lucent and the next layer (SW₂) is dark in thin sectioned ruthenium red fixed spores (Fig 10). The fracture planes due to the freeze-etching process was observed between the two cell wall layers only in TCA treated spores (Fig 41). Most spores tend to fracture between the 3rd layer (the rodlet mosaic (RM)) and the 4th layer (GM), exposing the outer face of the rodlet mosaic. The outermost layer which has the granular matrix (GM) exhibits a variable thickness.

Untreated spores (Fig 9) occasionally are fractured just outside the spore and some cells exhibit only an outermost granular matrix which is thin enough to assume the topography of the underlying rodlet mosaic.

The mature spore envelope schematic presented in Fig 38 is not in agreement with conclusions presented by Wildermuth et al. (88) for *S. coelicolor* and by Bradley and Ritzi (8) for *S. venezuelae*. The structure these authors identify as the fibrous sheath (FS in Fig 10) supposedly includes the rodlet mosaic. If the rodlet mosaic is actually ethanol soluble, as Bradley and Ritzi (8) suggest, then it should not exist in electron micrographs of thin sections since the resin embedded spores were dehydrated in ethanol.

The present work clearly indicates that: 1) the rodlet mosaic is not removed during the embedding process (Fig 12 and 13); and 2) there is at least one distinct layer of material (GM) residing outside
the mature spore rodlet mosaic. Residual material from the granular matrix (GM in Fig 11) remaining after spore maturation and release as single spores or short chains could account for what has often been called the "fibrous sheath" (FS in Fig 10).

The rodlet mosaic has usually been considered to be released from the mature spore (8, 30, 87). If this is true the rodlet mosaic would obviously not envelope the free mature spore, but rather be removed as part of the "fibrous sheath".

Freeze-etch replica images of S. coelicolor (Fig 8, 9, 41 and 42) show that the rodlet mosaic completely envelopes the mature spore, and are indicative of the interpretation in Fig 10 that the thin electron dense layer (arrows) surrounding the spore wall results from cross-sectioned rodlet mosaic.

Several lines of evidence uncovered during this study indicate that the Streptomyces coelicolor A3(2) rodlet mosaic is chitin: 1) similarity of rodlet precipitate infrared spectra to chitin spectra (Fig 34) (69); 2) thin layer chromatography identification of a single sugar (glucosamine) from 6 N HCl hydrolyzed rodlets (Table 6) (20) rodlet-like fibrils serve as a chitinase substrate (Table 5); 4) freeze-etch replica images of crab chitin (Fig 35) and spore rodlet mosaics (Fig 8) are very similar; 5) negatively stained crab chitin (Fig 36) and isolated rodlet-like polymer (Fig 27) have indistinguishable images; and 6) isolated rodlets (Table 4) and chitin isolated from other sources (24) have protein bound to the polymers.
Chitin from any source should have the same ultrastructural features. The mosaic of fibrils demonstrated in the freeze-etch of purified chitin (Fig 35) is remarkably similar to the mosaic appearance in replicas of intact Streptomyces, Aspergillus, Penicillium, and Trichophyton (31) aerial spores. Individual fibril diameters of the freeze-etched crab chitin (Table 1); of the Aspergillus spore mosaics (33); the Penicillium spore mosaics (32); and the Streptomyces coelicolor A3(2) freeze-etch spore mosaics (Table 1) are all about 5 nm.

Freeze-etch replicas of Penicillium and Aspergillus aerial growth demonstrate rodlet mosaics which appear very similar to those of the Streptomyces. These fungi also have a thin external matrix covering the rodlets (32, 33).

Chitin has not been previously demonstrated in Streptomyces or any of the Actinomycetales (2) probably because of a concentration problem. Precipitated rodlet-like polymer yield from Streptomyces coelicolor A3(2) aerial growth was not more than 0.1%. X-ray analysis and infrared spectroscopy are not sensitive enough to detect such low percentage components in whole cells or isolated walls. Rodlets, in whole cell samples, pressed in KBr windows would be present at a maximum of 0.00025% concentration.

Infrared (IR) spectra of isolated rodlet-like material and crab chitin (Fig 34) do not have the same peak resolution. Preparation of polysaccharides in KBr windows for IR spectral analysis tends to make the samples amorphous (75). The purified crab chitin particles
were larger aggregates than were the fibrils of the precipitated rodlets. Pressed crab chitin would probably have a lower degree of amorphism than would the precipitated rodlets. The higher degree of rodlet sample amorphism is evident in the comparatively broadened peak areas in Fig 34.

Modifying the surface-to-volume ratio of growth media with dilute agar (undissolved or dissolved) or acid washed diatomaceous earth appears to stimulate the landmarks of *Streptomyces coelicolor* A3(2) secondary metabolism. Spores exhibiting rodlet images and the water soluble pigment are produced when either the agar (undissolved or dissolved) or diatomaceous earth are added to shake flasks.

*S. Coelicolor* A3(2) did not produce the secondary metabolites when it was cultured on the nutrient agar medium and was lysed by expression of phage reproduction, probably the ØC31 phage genome (45). It seems possible that either control of rodlet (chitin) synthesis or coding for chitin synthesis is associated with the *S. Coelicolor* A3(2) phage genome ØC31, which is ordinarily integrated into the parent cell circular DNA polymer (45).

Rodlet-like fibers recovered from shake culture of *S. coelicolor* A3(2), grown in the 0.5% agar-containing medium, had a red-purple pigment associated with them after ethanol precipitation. Several lines of evidence indicate that the fibers isolated from the culture supernate are rodlet-fibers: 1) negative stained fibers appear like isolated rodlets and 2) molecular weight is approximately $2 \times 10^6$. Completely hydrolyzed samples produce one major spot which
co-chromatographs with D-glucosamine-HCl and the infrared spectrum of the intact rodlet-like fibers is very similar to the chitin and aerial spore-isolated rodlet infrared spectra.

The presence of the intact chitin-like fibers (free in the medium) in the agar-containing shake flasks poses an interesting problem. Crab chitin agar appears to be a *Streptomyces* growth supporting medium (36) and it seems that if chitin fibers are released by germinating spores in the agar-containing shake cultures the cells would hydrolyze the chitin and use the available glucose. It is possible that at least some of the released fibers are hydrolyzed.

One explanation could be that the rodlet-like fibers precipitated from agar-containing shake cultures are bound to a purple pigment, which may exhibit a blocking effect inhibiting exocellular chitinases. Alternately, the autolytic chitinases required for germ tube emergence may be spore wall bound.

*S. coelicolor* A3(2) spores failed to germinate and to produce detectable growth in glycerol-asparagine shake culture medium containing 1% purified crab chitin. Perhaps the chitin and likewise the released rodlet-like fibers inhibit germination under these conditions. Perhaps the high chitin concentration (1%) favored competitive binding with spore envelope associated chitinase, resulting in blockage of spore germination and mycelia production. The Hsu and Lockwood (36) chitin agar soil inoculum was from undetermined cell morphology and mycelia may have been present.
Shake flask conditions, used in the present work, and chitin agar plates, used in Hsu and Lockwood's work, are very different cultural conditions.

An identification key designed for species identification must have dichotomous features. "Presence or absence of cytoplasmic organelles" is the key (but not the only) feature regulating identification of cells as "bacteria" (54, 56). *Streptomyces* have a complex mesosomal membrane system, more complex than any of the other bacteria, but are definitely procaryotic and as such are bacteria (54). *Streptomyces* and many of the other actinomyces have muramic acid, unique to bacteria, in their cell walls.

*Streptomyces* have various other bacterial characteristics but also have several fungal-like features which are not found in other bacteria (Table 8): 1) mycelia with true branching; 2) formation of conidia (more recently called arthrospores (40)); 3) elaboration of the spore associated rodlet mosaic; and 4) formation of chitin during sporulation (present work).

The *S. coelicolor* A3(2) rodlet mosaic has been identified as chitin. A similar mosaic structure has been demonstrated in many *Streptomyces* and other actinomycetes (88) and in crab chitin (present work, Fig 35) as well as in various fungi (32). The rodlet mosaics from these sources may also be chitin.

Presence of the chitin rodlet mosaic enveloping mature *Streptomyces* spores may infer an acid resistant quality to the spore envelope. The N-acetyl moiety infers acid stability and hydrolysis resistance to N-acetyl-D-glucosamine and its polymers (25). The
<table>
<thead>
<tr>
<th>Streptomyces characteristic</th>
<th>Characteristic is similar to</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
</tr>
<tr>
<td>I. Muramic acid, alanine, glycine, glutamic acid and LL-DAP in cell walls (14)</td>
<td>+(14)</td>
</tr>
<tr>
<td>II. Procaryotic ultrastructure and amitotic nuclear division (54)</td>
<td>+(54)</td>
</tr>
<tr>
<td>III. Secondary metabolism associated with viral genome (45)</td>
<td>+(49)</td>
</tr>
<tr>
<td>IV. Mycelial growth with true branching (22)</td>
<td></td>
</tr>
<tr>
<td>V. Nonmotile conidia in chains (40)</td>
<td></td>
</tr>
<tr>
<td>VI. Rodlet mosaic (5 mm fibrils) on aerial growth (88)</td>
<td></td>
</tr>
<tr>
<td>VII. Aerial hyphae and spores contain chitin (Present work, Tables 4 &amp; 5)</td>
<td></td>
</tr>
</tbody>
</table>
hydrolysis conditions used in this work, promoted the isolated microscopic rodlet fibrils to form 1 mm long particulates. The macroscopic particles were destroyed by 6 N HCl at 115 C within 12 hr. The rodlet mosaic (Fig 39) is a relatively thin layer (about 5-10 nm) but the interlacing rodlet fibrils appear to create a tight basketweave effect, without visible perforations.

UDP-N-acetyl-D-glucosamine is a common precursor in chitin synthesis, murein synthesis, and synthesis of many antibiotics (29, 59, 67). The chitin rodlet mosaic in the Streptomyces may be linked to the formation of the other components mentioned above.

Mechanisms of the diatomaceous earth shake culture stimulus could be the increase in solid surface-to-liquid volume ratio (i.e., increase in substrate concentration sites) or could include other factors such as aeration effects and/or SiO₂ molecular effects.

The fact that Streptomyces coelicolor pigmentation and spore production were stimulated by the presence of diatomaceous earth in shake cultures could be of some value in increasing antibiotic production in other Streptomyces.
SUMMARY

Cytochemical analysis of *Streptomyces coelicolor* A3(2) indicated that the aerial growth rodlet mosaic is a polysaccharide. Statistical analysis of frequency distributions of individual rodlet lengths from control and ether reoriented spore mosaics, indicated that the rodlet fibrils are individual particulates, rather than evaginations in a continuous sheet of material.

A model of the mature spore envelope was developed from freeze-etch replicas, thin sections, and critical point dried *S. coelicolor* A3(2) mature spores.

The rodlet mosaic was found to be situated between the outer spore wall and an outermost granular matrix. The spore envelope layers from the inner surface to the external surface appear to be: plasma membrane, inner spore wall, outer spore wall, rodlet mosaic, and the granular matrix. The granular matrix had an uneven thickness and much of the matrix was frequently absent from the inter-spore spaces of mature spore chains.

*S. coelicolor* A3(2) mosaic rodlets were isolated by: 1) lysozyme and trypsin treatments; and 2) acetic acid refluxing and ethanol precipitation.
Complete acid hydrolysis of rodlets released one sugar, which co-chromatographed with D-glucosamine-HCl, and released 139% of acetic acid expected values. Cell associated rodlet mosaics and isolated rodlets were hydrolyzed with chitinase, and fingerprint infrared spectra identified the rodlets as chitin.

*S. coelicolor* A3(2) produced spores, antibiotic pigment, and released rodlet-like fibers when grown in shake cultures of glycerol-asparagine media containing 1% acid-washed diatomaceous earth or 0.5% agar agar.
APPENDIX A

LIQUID NITROGEN CRYO-IMPACTING (73)

The basic idea of liquid nitrogen cyro-impacting is that individual cells or masses are easily cracked under low-impact forces when frozen at very low temperatures (-196 °C).

Centrifuge washed cell pastes (<1 g) were placed in a precooled stainless steel 150 ml jar (D 150 ml jar, Societic Prolabo, Paris, France) containing liquid nitrogen and a 3.2 cm 18% chrome steel ball. The assembled jar, with the cells, was vertically reciprocated 700 rpm for 2 min. Broken cells were scraped from the walls of the jar with a liquid nitrogen-cooled spatula and stored in liquid nitrogen until used. The cells were not thawed, once they were frozen, until they were used for analysis or extraction.

Greater than 95% of the cell paste was disrupted within 2 min and no enzymes or other reagents were required for spore disruption.
APPENDIX B

CULTURE MEDIA

All media prepared as recommended by Shirling and Gottlieb (72).

Trace Salts Solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.1 g</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>0.1 g</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

(Stored at -20 °C as a stock solution)

Tryptone-yeast Extract Broth

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-Tryptone (Difco)</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Bacto-Yeast Extract (Difco)</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>1.0 l</td>
</tr>
</tbody>
</table>

pH adjusted to 7.0-7.2 (before autoclaving)
if necessary with 5 N-KOH

116
Glycerol-asparagine Agar

L-asparagine·H₂O 1.0 g
Glycerol 10.0 g
K₂HPO₄ (anhydrous) 1.0 g
Distilled H₂O 1.0 L
Trace salts solution 1.0 ml

The pH of this solution should be between 7.0-7.4. Adjust with 6 N HCl if necessary.

Agar agar (Difco) 20.0 g

Agar was liquified during the 17 min/121 C sterilization period and since the hot solution was somewhat stratified it was homogenized by moderate swirling.
APPENDIX C

LINEAR REGRESSION ANALYSIS

Given: Set of x, y coordinates; n, the # of coordinate pairs

To Find: (1) a (the slope)

(2) b ('Y' coordinate intercept)

(3) coefficient of determination {correlation} ($T^2$)

$$a = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}}$$

$$b = \bar{y} - ax, \text{ where } \bar{y} = \frac{\sum y}{n}; \quad \bar{x} = \frac{\sum x}{n}$$

$$T^2 = \frac{\left[ \sum xy - \frac{\sum x \sum y}{n} \right]^2}{\left[ \sum x^2 - \frac{(\sum x)^2}{n} \right] \left[ \sum y^2 - \frac{(\sum y)^2}{n} \right]}$$

LITERATURE CITED


