THE PRODUCTION OF FRAGILE CYSTS BY AN ABERRANT STRAIN OF AZOTOBACTER CHROOCOCCUM ISOLATED FROM SOIL

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THE PRODUCTION OF FRAGILE CYSTS BY AN ABERRANT
STRAIN OF AZOTOBACTER CHROOCoccus
ISOLATED FROM SOIL

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Gerald D. Cagle
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CHAPTER I

INTRODUCTION

The existence of bacterial cells capable of utilizing atmospheric nitrogen independent of a host plant has been known for some seventy-five years (38). The heterotrophic bacteria that are unequivocally capable of fixing nitrogen non-symbiotically are divided into two major groups: the anaerobic spore-formers of the genus Clostridium, and the aerobic encysting bacteria of the genus Azotobacter. There are many other bacteria capable of fixing small quantities of atmospheric nitrogen non-symbiotically; among these are members of the genera Aerobacter, Bacillus, Pseudomonas, Serratia, and Rhodosprillum, as well as several members of the class Cyanophyta, the blue-green algae.

Several bacterial types form specialized resting cells which are generally smaller than the vegetative cells. These specialized cells, commonly called endospores, lack the characteristic morphology which the vegetative cells possess, are essentially inactive physiologically, and are much more resistant to various deleterious agents (4). In contrast
to the endospore, the non-symbiotic nitrogen fixing bacteria, of the family Azotobacteraceae, form a resting cell which is quite different morphologically and physiologically: it is generally termed a cyst. The cyst produced by these organisms differs greatly from any other form of resting cell found in the bacteria. The Azotobacter cyst and the bacterial endospore are contrasting structures in that the cyst wall is formed de novo, probably by the incorporation of metallic ions into polymerized capsular material, while the endospore is formed primarily of internal cellular constituents. The cyst appears to retain all of the capsular material of the vegetative cell, and the vegetative cell itself, while the endospore is composed of a small, critical, portion of the vegetative cell.

The three regions of the Azotobacter cyst have been designated as the exine, intine, and the central body by Winogradsky (38). It has been demonstrated that the exine is responsible for the resistant properties of the encysted cell (23). A comparison of the resistant properties of the vegetative cell and the cyst has been conducted (2, 32) and it has indicated that the vegetative cell is much more susceptible to various deleterious agents than is the cyst. It has been shown that the Azotobacter cyst is resistant to
desiccation, ultraviolet irradiation, ionizing radiation, as well as mechanical stress (26, 27, 35). The purpose of this study is to determine if a strain of \textit{Azotobacter chroococcum} isolated from the soil in northern Louisiana produces cysts which are as resistant to deleterious agents as those produced by previously reported strains of \textit{Azotobacter} (27).

\textbf{Review of the Literature}

\textbf{Description of the genus.---}Beijerinck, in 1901, was the first to isolate members of the genus \textit{Azotobacter} from the soil (2). Prior to this time, however, Winogradsky had conceived the possibility of the existence of oligonitrophiles, and had succeeded in isolating the anaerobic spore-forming bacteria of the genus \textit{Clostridium}. During the experiments conducted by Winogradsky, members of the genus \textit{Azotobacter} were present in large numbers, but it remained for Beijerinck to isolate these organisms in pure culture.

\textit{Bergey's Manual of Determinative Bacteriology} (3) states that the genus \textit{Azotobacter} (meaning nitrogen rod) is distributed throughout the world. In general, the cells are large, pleomorphic, coccolid-shaped rods, Gram negative, non-sporeforming, and peritrichously flagellated. The members
of this genus are obligately aerobic, capable of nonsymbiotic nitrogen fixation and characterized by their ability to form cysts.

The morphology of the members of this genus is characterized by extreme pleomorphism. Bergey's Manual of Determinative Bacteriology (3) describes the vegetative aspects being approximately ellipsoid, and ranging in size from 2 μ to 6 μ in length by about 0.5 μ to 3 μ in width.

Probably because of the great degree of pleomorphism observed in this genus, a great volume of research has been published concerning the morphology of the organisms (21, 4, 16). Jensen (16) described the cells of typical cultures of Azotobacter. He noted that cultures included the following morphological types: bluntly shaped rods; spherical cells; small rods or spheres, precysts; and cysts. He also described large, swollen, irregular cells in cultures grown on media containing complex nitrogen sources, such as peptone.

On the basis of the varied descriptions given for the genus Azotobacter, and on readily observed pleomorphism, Lohniss and Smith (21) described a complex life cycle involving some thirteen different morphological stages including gonidia, buds, arthrospores, exospires, endospores, microcysts and fungoid forms. Their work implied the rise of different
morphological varieties in axenic cultures of Azotobacter; there are still adherents to their point of view (14). However, many of their observations have not been substantiated by subsequent workers and some of their observations have been attributed to the use of impure cultures. In 1954, Jensen (16) indicated that the morphology of Azotobacter is so variable that attempts to isolate a single morphological type in pure culture had not been successful to the above mentioned date. Following the work of Lohnis and Smith (21), Eisenstark, McMahon, and Eisenstark (9) studied the morphology of a pleomorphic strain of A. agilis utilizing phase contrast microscopy and concluded from their data that four major morphological forms of A. agilis existed. When cultures were grown on nitrogen-free media, these workers observed small rods, large rods, coccoid forms and elongated branched forms. Later, Eisenstark, Ward, and Kyle (10) detected the growth of large ovoid forms when Azotobacter cells were cultured in standard nutrient broth. The concept of the existence of gonidia in the life cycle of Azotobacter, proposed by Lohnis and Smith (21), has been renewed by the more recent work of Bisset and Hale (6). In 1953, these workers reported the production of cellular elements arising from the rupture of mature vegetative cells in cultures of
Azotobacter. They called these cellular elements gonidia, but noted that they were not capable of independent growth. These structures could, however, under certain conditions, revert to the typical Azotobacter cell. Further evidence of the presence of gonidia in Azotobacter was furnished by Laurence (18) who reported that in cell-free filtrates of aged cultures, small particles were observed that upon incubation in fresh culture media were capable of reversion to typical cellular forms. Utilizing carbon shadow casting techniques, Van Schreven (32) studied the morphology of penicillin-treated cells. He observed in these cells, small particles which he considered to be gonidia or dwarf reproductive cells. Van Schreven reported that the media in which Azotobacter is grown profoundly affects the morphology of the organism.

Metabolism.—The metabolism of Azotobacter is interesting in that these bacteria oxidize a wide variety of substrates and also have the highest respiratory quotient observed in any living organism (30). The carbon metabolism is unusual in that several metabolic pathways operate concurrently in the same cell. There is direct evidence that the degree of operation of any pathway present is under direct physiological
control by the organism (37). It has been suggested (22) that the pentose-phosphate pathway is operative in Azotobacter. Isotopic studies by Still and Wang (30) have indicated that the pentose-phosphate pathway is not a major pathway for the oxidation of carbon compounds in Azotobacter, however. Additional isotopic studies by these workers indicates that the Entner-Doudoroff pathway is the primary pathway of carbon metabolism in members of the genus Azotobacter. A possible series of reactions depicting the utilization of a hexose is given (13):

\[
\begin{align*}
\text{Glucose} + \text{ATP} & \rightarrow 6\text{-phosphogluconate} \\
\text{TCA Cycle} & \rightarrow \text{CO}_2 + 4(CH_2O) \\
\text{Pyruvate} & \rightarrow \text{2-keto-3-deoxy-6-phosphogluconate} \\
& \rightarrow \text{1-phosphoglyceraldehyde}
\end{align*}
\]
The metabolic pathway of glucose as measured by radiorespirometric experiments indicates that the carbon metabolism of *Azotobacter* resembles that of the pseudomonads, in that it concurrently utilizes the pentose-phosphate pathway and the Entner-Doudoroff pathway. Analysis of cellular constituents of bacteria grown in C\(^{14}\)-containing media indicates that the Entner-Doudoroff pathway is the major path for the oxidation of carbon compounds in *Azotobacter* (22).

The endogenous metabolism of the cyst is apparently very low. Exchange with the environment of certain ions and other growth factors does occur, however (33). According to Stevenson and Socolofsky (28) and Sobke, Charba, and Foust (24), poly-\(\beta\)-hydroxybutyric acid accumulation is necessary for the encystment process to occur and the amounts of this material that are stored within the central body are directly proportional to the length of time for which the cyst is viable.

**Capsule composition.**—The necessity of the capsule in the encystment of *Azotobacter* has been demonstrated (11). The presence of a true capsule and a slime layer have been reported by Cohen and Johnstone (8) in cultures of *A. vine-

landii and have indicated that these two portions of the cell are composed of the same material. Chemical analysis of the extracellular polysaccharide has indicated that the capsule is composed of polymers of galacturonic acid, glucose, rhamnose, and a minute amount of a compound identified as mannuronolactone; the ratio of components being 43:2:1. These workers have noted very small differences in the composition or ratio of capsular components when various strains of Azotobacter were examined (28). Subsequent experiments by Cohen and Johnstone (7) have indicated that the capsular material of A. agilis differs markedly from A. vinelandii. They have reported that the capsule of A. agilis contains no mannuronolactone or uronic acid, only glucose and rhamnose were reported in a ratio of 1.0:0.7.

Azotobacter cyst.—The most unique form in the life cycle of the Azotobacter is the cyst. Jones in 1920 (17) was the first to report the observation of thick-walled cells, which he called arthrosorpses, in fourteen day-old cultures of Azotobacter. Fifteen years later, Batchinskaya (1) reported the formation of a two-layered capsule around the cells in aging cultures of Azotobacter, noting the inner layer to be slimy while the outer layer was rigid.
Winogradsky, in 1938, published the first description of the encystment process and gave the first accurate cytological characterization of the cyst (38). He reported that encystment occurs in a period of a week or less when the cells of *Azotobacter* are grown on media containing carbon sources such as butanol, ethanol, butyrate, and other short-chained organic compounds.

Concerning the encystment process, Winogradsky (38) reported that after two or three days of growth some of the bacillary vegetative cells were seen to evolve into rounded forms which became coccoid and reduced in size. The rounding and compaction sequence continued through the fifth or sixth day when the coccoid forms, sometimes referred to as pre-encysting forms, developed into mature cysts. As a result of these studies, Winogradsky named three distinct anatomical areas of the cyst. Using a violamine stain, Winogradsky designated an area which stained black to brown as the central body, this area being analogous to the vegetative cell. The central body is surrounded by a dense double-layered capsule, the inner layer of which stained colorless to yellow, was termed the intine. The outer layer of cyst material was stained black and was designated the exine by Winogradsky. More recently, Vela and Wyss (34) developed a
polychromatic cyst stain giving excellent differentiation to the morphological areas of the cyst. When this stain is used a red to brown color is imparted to the exine, and a light green color is imparted to the central body, while the intine remains colorless.

Wyss, Neumann, and Socolofsky (39) have employed electron microscopy in observing the morphological changes which occur in the cell during encystment. Information obtained from the studies by these workers has indicated that vegetative cells and pre-encysting forms have a morphology similar to that described by Winogradsky. Observation of four and five-day-old cysts indicated that the exine is gradually deposited about the periphery of the capsule and continues to be deposited until a thick, laminated exine encompasses the cell. Subsequent studies by Tchan and Birch-Andersen (27) indicate that cyst morphology is in keeping with that reported by Wyss, Neumann, and Socolofsky.

**Conditions favoring encystment.**—Winogradsky reported the cultural conditions necessary for encystment to occur (39). Socolofsky and Wyss (26) have reported that encystment does not occur in media containing carbon sources such as mannitol or glucose and that encystment occurs almost com-
pletely when butanol is provided as the sole carbon source.

Layne and Johnson (19, 20) have reported the production of *Azotobacter* cysts in response to low mineral and carbon concentrations. The results obtained by these workers have been the subject of considerable controversy, however.

Stevenson and Socolofsky (28) have reported that cyst formation is partially dependant upon the accumulation of certain concentration of poly-β-hydroxybutyric acid in encysting cells. Stewart, Olson and Wyss (29) have reported that the concentrations of magnesium and copper are important in cyst formation. Studies by Eklund, Pope and Wyss (11) have indicated that the presence of extracellular polysaccharide is necessary for encystment to occur. Subsequent studies by Eklund and Wyss (12) have indicated that unencapsulated cells grown in the presence of ammonium ion or phage-induced depolymerase were incapable of cyst formation.

**Resistant properties of the cyst.**—The *Azotobacter* cyst has been described as being resistant to many deleterious effects. Among the resistant properties studied, possibly the resistance to heat has yielded the most widely varying results. Socolofsky and Wyss (27) have reported the cyst
to be almost as susceptible to heat as the vegetative cell. In contrast, Grabosky and Giambiagi (14) have reported the cyst to be quite resistant to heat, attributing the resistance not to the cyst *per se*, but rather to the presence of heat resistant corpuscles within the cyst. Bisset, Baird-Parker, and Hale (5) have reported the isolation of four Gram positive forms of *Azotobacter* found to be viable after fifteen minutes of exposure at 20° C, and further that two of the four isolates were viable after three minutes of treatment at 100° C.

Most members of the genus are quite sensitive to changes in pH, very few strains are able to survive at pH levels below 6.

Studies on the resistance of *Azotobacter* vegetative cells and cysts to ultraviolet irradiation has revealed considerable differences between these two morphological forms. Socolofsky and Wyss (27) have reported that encysted cells require twice as much radiation as do the vegetative cells in order to produce the same extent of killing. An even greater difference in the resistant properties of the vegetative cell and cyst is apparent when the cells are subjected to ionizing (gamma) radiation. Socolofsky and Wyss (27) have reported that the cyst requires seven times the amount
of gamma radiation necessary to inactivate the vegetative cell. Vela and Wyss (35) have shown soil Azotobacter populations to be more resistant to gamma radiation than laboratory cultures, and have asserted that survival of these organisms in nature is dependent upon formation of these specialized resting forms.

The resistance to sonication, a form of mechanical stress, has been reported by Socolofsky and Wyss (27) and their results indicate that four minutes of sonication are necessary to effect ninety per cent inactivation of the vegetative cells, while more than sixty minutes of sonication were necessary to effect the same percentage inactivation in encysted cells.

The most apparent difference in the resistant properties of the vegetative cell and cyst is the response to desiccation. Encysted cultures in our own laboratory have been maintained for over seven years in stored soil. Vincent gives reference to the preservation of viable encysted cells for periods of ten years (36). Socolofsky and Wyss (27) reported that at the end of twelve days of mild desiccation, only one per cent of the nonencysted cells remained viable, while one hundred per cent retained viability in the same time. This pro-
procedure has been employed by Stevenson and Socolofsky (28) in the differentiation of vegetative cells and encysted cells.

As previously noted, the resistance of the cyst apparently resides in the protection provided to the central body by two coat components or capsular layers. Parker and Socolofsky (23) have reported that the resistant properties of the encysted cell are due to the laminated structure of the cyst exine. These workers have shown that the amount of exine is proportional to the degree of resistance exhibited by the encysted cell. Parker and Socolofsky have also reported that in cysts whose exine is partially degraded, and in encysted cells which are germinating, that as the exine is gradually removed, the resistance of the cyst disappears. Further studies have indicated that the central body of the cyst may be removed, and this structure shows no pronounced resistance to deleterious agents.

Germination.--When Azotobacter cysts are placed in a favorable environment, they undergo a series of changes which transform them into the vegetative form. Jones in 1920 (17) first reported the germination of the structures which he termed arthrospores, but gave no description of the process. Winogradsky (39) was the first to publish a
description of the germination process.

Electron microscopic studies by Wyss, Neumann, and Socolofsky (39) have indicated that during the germination process there occurs an enlargement of the central body, with a concurrent decrease in the amount of intine present. Perhaps this indicates that the intine serves as an energy source for the germination process to occur. Tchan and Birch-Andersen (31) have reported that the central body is displaced to an eccentric position within the cyst during germination. As germination progresses, the area of exine rupture becomes conical and enlarged, suggesting that this area serves to accumulate enzymes responsible for germination.

The role of metal ions.—The formation of the cyst by members of the Azotobacteraeae depends upon the presence of a capsule (11). In addition to the necessity of extracellular polysaccharide, the presence of sufficient metallic ions for encystment to occur has been reported (27, 15). Socolofsky and Wyss (26) have shown that chelating agents, such as ethylenediaminetetracetic acid (EDTA), are efficient in destroying the integrity of the cyst wall. These chelating substances act upon the cyst to remove certain divalent metallic ions which are associated with the integrity of the cyst.


33. Vela, G. R., unpublished notes, Department of Biology, North Texas State University, Denton, Texas, 1968.


CHAPTER II

MATERIALS AND METHODS

Organisms and Media

The organisms used in this study are Azotobacter chroococcum, strain 75-1, obtained from the Department of Microbiology, University of Texas at Austin, and an organism identified as Azotobacter chroococcum, strain NTI (North Texas Isolate), according to the criteria given in Bergey's Manual of Determinative Bacteriology.

All cells were grown in media containing Burk's nitrogen-free salts (4), supplemented with glucose or n-butanol in order to obtain cells or cysts, respectively. The composition of Burk's nitrogen-free salts solution is given in Table I.

TABLE I

COMPOSITION OF BURK'S NITROGEN-FREE SALTS SOLUTION

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (Grams/Liter)</th>
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<tr>
<td>KH$_2$PO$_4$</td>
<td>0.2</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.8</td>
</tr>
<tr>
<td>MgSO$_4$ · 7 H$_2$O</td>
<td>0.2</td>
</tr>
<tr>
<td>CaCl$_2$ · 2 H$_2$O</td>
<td>0.085</td>
</tr>
<tr>
<td>FeSO$_4$ · 7 H$_2$O</td>
<td>0.005</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$ · 2 H$_2$O</td>
<td>0.0003</td>
</tr>
</tbody>
</table>
The above medium was supplemented with 1.5 per cent agar when solid plates of media were used to cultivate the organisms. The medium was prepared by standard sterilization at 121° C, for 15 minutes in the autoclave.

Viable Cell Counts

Viable cell counts were made by the standard spread plate technique, utilizing cold, sterile distilled water dilution blanks. Plates of media used for the enumeration of viable cells were prepared by supplementing Burk's nitrogen-free salts with 1 per cent glucose and 1.5 per cent agar. Each dilution was plated in triplicate and incubated for 48 hours at 30° C before colony counts were made.

Optical Density Measurements

The optical density (OD) of liquid cultures was measured at a wavelength of 520 mμ using a Bausch and Lomb Spectronic 20 colorimeter-spectrophotometer.

Sonication

Sonication experiments were conducted using a Branson Sonifier, with an audio frequency current of 9.2 to 9.3 amperes, at 20,000 cycles per second. Cyst suspensions, after removal from the growth medium, were placed in cold
sterile distilled water until sonicated. When cysts were sonicated, the temperature of the suspension was maintained at 3-5 °C to prevent the rupture of cysts due to heat produced by action of the sonifier. At various intervals, samples were removed, diluted appropriately and observed, at a wavelength of 520 μm on a Bausch and Lomb Spectronic 20.

Ultraviolet Irradiation

Ultraviolet inactivation of the two encysted organisms was accomplished, using a 115 watt minerallight, placed 34 centimeters above the surface of a liquid cyst suspension one centimeter in depth. At various time intervals samples were removed from the container, placed in cold sterile distilled water dilution blanks, and plated onto Burk's nitrogen-free medium, supplemented with one per cent glucose and one and one-half per cent agar. All ultraviolet inactivation studies were performed in the dark to prevent incidental photoreactivation.

Determination of pH

The pH of the growth medium was measured directly in culture flasks utilizing a Corning model 10 combination electrode pH meter.
Determination of Encystment and Resistance to Desiccation

The degree of encystment was determined according to a method developed by Socolofsky and Wyss (1), and by Stevenson and Socolofsky (2). According to these workers, a cyst is considered to be that morphological form which remains viable after a period of four days of mild desiccation. Utilizing this technique, encysted cells were impinged upon the surface of washed, sterile filter membranes, pore diameter 0.45 μ, and placed in a sterile petri plate in an incubator at 30°C. At intervals of two days, cysts were removed from the membranes with the aid of a magnetic stirrer and a rotary shaker. Experiments in this laboratory have indicated that the cysts could be quantitatively removed when this method is employed. Encystment was also determined microscopically using a polychromatic cyst stain developed by Vela and Wyss (3).

Column Chromatography

Column chromatography was employed in the partial separation of cell free filtrates. Sephadex grade G-75 and grade G-200 columns were prepared by rehydrating the dextran in 0.1 M phosphate buffer. Samples of the cell
free filtrate were placed onto filter discs, located at the top of the column. These discs were used in order to insure even distribution of the filtrate throughout the length of the column. An automatic collecting device was used to collect each of the fractions and each fraction was then assayed for the presence of transforming activity.
CHAPTER BIBLIOGRAPHY


CHAPTER III

RESULTS

This study may be divided into three parts. The first facet is concerned with the isolation of an aberrant strain of the species Azotobacter chroococcum, which upon encystment, forms cysts not typically resistant to mechanical disruption. The second phase concerns itself with a comparison of the resistant properties of Azotobacter chroococcum, strain 75-1, and the newly isolated organism, Azotobacter chroococcum, strain NTI. The final portion of this study is concerned with the effect of an unidentified principle found in liquid culture filtrates of the new isolate on the structural integrity of the normally sturdy Azotobacter chroococcum, strain 75-1, cyst.

While examining cysts from cultures of Azotobacter isolated from various soils, it was noted that one of the strains isolated produced a cyst which was inordinately susceptible to mechanical stress. This organism was identified according to the criteria given in Bergey's Manual of Determinative Bacteriology and assigned to the species Azotobacter chroococcum. This isolated organism was further designated as a
strain of this species because of the fragility of the cysts when these were compared to those of *Azotobacter chroococcum*, strain 75-1.

The organism used in this study was isolated from a single soil sample obtained from northern Louisiana. Table II indicates the number of *Azotobacter* colonies isolated and the number of *Azotobacter chroococcum*, strain NTI, found.

**TABLE II**

RELATIONSHIP OF SOIL *AZOTOBACTER* TO *AZOTOBACTER CHROOCOCCUM*, STRAIN NTI, ISOLATED FROM SOIL

<table>
<thead>
<tr>
<th>Azotobacter, Plate Colony Number</th>
<th>Azotobacter chroococcum, Strain NTI, Plate Colony Number</th>
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<tbody>
<tr>
<td>1-25</td>
<td>0</td>
</tr>
<tr>
<td>26-50</td>
<td>0</td>
</tr>
<tr>
<td>51-75</td>
<td>1</td>
</tr>
<tr>
<td>76-100</td>
<td>0</td>
</tr>
<tr>
<td>101-125</td>
<td>0</td>
</tr>
<tr>
<td>126-150</td>
<td>0</td>
</tr>
<tr>
<td>151-175</td>
<td>2</td>
</tr>
<tr>
<td>176-200</td>
<td>1</td>
</tr>
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<td>201-225</td>
<td>0</td>
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<tr>
<td>226-250</td>
<td>1</td>
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<tr>
<td>251-275</td>
<td>2</td>
</tr>
<tr>
<td>276-300</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>300</td>
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</table>

Originally the organism designated *Azotobacter chroococcum*, strain NTI, was isolated some thirty days after obtaining
the soil sample and was reisolated from the same soil sample at the end of four and six months. Of the Azotobacter colonies isolated from this soil sample, approximately two per cent yielded the organism designated *Azotobacter chroococcum*, strain NTI. The seven soil isolates, noted in Table II, were studied further and all were shown to be identical organisms. Cursory comparison of these data to previous studies showed that the organism could not be readily found in a number of other soils that were examined.

Studies on the media in which the aberrant organism were grown revealed that the noted fragility of the cysts was not dependent on pH nor on the age of the culture. Experiments were performed to determine the pH of liquid cultures during the growth cycle of *Azotobacter chroococcum*, strain NTI. The pH of liquid growth cultures was measured periodically and compared to the pH of liquid cultures of typical *Azotobacter* species. Data from these experiments indicate that no appreciable differences exist in the pH of the two culture media of the organisms during their growth cycle.

Figure 1 is a photomicrograph of *Azotobacter chroococcum*, strain 75-1, as grown in the laboratory on Burk's
nitrogen-free medium, supplemented with 0.2 per cent n-butanol and 1.5 per cent agar.

Fig. 1- *Azotobacter chroococcum*, strain 75-1, cyst, 4000 X
The cysts pictured in Figure 1 were taken after inoculation of vegetative cells onto Burk's plates with n-butanol as the sole carbon source. Figure 2 illustrates the new isolate cultured under identical conditions.

Fig. 2- *Azotobacter chroococcum*, strain NTI, cyst, 4000 X
It is evident that the cysts of these two organisms are very similar. Apparent in both Figures 1 and 2 are the distinct anatomical areas of the cyst: the exine, intine, and central body. Figure 3 shows the same cyst suspension viewed in Figure 1 after fifteen minutes of sonication.

Fig. 3- *Azotobacter chroococcum*, strain 75-1, cyst, 4000X
In comparing Figures 1 and 3, it appears that little or no disruption has occurred in the cyst suspension after fifteen minutes of continuous sonication. On the other hand, Figure 4 is a photomicrograph of cysts of the newly isolated organism which have been sonicated for a period of only five minutes.

Fig. 4- Azotobacter chroococcum, strain NTI, cyst, 4000 X
When Figures 1 and 4 are compared a vast difference is apparent after sonication of the cyst suspension of the newly isolated organism. It is apparent that very few intact cysts remain after this short period of sonication, while after a much longer period of sonication almost all of the cysts of *Azotobacter chroococcum*, strain 75-1, remain intact. A graphic comparison of the resistance of the two encysted organisms to mechanical stress, as measured by sonic treatment, is given in Figure 5.

![Graph showing resistance to mechanical stress](image)

*Fig. 5—Comparative resistance of encysted organisms to mechanical stress.*
The measurements observed in Figure 5 indicate the loss of optical density of a cyst suspension as a function of cell disruption by ultrasonic waves. From the data obtained, an optical density change of less than 0.02 absorbancy units is noted for a cyst suspension of *Azotobacter chroococcum*, strain 75-1, after thirty minutes of continuous sonication. If the same optical density measurements were performed on the cyst of the newly isolated organism, the time interval necessary to effect the same optical density change would be on the order of approximately fifteen seconds. It is inferred from the data presented in Figure 5, in addition to that presented in the photomicrographs, that the cysts of *Azotobacter chroococcum*, strain NTI, are much more susceptible to sonic disruption than those of *Azotobacter chroococcum*, strain 75-1.

Differences in the resistant properties of the two encysted organisms to ultraviolet radiation also revealed different rates of survival. Figure 6 depicts the ultraviolet survival curves for the cysts of the two organisms. The data presented in this graph indicate that after fifteen seconds of ultraviolet irradiation approximately forty eight per cent of the cysts of *Azotobacter chroo-
coccum*, strain NTI, are inactivated, while only eight
Fig. 6—Comparative resistance of encysted organisms to ultraviolet radiation.

per cent of the cysts of *Azotobacter chroococcum*, strain 75-1, are inactivated during the same radiation period. When observing the percentage survival after thirty seconds of radiation, it is observed that some sixty three per cent of the cysts of the newly isolated organism are inactivated while some thirty three per cent of the cysts of *Azotobacter chroococcum*, strain 75-1, are inactivated. For radiation periods in excess of thirty seconds the data presented here is not subject to accurate interpretation, although qualitative differences in the rate of survival are noted. It
appears that the cyst of *Azotobacter chroococcum*, strain NTI, is markedly more susceptible to ultraviolet radiation than the cyst of *Azotobacter chroococcum*, strain 75-1, under the conditions of our experiment.

Studies on the resistance of the cysts of the two organisms to desiccation yielded almost identical results. Results in Figure 7 indicate the comparative resistance of the cysts of the two organisms with regard to desiccation.

![Diagram](image)

**Fig. 7**—Comparative resistance of encysted organisms to desiccation.
previously, Stevenson and Socolofsky (3) determined that the
cyst was that morphological form that remained viable after
a period of four days of mild desiccation. Using this
method, cysts were impinged on filter membranes and were
desiccated for periods of two, four, six, and eight days.

As observed in Figure 7, the surviving fraction at the end
of each two-day period does not change appreciably in the
case of either organism. In each instance, almost one hundred
per cent of the encysted population remains viable.

From the experiments described here, it has been deter-
mined that the resistance of the two cysts differs with
respect to some deleterious agents. An electron microscopic
examination of ultra-thin sections of five day-old encysted
organisms grown on Burk's nitrogen-free salts supplemented
with 0.2% n-butanol and 1.5% agar revealed that the cysts
of *Azotobacter chroococcum*, strain 75-1, exhibit morphological
details believed to be typical of the *Azotobacter* cyst; the
exine, intine, and central body are easily identified. The
anatomy of this cyst is in accord with previous reports by
other workers who have examined the *Azotobacter* cyst (4).
The exine of these cysts has a typically thick, laminated
substructure; it appears that this exine imparts many of
the resistant properties to the encysted organism (1).
Figure 8 is an electron micrograph of the cyst of *Azotobacter chroococcum*, strain 75-1, magnified some 11,000 times.
Of particular interest in Figure 8 is the ultrastructural appearance of the intine of the organism. Like the exine, the intine appears to be lamellar in structure. It is divided into an outer layer which is granular and moderately electron dense and an inner layer which appears to be more electron dense. The central body contains, in addition to the large amount of lipid, electron dense particles described by Wyss, Neumann, and Socolofsky (2), but not identified.

When the ultrastructural anatomy of the cyst of *Azotobacter chroococcum*, strain 75-1, is compared to that of the newly isolated organism, certain differences are readily apparent. In observing the cyst of the newly isolated organism, *Azotobacter chroococcum*, strain NTI, in Figure 9, perhaps the first difference which is apparent is the distinct lack of structural integrity in the exine of the cyst. While the resistant properties of the normal cyst, pictured in Figure 8, are associated with a lamellar exine, this lamellar structure is almost completely lacking in the cyst of *Azotobacter chroococcum*, strain NTI. It appears that in this organism that the typical lamellar exine has been replaced by an exine which is very convoluted in appearance. In the intine, differences of structure of the two cysts also exist. The intine area of the cyst of *Azotobacter chroococcum*,
strain NTI, appears to lack the ordered arrangement of the intine area of the cyst of *Azotobacter chroococcum*, strain 75-1. Figure 9, shown below, is an electron micrograph of the cyst of *Azotobacter chroococcum*, strain NTI, magnified 11,000 times.

*Fig. 9*—*Azotobacter chroococcum*, strain NTI, cyst, 11,000 X
In addition to the noted lack of intine arrangement, the
cyst of *Azotobacter chroococcum*, strain NTI, appears to
also lack the lamellar structure of the intine area, which
is apparent in the cyst of *Azotobacter chroococcum*, strain
75-1, shown in Figure 8. With regard to structure, there
appears to be very little difference in the appearance of
the central bodies of the two encysted organisms.

The final part of this study is involved with the
effect of a culture filtrate component of the newly isolated
bacteria on cysts of *Azotobacter chroococcum*, strain 75-1.
Previous experiments have indicated that cysts of *Azoto-
bacter chroococcum*, strain NTI, are quite susceptible to
mechanical stress. Experiments were designed to determine
whether or not this characteristic fragility could be trans-
ferred to cysts which are normally resistant to mechanical
stress. Cultures of Burk's nitrogen-free salts, supplemented
with dextrose, were incubated for four days on a rotary
shaker at 30 C, after inoculation with *Azotobacter chroo-
coccum*, strain NTI. The resultant cells were then cen-
trifuged and the culture fluid filtered to remove any re-
sidual cells. Cysts of *Azotobacter chroococcum*, strain 75-1,
which are resistant to mechanical stress had been cultured
on Burk's nitrogen-free plates supplemented with 0.2% n-butanol
and 1.5% agar. These cysts were removed from the plates and suspended in a small amount of the cold cell-free filtrate of *Azotobacter chroococcum*, strain NTI. Table III illustrates the activity of the untreated culture filtrate as measured by cyst conversion. This measurement is defined as that time interval which will result in the transfer of fragility to cysts of *Azotobacter chroococcum*, strain 75-1, which are normally resistant to mechanical stress.

**TABLE III**

**CONVERSION OF AZOTOBACTER CHROOCOCCUM, STRAIN 75-1, BY UNTREATED CULTURE FILTRATE**

<table>
<thead>
<tr>
<th>Time(minutes)</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>+</td>
</tr>
<tr>
<td>40</td>
<td>+</td>
</tr>
</tbody>
</table>

From the data presented in Table III, it is noted that the shortest time interval in which the substance or substances in the culture filtrate could convert the cysts of *Azotobacter chroococcum*, strain 75-1, to the abnormally fragile form seen in *Azotobacter chroococcum*, strain NTI, was approximately thirty minutes. This period of incubation is necessary to bring about the conversion of normally resistant
cysts to fragile cysts, as measured by sonication. The following procedure was then devised to isolate the active portion of the cell-free filtrate responsible for cyst conversion. A twelve-inch length of dialysis tubing was filled with culture filtrate of the new isolate and then dialyzed against 0.1 M phosphate buffer for twenty-four hours, with several changes of the buffer. Following this dialysis, it was found that the active conversion substance was capable of permeating the dialysis membrane. The chemical procedures utilized in partially purifying this material are illustrated in Table IV.

### TABLE IV

**CHEMICAL PROCEDURES UTILIZED IN PURIFYING ACTIVE FRACTION OF CULTURE FILTRATE**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialysis (dialysate)</td>
<td>+</td>
</tr>
<tr>
<td>Ether extraction</td>
<td>-</td>
</tr>
<tr>
<td>Water soluble fraction</td>
<td>+</td>
</tr>
<tr>
<td>Activated charcoal filtrate</td>
<td>+</td>
</tr>
<tr>
<td>Hot methanol extraction</td>
<td>-</td>
</tr>
<tr>
<td>HCl treatment, pH 1.0</td>
<td>-</td>
</tr>
<tr>
<td>Petroleum ether extraction</td>
<td>-</td>
</tr>
<tr>
<td>Pyridine extraction</td>
<td>±</td>
</tr>
</tbody>
</table>
Following pyridine extraction, as seen in Table IV, it was noted that the conversion of normal cysts to fragile cysts was almost immediate. This indicates the chemical procedure was effective in concentrating the active conversion principle.

Other means were also utilized to partially separate the active portion of the cell-free filtrate of the new isolate. Column chromatography, utilizing Sephadex G-75 and G-200, both poly-dextrans, were employed to separate the active fraction on the basis of molecular size. Table V gives the results of this separation, as measured by cyst conversion.

**TABLE V**

CONVERSION OF *AZOTOBACTER CHROOCOCCUM*, STRAIN 75-1, BY CHROMATOGRAPHICALLY PURIFIED CULTURE FILTRATE

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>G-75 flow rate 1.2 ml/min - activity</th>
<th>G-200 flow rate 0.5 ml/hr - activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16-40</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
In each case the flow rate was determined using blue dextran. The flow rate of each column is noted in Table V. Following partial purification of the conversion principle using column chromatography, it was also noted that the action of the material was immediate. This indicates that this method of concentration of the active material was also effective.
CHAPTER BIBLIOGRAPHY


CHAPTER IV

DISCUSSION

Previous investigations concerning the resistance of the Azotobacter cyst to various deleterious agents have concluded that the cyst is a resistant structure and is capable of withstanding greater amounts of mechanical stress, ultraviolet irradiation, gamma irradiation, and desiccation than the vegetative cell (1). This study has been concerned with the resistant properties of the cyst of an organism isolated from soil in northern Louisiana. This organism was designated Azotobacter chroococcum, strain NTI, according to the previously noted criteria. After the isolation of this organism, it was determined that this particular organism was quite susceptible to slight mechanical stress. Experiments were undertaken to quantitate this susceptibility.

The cyst of Azotobacter chroococcum, strain NTI, was subjected to treatment with ultrasonic waves, a form of mechanical stress; ultraviolet irradiation; gamma irradiation; and desiccation. The results of these studies compared to those for a resistant cyst of the same tax-
onomic species. The first such agent to which the cyst was subjected was sonication. As noted from the data presented in Figure 5, the cyst of *Azotobacter chroococcum*, strain NTI, appears to be approximately three times as sensitive to sonic disruption as the cyst of *Azotobacter chroococcum*, strain 75-1. Also apparent in these resistance studies is the time period necessary to effect rupture of the cyst suspension of each organism. The data presented here indicates that the cyst of *Azotobacter chroococcum*, strain 75-1, requires more than six times the sonication period necessary to rupture the same amount of *Azotobacter chroococcum*, strain NTI. From the response to sonication of the two encysted organisms, it may be concluded that a distinct difference does exist in the resistant properties of each.

When the two encysted organisms were compared in response to ultraviolet irradiation, even greater differences were apparent. Cultures which had been incubated identically were irradiated for varying periods of time. The data presented in Figure 6 indicates that after fifteen seconds of irradiation, more than six times the number of cysts of the newly isolated organism are inactivated as compared to the cyst of the resistant encysted organism. This data is
in keeping with data presented previously by Socolofsky and Wyss (1) for the normally resistant Azotobacter cyst. Figure 6 indicates that only eight per cent of the population of the normal cyst is inactivated during the first fifteen seconds of ultraviolet radiation. When this amount of inactivation is compared to the cyst of the newly isolated organism, some eight times the number of cysts are inactivated. Following thirty seconds of irradiation, fifty-three per cent of the cysts of Azotobacter chroococcum, strain NTI, are inactivated while some thirty-three per cent of the cysts of Azotobacter chroococcum, strain 75-1, are inactivated. Irradiation periods in excess of thirty seconds do not yield quantitative results. From the data presented, however, it is noted that qualitative differences do exist and the amount of inactivation is considerably greater in the case of Azotobacter chroococcum, strain NTI. From this data it is evident that differences in the resistant properties of the two cysts does exist with respect to ultraviolet irradiation.

The resistance of the cysts of the two organisms was also determined in response to desiccation. Cysts, grown on Burk's nitrogen-free media supplemented with 0.2% n-butanol and 1.5% agar, were placed onto filter membranes
and subsequently removed at two day intervals. The cyst of *Azotobacter chroococcum*, strain NTI, had been found to be susceptible to certain deleterious effects, and it was suspected that this cyst might also be susceptible to desiccation. From the data obtained, and presented in Figure 7, it appears that the cyst of the newly isolated organism is as resistant to desiccation as the cyst of *Azotobacter chroococcum*, strain 75-1. It is noted, however, that the cyst must possess some resistance to desiccation in order to survive. The *Azotobacter* cyst has been noted to be one of the most resistant biological forms to desiccation. Vincent, Humphrey, and North (3) give reference to the fact that viable encysted cells have been maintained on silica gel for periods in excess of ten years. Vela and Wyss (2) have asserted that the cyst is the primary form in which the azotobacter exist in the soil, and that their existence is dependant upon cyst formation. As desiccation is the deleterious agent to which the cyst is most often subjected, it appears that this particular organism is effective in maintaining viability in its own ecological niche. Deleterious effects such as sonic treatment and types of irradiation probably effect the cyst in nature to a smaller degree than does desiccation.
Preliminary studies showed that some material in the cell-free filtrate of *Azotobacter chroococcum*, strain NTI, was responsible for fragilizing the cyst of *Azotobacter chroococcum*, strain 75-1. The specific action of the culture filtrate has not yet been resolved. Table III indicates that the action of the fragilizing material is dependant, in part, upon time. Efforts to isolate the active material, by column chromatography and chemical procedures, were successful in partially purifying a small group of substances which were immediate in their action of converting normal cysts to fragile cysts. The determination of the chemical structure of these materials responsible for cyst conversion is not within the scope of this study. However, the active substance in the culture filtrate is an interesting one and could lead to a better understanding of the events which transpire during the cyst germination process upon structure elucidation.

From the knowledge gained during attempts to separate the active material in the culture filtrate, several general statements may be made concerning its nature. It was noted that the active material was dialyzable. It may be assumed that the material was of small size and low molecular weight. This hypothesis is further substantiated by the rate of
flow of the substance through Sephadex columns, as noted in Table V. Knowledge gained from chemical procedures, and presented in Table IV, indicate that the active material is not readily soluble in ether. The active material is soluble in pyridine, indicating that the material is of an organic nature and not an altered ion or radical formed in the growth media of the bacterium.
CHAPTER BIBLIOGRAPHY


CHAPTER V

SUMMARY

An organism isolated from soil in northern Louisiana and shown to be of the species *Azotobacter chroococcum* was found to produce a cyst which is characterized by extreme fragility when compared to cysts of a typical strain of the same taxonomic species. The new isolate, designated *Azotobacter chroococcum*, strain NTI, was found to be as resistant as the normal cysts to desiccation, but were found to be quite sensitive to mechanical stress and more susceptible to ultraviolet radiation. Electron microscopy revealed the cysts of the newly isolated organism lack a laminar exine and general compactness of this structure. Certain anatomical differences were also noted in the cyst intine area. Electron microscopic anatomy indicated little or no difference in the appearance of the central body of the cysts.
BIBLIOGRAPHY

Books


Articles


Vela, G. R., unpublished notes, Department of Biology, North Texas State University, Denton, Texas, 1968.


