A STUDY OF TRANSFORMATION IN *BACILLUS CEREUS* VAR. *MYCOIDES*

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A STUDY OF TRANSFORMATION IN *BACILLUS CEREUS*

VAR. *MYCOIDES*

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CHAPTER I

INTRODUCTION

In 1886 Flügge described a Gram-positive bacillus that formed colonies of two distinct morphologies; the individual colonies had either dextral (clockwise) or sinistral (counterclockwise) curving terminal filaments, but never both. He named the organism *Bacillus mycoides*.

In 1932 Lewis found that *B. mycoides* readily dissociated from the typical rhizoid colony into various "smooth" forms and that the rate of dissociation depended on several factors, the most important of which were the temperature of incubation and the growth medium. The rate of dissociation was generally retarded by depleted media, metabolic products, low temperatures and inhibitory chemicals. Once dissociated, the organism did not revert to its original rhizoid state.

Gause (1939) considered the dextral form an inverse mutant of the sinistral form. He stated that the dextrality and sinistrality in *B. mycoides* is a hereditary feature inasmuch as dextral forms are always obtained from dextral forms and sinistral forms from sinistral after numerous transfers on agar.

In their study of this organism, Smith, Gordon and Clark (1952) found that *B. mycoides* was physiologically identical with *Bacillus cereus* and differed only in that the rods of the rhizoid organism were slightly thinner and were in long chains twisted together to form

strands. Because of this similarity the name of the organism was changed to B. cereus var. mycoides. These investigators supported Gause's observation that during continuous subculturing on agar, a dextral form always gave rise to dextral colonies and sinistral forms always produced sinistral colonies.

However, they found that when either type of rhizoid strain was serially transferred in broth over a period of time (usually between eight to ten days), dissociation occurred giving rise to a smooth strain that was identical to B. cereus. These non-rhizoid forms of B. mycoides were very stable; in attempts to stimulate reversion, the cultures were grown on different media under varying conditions of pH, oxygen tension, temperature, humidity and surface tension and in the presence of immune sera or bacteriophage but no rhizoid colonies were ever recovered. It was this irreversible dissociation from rough to smooth types which prompted this study of transformation in B. cereus var. mycoides.

The phenomenon of transformation which permits certain strains of bacteria grown in the presence of killed cells, culture filtrates or extracts from related strains to acquire and propagate certain properties of the related strains was first discovered by Griffith in 1928. He found that an attenuated, non-encapsulated, rough variant of Pneumococcus would become transformed to a fully encapsulated and virulent strain of a heterologous type by in vivo passage in mice of viable rough cells and dead cells of the heterologous smooth strain. In 1944, Avery, MacLeod, and McCarty, who also used a rough and smooth Pneumococcus system, discovered that deoxyribose nucleic acid (DNA) was the substance that induced transformation. Since these two discoveries, a great many studies have been made on the transformability of morphological and biochemical

characteristics in different species of the genus Bacillus.

The first such experiment was reported by Spizizen in 1958; three auxotrophic mutants (indole$^-$) of Bacillus subtilis, strain 168, were transformed to nutritional independence with DNA isolated from a wild-type B. subtilis, strain 23. Spizizen and Anagnostopoulos (1960) determined the physiological conditions required for optimal transformation of an indole$^-$, sucrose$^-$ recipient strain of B. subtilis. The transformed organism (suc$^+$) was found to contain an inducible sucrase when plated on sucrose agar.

Jannes (1962) used glycine spheroplasts of Escherichia coli both as receptor cells and for the preparation of transforming DNA in an attempt to transform streptomycin resistance. Transformation could only be demonstrated if, after incubation with donor DNA, the cells were grown on an assay medium containing a low level of antibiotic. Therefore, in an attempt to raise the level of antibiotic resistance, he prepared a high molecular weight DNA from protoplasts of a streptomycin-resistant mutant of B. subtilis, strain 23. Streptomycin resistance was transferred to a sensitive strain of B. subtilis; yeast autolysate was required in the medium when resistance was transferred.

Stocker (1963) found that a non-motile (non-flagellated, fla$^-$), tryptophanless (try$^-$) strain of B. subtilis was transformed to fla$^+$ and to try$^+$ by wild-type DNA, but that these phenotypic expressions did not result until three or more hours after uptake of donor DNA.

Nester and Stocker (1963) transformed an auxotrophic strain of B. subtilis, SB1, which has a requirement for histidine (his$^-$) and tryptophan (try$^-$) to his$^+$ and try$^+$ in their study of the resistance of competent cells to penicillin.

Marmur, Seaman and Levine (1963) examined the DNA from various species of the Bacillaceae for base composition, ability to carry out interspecific transformation, and formation of molecular hybrids *in vitro*. They found that the minimal requirement for genetic compatibility among the different species and for DNA interaction is the similarity of the guanine plus cytosine contents of the DNA and that from this guanine plus cytosine content it is possible to predict which species of *Bacillus* might yield DNA capable of transforming other species. In their study, the species that had a *B. subtilis*-like DNA base composition (i.e., *B. natto* and *B. subtilis* var. *aterrimus*) transformed *B. subtilis* with a very high efficiency. The following genetic markers were used in the transformation: $ind^- \longrightarrow ind^+$ (indole); $arg^- \longrightarrow arg^+$ (arginine); $E^s \longrightarrow E^r$ (erythromycin sensitivity and resistance). They found the guanine plus cytosine content of *B. cereus* to be 33 per cent compared to 43 per cent in *B. subtilis* and the per cent of transformation between these two organisms to be zero.

Kawamata, Shigeta and Kunita (1957) have been the only workers to report genetic transfer between genera. They reported that a strain of *Staphylococcus aureus*, which had been artificially adapted to penicillin, could be sensitized by incubating the cells with the nucleic acid fraction of *Bacillus circulans*, which was highly sensitive to the antibiotic.

McDonald and Matney (1963) found two strains of *B. subtilis*, 168 and P1, which would grow at 55 C (55^+) on complete media. Strain $168S^r$ would not grow at temperatures above 50 C (55^-). When $168S^r$ bacteria were grown in the presence of DNA extracted from 168 (55^+), the ability to grow at 55 C was transformed at a frequency of 10^{-4}. An incubation period of 3 to 4 hours at 37 C was necessary for phenotypic expression

of the 55^{+} character. Only 10 to 20 per cent of the transformants retained the high-level streptomycin resistance (S^{r}) of the recipient indicating close linkage between the S^{s} and 55^{+} loci.

Felkner and Wyss (1964) transformed a streptomycin-sensitive strain of B. cereus 569 to streptomycin resistance. They found a "competency factor" which could make non-competent cells competent--that is, increase the ability of cells to take up exogenous donor DNA, incorporate the DNA into their own DNA, and become transformed. A similar factor had previously been reported in a Pneumococcus system by Tomasz and Hotchkiss (1964) who called the factor "activator" and described it as a macromolecular cell product, probably a protein.

The transfer of streptomycin resistance to streptomycin-sensitive organisms has been used quite extensively as an assay system in transformation studies. Alexander and Leidy (1953) found that resistance to streptomycin, at a concentration exceeding 1000 μg/ml, has been induced in sensitive strains of Hemophilus influenzae by exposure to DNA-containing extracts isolated from a strain of resistant H. influenzae. The donor DNA is essential for the process which brings this change and the reaction can be prevented by destruction of the DNA by deoxyribonuclease. The transformed resistant trait was heritable.

Perry and Slade (1961) exposed 43 strains of streptococci to DNA extracted from streptomycin-resistant streptococci. Transformation to streptomycin resistance was obtained with streptococci belonging to serological groups F, H and O and with one serologically unclassifiable strain. These investigators found that substances which affect cell permeability prevented the occurrence of transformation. Their radioisotope studies with ^{32}P-labeled DNA showed that nontransformable

strains incorporated amounts of DNA comparable to the transformable strains.

Jablonski and Poplawski (1961), using DNA isolated from a streptomycin-resistant Brucella mutant, were able to transfer resistance to a susceptible strain in the presence of sheep serum. Komarov (1962) obtained streptomycin-resistant S. aureus cells by incubating a 12 to 16 hour culture of streptomycin-sensitive cells with DNA isolated from a resistant strain in nutrient broth for 30 minutes.

Ephrussi-Taylor (1962) transformed streptomycin-sensitive pneumococci to streptomycin resistance. However, she found that these transformed cells did not become indifferent to streptomycin until 150 to 160 minutes after DNA fixation. She theorized that resistance develops in two steps: first, the bacteria are altered so that streptomycin is no longer bactericidal and secondly, the cells then become completely indifferent to streptomycin. Nester and Stocker (1963) have also demonstrated that there is a lag and nonmultiplication of transformants up to 3 to 5 hours after the addition of DNA because competent cells are resistant to penicillin during this period; their conclusion is based on the premise that penicillin is only effective against actively growing cells.

This study was conducted to determine if dextral and sinistral strains of B. cereus var. mycoides could be transformed either from (1) a smooth dissociant to the original rhizoid state; or (2) from streptomycin sensitivity to streptomycin resistance.

CHAPTER II

MATERIALS AND METHODS

Test Organisms

The cultures used in these studies included two strains of B. cereus var. mycoides, two strains of B. cereus, and two strains of B. subtilis. Sources are indicated in the Appendix. For convenience, the designation B. mycoides will be used to designate B. cereus var. mycoides.

Maintenance of stock cultures. The test organisms were stored on nutrient agar slants at 4 C except for the cultures of B. mycoides. A new procedure for storing these organisms was developed which facilitated the ease of their transfer for subculturing.

A sterile strip (1/2" X 1-1/2") of Millipore filter membrane was laid on the surface of an agar slant (Figure 1). The inoculum was streaked on the membrane. B. mycoides grows as a smooth colony on a membrane surface and the growth can be much more easily removed by an inoculating needle or loop than the typical rhizoid growth which adheres very tightly to an agar surface. Cells transferred to nutrient agar from the membrane form typical rhizoid colonies (Figure 2).

Repeated transfer of B. mycoides BmS6 [rhizoid (R^+), streptomycin-sensitive (S^s)] at two-day intervals for 30 days and then for two-week intervals for 30 days indicated that this method of storage did not cause dissociation of the organism to the smooth (R^-) colony type.

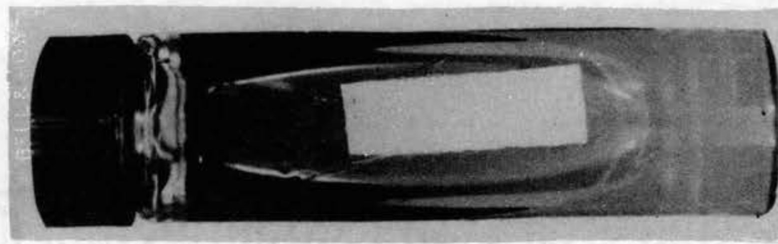

Figure 1: Membrane filter slant for stock cultures

Figure 2: Dextral form of B. cereus var. mycoides BmD2 (R^+) after 14 hr growth at 37 C on nutrient agar

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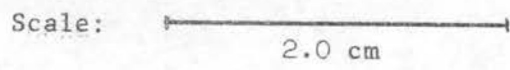

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Media

With the exception of penassay medium, the defined media used in this study were modifications of Spizizen's minimal medium (1958) which contained: $(NH_4)_2SO_4$, 0.2 per cent; K_2HPO_4, 1.4 per cent; KH_2PO_4, 0.6 per cent; sodium citrate, 0.1 per cent, $MgSO_4 \cdot 7H_2O$, 0.02 per cent and glucose, 0.5 per cent.

The minimal salts medium (MS) contained the above ingredients plus 0.3 per cent acid-hydrolyzed casein and was further modified by adding either 0.1 per cent yeast extract (MS + 0.1 per cent YE) or 0.01 per cent yeast extract (MS + 0.01 per cent YE).

Felkner and Wyss (1964) supplemented Spizizen's medium with 0.004 per cent tryptophan, 0.05 per cent casein hydrolysate and 10^{-4}M $FeSO_4(NH_4)SO_4$; this medium has been designated, M-1 medium. M-2 medium was the same as the M-1 except tryptophan was reduced 8-fold and casein hydrolysate 5-fold.

Penassay medium contains 0.15 per cent beef extract, 0.15 per cent yeast extract, 0.5 per cent peptone, 0.35 per cent NaCl, 0.368 per cent K_2HPO_4, 0.132 per cent KH_2PO_4, and 0.1 per cent glucose.

The minimal media were autoclaved at 121 C for 15 minutes and the penassay medium at 121 C for 25 minutes. The glucose was sterilized separately and added aseptically. Agar (2 per cent) was added when a solid medium was needed.

Commercially prepared nutrient broth, nutrient agar and brain heart infusion broth (Difco) were hydrated and autoclaved at 121 C for 25 minutes.

Preparation of Dissociated and Resistant Organisms

A dextral strain of *B. mycoides*, BmD2 (R^+), was used to inoculate 500 ml of brain heart infusion broth and the culture was incubated for

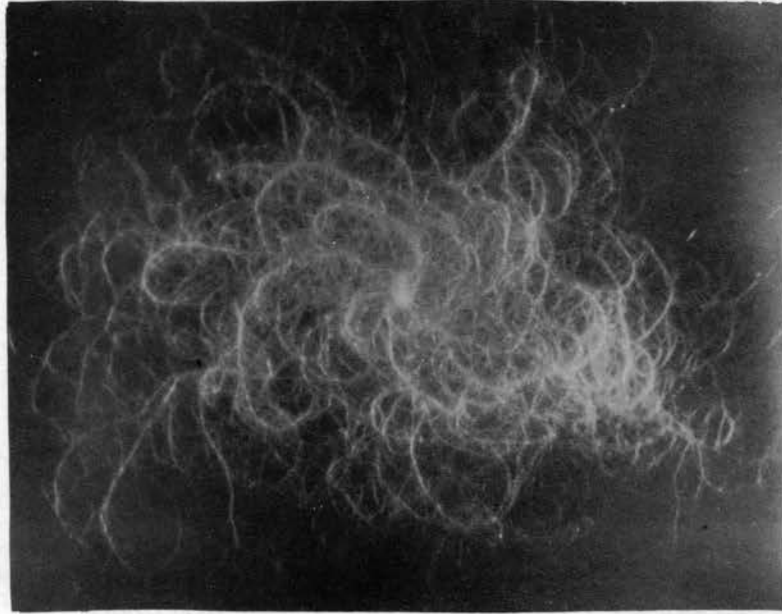
one week at 30 C. At the end of this incubation period, a 0.1 ml sample was spread on an agar surface to check for any morphology changes. This sampling was continued at two-day intervals until a smooth dissociant was isolated.

The gradient plate method (Szybalski, 1952) was used to develop a streptomycin-resistant strain from B. mycoides BmS6 (Figure 3A). Streptomycin resistance was developed to a maximum level of 1000 μg/ml. The streptomycin-resistant strain (BmS6, R^+, S^r) grows slowly in the presence of streptomycin (Figure 3B). Even in the absence of streptomycin, it does not grow as rapidly as the streptomycin-sensitive parent (Figure 3C).

Preparation of Deoxyribonucleic Acid (DNA)

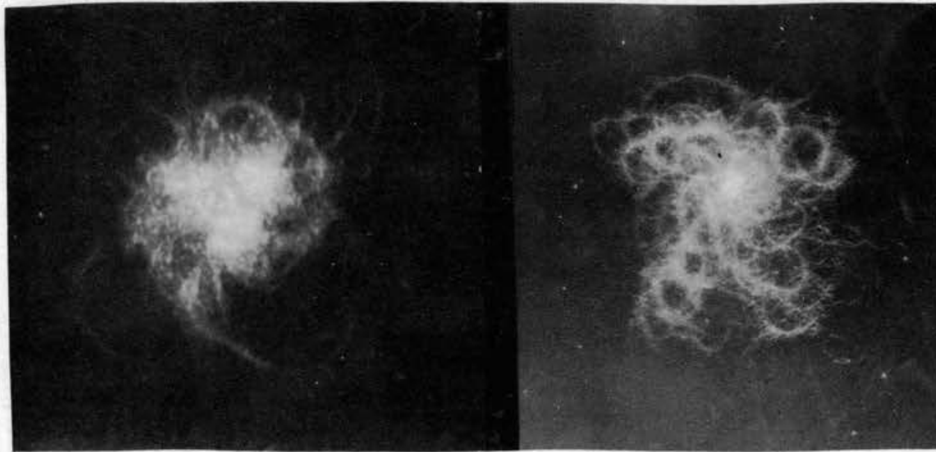
Two methods were used in preparing transforming deoxyribonucleic acid: (1) the chloroform-isoamyl alcohol procedure described by Marmur (1961); and (2) the phenol procedure developed by Saito and Miura (1963).

In the first method, a 10 liter batch culture was grown overnight in nutrient broth containing Dowex Antifoam A to prevent foaming. These cells were harvested using the Sharples centrifuge operating at 45,000 rpm. The cells were then washed with 100 ml of a saline-versene solution (0.15M versene - 0.1M saline, pH 8.0), centrifuged and resuspended in 50 ml of the saline-versene solution. Muramidase (10 mg/ml) was added to a final concentration of 100 μg/ml and the mixture incubated at 37 C for 90 minutes. Sodium lauryl sulfate (30 per cent) was added to a final concentration of 3 per cent and the mixture allowed to stand until lysis was complete as determined microscopically. Then sodium perchlorate (5M) was added to a final concentration of 1M and the mixture incubated at room temperature for one hour to dissociate



A.

Scale ⊢—— 2.0 cm ——⊣



B. C.

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Figure 3: Colonial morphology of B. cereus var. mycoides BmS6 (sinistral)

A. Streptomycin-sensitive strain, 14 hr at 37 C on nutrient agar
B. Streptomycin-resistant strain, 14 hr at 37 C on nutrient agar plus 500 μg/ml streptomycin
C. Streptomycin-resistant strain, 16 hr at 37 C on nutrient agar

the protein from the nucleic acids. The lysed cell suspension was shaken with an equal volume of chloroform-isoamyl alcohol (20:1) for 30 minutes to deproteinize the mixture. After centrifugation, the upper layer was decanted and the nucleic acids were precipitated by layering two volumes of cold 95 per cent ethanol on the solution. The nucleic acids were removed by spooling on a glass rod, dissolved in 0.015M NaCl-0.0015M sodium citrate solution, and 1.5M NaCl-0.15M sodium citrate solution was added to give a final diluent concentration of 0.15M NaCl-0.015M sodium citrate. The deproteinization and dissolving process was repeated until no more protein was precipitated. The final DNA-citrate-saline solution was used as a stock supply and stored at 4 C.

In the second method, a 10 liter batch culture was also grown overnight in nutrient broth and the cells harvested using the Sharples centrifuge. The cells were washed with the 0.15M versene-0.1M saline solution (pH 8.0) and then muramidase (2 mg/g of wet-pack cells) was added. When the cells began to lyse, they were immediately frozen in an acetone-dry ice bath. They were then thawed in a 60 C waterbath and 50 ml of Tris buffer-sodium lauryl sulfate solution (pH 9.0) were added and the quick freeze-thawing procedure was repeated. The lysed cell suspension was then shaken with an equal volume of phenol (88 to 90 per cent) at 4 C for 20 minutes to deproteinize the mixture. After shaking, the mixture was centrifuged and the top layer decanted. The procedure for recovering and storing the DNA was the same as in the first method.

The DNA concentration of both preparations was determined by measuring their absorbancies at 260 and 280 mμ using a Beckman DU spectrophotometer and then quantitating from the nomograph prepared by E. Adams and distributed by California Corporation for Biochemical Research,

Los Angeles, California.

Characterization of DNA

Determination of DNA, RNA, and protein content of donor DNA. The amount of DNA was determined by the colorimetric diphenylamine method of Burton (1956) using salmon sperm DNA as the standard, and the RNA content by the orcinol method (Ashwell, 1957) using D-ribose as the standard. Protein was detected by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Melting point (T_m) determination. Sufficient B. mycoides DNA was dissolved in a 10^{-3}M NaCl-10^{-2}M versene solution to give an absorbancy of 0.170 at 260 mμ using the optics of a Beckman DU spectrophotometer and a Gilford photometer (Szybalski and Mennigmann, 1962). Three ml of this mixture were placed in a glass-stoppered quartz cuvette, heated in the Gilford spectrophotometer chamber, and the changes in absorbancy with increasing temperature were recorded on the Gilford Model 2000 multiple sample absorbance recorder. The measurements were made until the absorbancy remained constant over a 5 to 10 C change in temperature.

Development of Competent Cells

Attempts to develop competency were carried out in three steps: (1) An initial medium was inoculated from the stock culture of the organism and was incubated for 12 to 14 hours at 37 C; (2) a second medium (5 ml) was inoculated with 0.1 ml of the 12 to 14 hour culture and incubated four hours at 37 C with constant shaking; and (3) a third medium (9 ml) was inoculated with 1.0 ml of the 4-hour culture and incubated 60 to 90 minutes with constant shaking. A different medium was usually used during each incubation interval.

In an attempt to find a suitable media sequence for competency

development in B. mycoides, the series indicated in Table I were used:

TABLE I

MEDIUM USED DURING INDICATED INCUBATION PERIOD

	12 to 14 hours	4 hours	60 to 90 minutes
1.	Nutrient agar overlaid with 10 ml of nutrient broth	Nutrient broth	Nutrient broth
2.	Nutrient agar overlaid with 10 ml of nutrient broth; or penassay agar overlaid with 10 ml of penassay broth	MS + 0.1 per cent YE	MS + 0.01 per cent YE
3.	Nutrient agar overlaid with 10 ml of nutrient broth; or penassay agar overlaid with 10 ml of penassay broth	M-1 medium	M-2 medium

Prolonged incubation in broth tends to cause dissociation of B. mycoides from rough to smooth colonial morphology. Therefore, all of the 12 to 14 hour cultures were grown on either nutrient or penassay agar which had been overlaid with 10 ml of the corresponding broth to prevent dissociation. A single-point inoculation was made on nutrient agar after each 12 to 14 hour incubation to check for any morphology changes.

Transformation Procedure and Method of Assay

The procedure indicated in Figure 4 was used in attempting to transform B. mycoides.

The total count assay was made using Millipore filters on nutrient agar because, as previously stated, B. mycoides grows as smooth colonies on membrane filters. Colonies from these filters were replicated onto nutrient agar (Lederberg, 1952) using velveteen to determine the morphology of the colonies. After replication, the membranes were flooded with 10 ml of a 0.001 per cent malachite green dye solution to stain the filter

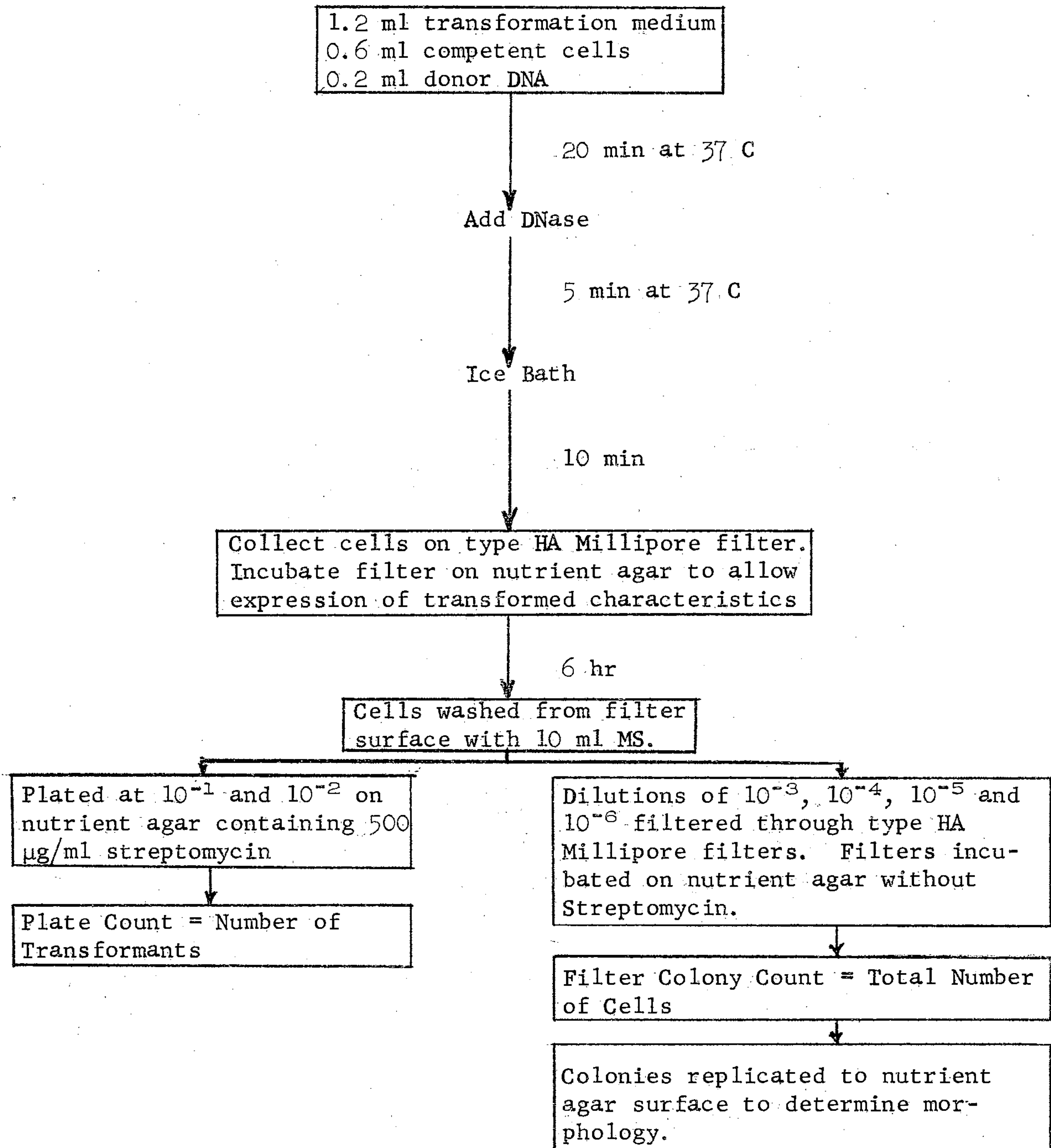

Figure 4: Transformation procedure

background and permit easier counting of the unstained colonies (Millipore Filter Corporation, 1961).

Incorporation of Radioactivity from ^{14}C-2-Thymine into Transforming DNA

A 12-hour culture of *B. mycoides* BmS6 (R^{+}, S^{r}) was grown at 37 C on nutrient agar containing 5 μg/ml of thymine and overlaid with 10 ml of nutrient broth containing 5 μg/ml of thymine. This culture was used as the inoculum for 300 ml of nutrient broth containing ^{14}C-2-thymine. The labeled mixture was incubated for 14 hours in a 37 C water bath with forced aeration. The radioactivity of the labeled broth was measured before the inoculum was added and after the cells had been removed by centrifuging. This measurement was made by adding 0.1 ml of the broth to 10 ml of scintillating fluid (Bray, 1960) and counting on a Nuclear Chicago scintillation counter.

Deoxyribonucleic acid was prepared from the labeled cells by the method of Saito and Miura (1963). The radioactivity of the DNA dissolved in citrate-saline solution was measured by adding 0.1 ml of the solution to 10 ml of Bray's scintillating fluid and counting on the scintillation counter.

Incorporation of Radioactivity from ^{14}C-Labeled DNA into DNA of Competent Cells

To determine any incorporation of the labeled DNA into the recipient cell DNA, it was necessary to: (1) measure the radioactivity of the competent cells after incubation with labeled DNA to determine the amount of uptake, and (2) measure the amount of transformation resulting from incorporation of the labeled DNA.

A culture of *B. mycoides* BmS6 (R^{+}, S^{s}) was grown to competency by the method described. The labeled DNA was added to the competent cells

and the radioactivity of the supernatant solution and of the cells at zero time and after 20 minutes incubation at 0 and 37 C was measured.

Prior to measurement of radioactivity, the cells were centrifuged from solution and washed three times with MS medium--the original supernatant and first wash solutions were kept for counting. The cells were then suspended in 1 ml of MS medium and lyophilized (Kihara, Ikawa and Snell, 1961). Hyamine (1.0 ml) was added to the dried cells to dissolve the cellular material. Bray's scintillating fluid (10 ml) was added to this mixture and the activity measured on the scintillation counter.

The original supernatant and first wash solutions were evaporated to approximately 1.0 ml in an oven at 100 C. Ten ml of scintillating fluid were added and the activity measured. Dilutions were made of the mixture incubated at 0 and 37 C for 20 minutes. Aliquots of these dilutions were plated according to the transformation procedure previously described.

CHAPTER III

RESULTS AND DISCUSSION

Since the objectives of this study involve the transformation of characteristics of B. mycoides, several preliminary investigations were made in an attempt to determine the dominant traits of this organism. Several soil samples were tested in an endeavor to determine if either the sinistral or dextral form of B. mycoides predominates in nature. Gause (1939) states that the sinistral is the usual form and that the dextral is very unusual and is probably a mutant of the sinistral; Murray and Elder (1949) found that the ratio of sinistral to dextral was approximately 3:1 in all cases examined. However, the data from this study indicate that neither the sinistral nor dextral form is dominant, and occur in an approximate ratio of 1:1--out of 144 colonies observed, there were 67 sinistral and 77 dextral.

Because there is a lack of dominance in either sinistral or dextral filamentation and because sinistral forms always give rise to sinistral forms and dextral forms to dextral, it appears that the direction of filament curvature in a given strain is heritable. However, an investigation was made to ascertain whether the growth medium could be a determining factor in the direction of filamentation. The results confirm those reported by Hastings and Sagen (1933) in that, on firm media, growth spreads from the point of seeding in the form of filaments which curve either clockwise or counterclockwise and produce a symmetrical

pattern. On less firm media (containing 0.7 per cent agar or lower) the growth is diffuse.

It was also found that the filaments will follow lines of stress in a medium, i.e. along the edge of a crack in the agar surface or in the indentations made by drying of the media.

Both rhizoid forms of B. mycoides will grow as smooth colonies on Millipore filter membranes, No. 2 Whatman filter paper and Glassine powder paper. These findings indicate that formation of a rhizoid colony may be a function of the moisture on the surface of the growth medium or their ability to slide over a smooth surface. Transfers from any of these aberrant forms to newly prepared media results in the original rhizoid growth pattern. These results indicate that the physical properties of the growth medium have no permanent effect on the growth pattern of B. mycoides.

Transformation of Smooth Dissociants to the Original Rhizoid State

A smooth dissociant, B. mycoides BmD2 (R^-), was prepared from B. mycoides BmD2 (R^+) by prolonged growth in broth. Deoxyribonucleic acid was prepared from BmD2 (R^+) and a transformation experiment performed by the procedure described in the Materials and Methods section. None of the smooth dissociants were transformed to the original dextral filamentous growth.

Since this was the first attempt at transforming B. mycoides, there were several factors which could account for the lack of transformation, i.e. non-competent recipient cells, temperature and length of incubation period of the transforming mixture, and failure to incorporate donor DNA.

Very little is known about the development of competency in a cell, and competence will appear only under narrow cultural conditions. Perry

and Slade (1961) found that factors decreasing the permeability of a cell decrease the uptake of donor DNA. Anagnostopoulos and Spizizen (1961) found that vegetative cells of B. subtilis became optimally competent for transformation when grown in simple media and at a particular stage of the growth cycle. Schaeffer (1964) states that the experience gained with one species is useless with another--rich media are required by some species whereas others need only a minimal medium; serum albumin is required for competence in pneumococcus, but is inhibitory in B. subtilis; and shaking either increases or decreases competence of different strains.

Since the conditions for developing competency in B. mycoides are unknown, several parameters were varied in the cell preparation and transformation procedures in an attempt to determine the conditions that would permit transformation of this species. To determine if the age of the culture influenced competency development, aliquots (0.1 ml) were removed from the overnight culture for the second step in growing recipient cells to competency and were incubated for 2, 3, 4, 5, and 6 hours before being transferred to the third growth cycle. Two mixtures were made from each of these different-aged cells with donor DNA; one mixture was incubated at 30 C and the other at 37 C to determine the effect of temperature. No transformation resulted from any of these mixtures.

Different media series were utilized in an attempt to develop competency. B. mycoides BmD2 (R^-) would not grow on Spizizen's minimal medium (1958). Therefore, in order to decrease the enrichment in the media during each cycle of competency development (a factor which apparently enhances the development of competency) brain heart infusion broth or penassay broth was used as the initial medium and the subsequent

cultures grown in nutrient broth. This stepdown procedure did not make the recipient cells competent, as evidenced by the lack of transformation.

All of the initial experiments in transformation of B. mycoides were attempts to transform a smooth dissociant to its original rhizoid state. Since Smith, et al. (1952) have found the dissociation from rough to smooth to be irreversible, it is possible that a segment might have been deleted from the organism's genome causing the dissociation. The frequency of transformation might be negligible since an identical segment of the donor DNA would have to be incorporated into the recipient DNA to restore the original rhizoid growth pattern. Therefore, another transforming system, which had been successfully used in other organisms, was developed using a sinistral form of B. mycoides, BmS6, to ascertain if another characteristic of B. mycoides can be transformed.

Transformation of Streptomycin-Sensitive Cells to Streptomycin Resistance

A streptomycin-resistant strain B. mycoides BmS6 (R^+, S^r) was developed by the gradient plate method and used as a source of donor DNA. Several transformation experiments were performed with this system using both B. mycoides BmD2 (R^+, S^s) and BmS6 (R^+, S^r) as recipient cells. The recipient cells were grown using nutrient broth as the growth medium for each step of "competency" development, or by growing the overnight culture in nutrient broth followed by 4-hour growth in MS plus 0.1 per cent yeast extract and 90-minute growth in MS plus 0.01 per cent yeast extract. No streptomycin-sensitive cells were transformed to streptomycin resistance.

In initial experiments, dilutions from the transformation mixtures using the streptomycin marker were plated on transformation assay medium

(nutrient agar containing 500 μg/ml streptomycin) immediately after incubation with DNA. Ephrussi-Taylor (1962) and Nester and Stocker (1963) have found that there is a latency period after the donor DNA is taken up by the recipient cell during which time the donor DNA is integrated into the recipient DNA and the character being transformed becomes expressed. Streptomycin-sensitive cells do not become immediately indifferent to streptomycin after transformation and therefore this early plating could have killed all of the transformed cells. In later experiments the transformation mixture (recipient cells plus donor DNA) was filtered through a Millipore filter which was placed on a medium capable of supporting growth to allow expression of streptomycin resistance before plating on the assay medium. No transformation resulted.

Competent B. mycoides BmD2 (R^+, S^s) and BmS6 (R^+, S^s) were incubated with varying amounts of donor DNA (a 4-fold range in concentration) to determine if the concentration of DNA affected transformation. No transformation resulted. Hotchkiss (1957) has found that there is a DNA level above which the number of transformants cannot be increased by adding more DNA and also that "pure" DNA may contain undetected DNA particles which do not carry the particular marker being measured and are inhibitory.

Effect of Addition of "Competency Factor" from Known Transformation Systems

Tomasz and Hotchkiss (1964) described an extracellular macromolecule produced by pneumococcal cells which induced competency. They called this factor activator and found that the product could transfer competency to cells which were growing under conditions of incompetence by either adding a growing activator culture to the incompetent cells or by diffusion of the activator-containing medium through a Millipore filter

into the incompetent culture.

Since the transformation of the auxotroph B. subtilis 168 from indole$^-$ to indole$^+$ can be quite easily accomplished, a culture of B. subtilis 168 (indole$^-$) cells was made competent by the method of Spizizen (1958). A 0.6 ml aliquot of these competent cells was added to a culture of B. mycoides BmS6 (R^+, S^s) during the 90-minute growth cycle of competency development and also to a transformation mixture containing B. mycoides BmS6 (R^+, S^s) cells which had been grown in nutrient broth through the three stages of competency development and DNA isolated from B. mycoides BmS6 (R^+, S^r). This was done to determine if B. subtilis 168 produced a competency factor which would confer competency to the streptomycin-sensitive B. mycoides cells permitting them to be transformed to streptomycin resistance. Presumably, such a factor is not produced or if it is, it does not induce competency in B. mycoides since none of the cells were transformed.

As a control to determine if the B. subtilis 168 cells were actually competent and thus capable of producing a competency factor, DNA was isolated from the prototroph B. subtilis W23 and added to a 0.6 ml aliquot of the competent B. subtilis 168 cells. Dilutions were made from this mixture and plated on minimal medium to determine the number of B. subtilis 168 transformants from ind$^-$ to ind$^+$ and on penassay agar to determine the total number of viable cells. The results of this experiment are shown in Table II.

A filtrate obtained by filtering a culture of competent B. subtilis 168 cells through a Sweeny filter was added to a "competent" B. mycoides

TABLE II

ATTEMPTED TRANSFORMATION OF B. MYCOIDES CELLS GROWN IN THE PRESENCE OF COMPETENT B. SUBTILIS CELLS

Transforming Mixture		No. of Colonies on Indicated Media:			
Recipient Cells	Donor DNA*	Minimal	Nutrient Agar +500 $\mu g/ml$ Streptomycin	Nutrient Agar	Per Cent Transformation
B. subtilis 168	B. subtilis W23	1.14×10^3	-	2.09×10^8	5×10^{-4}
B. subtilis 168	B. mycoides BmS6 (R^+, S^r)	0	0	2.89×10^8	-
B. subtilis 168 and B. mycoides BmS6 (R^+, S^s)	B. subtilis W23	1.80×10^5**	0	2.00×10^6 (B. mycoides) 2.17×10^8 (B. subtilis)	- -
B. subtilis 168 and B. mycoides BmS6 (R^+, S^s)	B. mycoides BmS6 (R^+, S^r)	8.70×10^5	0	3.00×10^6 (B. mycoides) 2.77×10^8 (B. subtilis)	-
B. mycoides BmS6 (R^+, S^s)	B. mycoides BmS6 (R^+, S^r)	-	0	1.64×10^8	-

* DNA concentration = 132.5 $\mu g/ml$

** No differentiation could be made between B. mycoides and B. subtilis colonies

BmS6 (R^+, S^s) plus BmS6 (R^+, S^r) DNA mixture to further investigate the possibility of the production and transfer of a competency factor by competent B. subtilis 168 cells. Again, no streptomycin-resistant B. mycoides cells grew.

Felkner and Wyss (1964) found that B. cereus $569S^s$ produced a "competence factor" which is transient, that is, the cells are competent and producing the factor during the 90-minute growth interval of competency development but lose their competency 30 minutes after this cycle is completed. These investigators found that non-competent cells were rendered transformable by addition of the factor, but that an optimum concentration of the factor is necessary for transformation to occur.

Several experiments were performed using both competent cultures of B. cereus 569 and sterile filtrates from competent cultures to determine if the "competence factor" produced by this organism would stimulate competency in B. mycoides BmS6 (R^+, S^s) but no streptomycin-resistant transformants were produced. Either the factor is non-transferrable to B. mycoides or the concentration of the factor was not adequate for the transformation of streptomycin-sensitive B. mycoides to streptomycin resistance. Experiments to clarify this latter possibility were performed. Even when the ratio of competent B. subtilis 168 cells or competent B. cereus 569 cells to B. mycoides BmS6 (R^+, S^s) in the incubation mixture was 100:1, no transformation resulted.

DNA was prepared from B. cereus $569S^r$ and added to aliquots of the B. cereus 569 and B. mycoides mixtures to serve as a control for the competency of the B. cereus 569 cells. Aliquots of the mixture were plated on brain heart infusion agar containing 500 μg/ml streptomycin to determine the number of transformants. The total viable cell count

was obtained from dilutions plated on brain heart infusion agar. The per cent of transformation from streptomycin sensitivity to streptomycin resistance in B. cereus 569 was quite low, ranging from 0.0002 to 0.0008 per cent.

Incorporation of Radioactivity from ^{14}C-2-Thymine into Recipient Cells

As evidenced by the lack of transformation in the different systems, it is apparent that the two strains of B. mycoides studied are either heritably incompetent or the optimum conditions necessary for their transformation were not found.

To determine if the B. mycoides cells which had been theoretically brought to competency were taking up donor DNA, a culture of B. mycoides BmS6 (R^+, S^r) was grown in a medium containing ^{14}C-2-thymine to label the DNA. This labeled DNA was extracted and used in a transformation experiment with B. mycoides BmS6 (R^+, S^s) as the recipient cells. The donor DNA had a radioactivity of 2,148 cmp. The radioactivity of the recipient cells was measured immediately after addition of the labeled DNA and again after a 20-minute incubation period of the recipient cells and the labeled DNA at 0 C and 37 C. The results are listed in Table III.

TABLE III

RADIOACTIVITY IN RECIPIENT CELLS AFTER EXPOSURE TO LABELED DONOR DNA

Incubation Temperature (C)	Time of Exposure to Labeled DNA (min)	Radio-Activity (cpm)	Counting Efficiency (%)
0	20	334	67
37	0	425	74
37	20	361	72

The background count of the scintillation counter was 260 cpm (70 per

cent efficiency).

Since the radioactivities of the different cell suspensions are so close to the background count, interpretation of the results is speculative. However, since the cells had been washed three times to remove all extraneous matter (including any labeled DNA which may be adsorbed to the surface of the cell) it appears that there was slight uptake of labeled DNA by cells incubated at 37 C compared with the 0 C control. But, even though there may have been uptake, the transformation experiment run concurrently yielded no transformants. This indicates that the labeled DNA was not being integrated into the recipient cell DNA.

Characterization of DNA from B. mycoides

One of the complications of transformation experiments is that the donor DNA may be impure. Along with molecules containing the transforming marker, other molecules which lack the marker may enter the cell and interfere with transformation. Zamenhof et al. (1957) purified the DNA of H. influenzae to the point where it contained less than 0.5 per cent (relative to DNA) of proteins, immunologically active substances, and ribonucleic acids. No loss of biological activity occurred during the removal of impurities which supports the theory that the transforming principle is DNA.

The DNA extracted from B. mycoides BmS6 (R^+, S^r) was not treated with ribonuclease to remove the RNA. The final DNA-citrate-saline solution contained 0.004 mg/ml of nucleic acids and 0.03 mg/ml of protein as determined from the nomograph of E. Adams using the absorbancies measured at 260 and 280 mμ. The concentrations of the various constituents of the final DNA preparation as determined by colorimetric methods are as follows: DNA, 78 μg/ml; RNA, 84.5 μg/ml; and protein, 170 μg/ml.

Cesium chloride density gradients run on the DNA preparation indicate that the DNA has a heterogeneous composition. This is verified by the relatively high protein content indicated above.

A melting point denaturation curve was prepared for B. mycoides DNA and also for B. subtilis DNA, as a control, using the Beckman DU spectrophotometer. A 40 per cent increase in absorbancy at 260 mμ (A_{260}) with a sharp transition in the curve indicates a high degree of purity and homogeneity of the DNA being tested. There is a sharp transition in the curve for the B. mycoides DNA, but only a 23 per cent change in A_{260} (not corrected for thermal expansion) which further indicates heterogeneity of composition. The melting temperature (T_m) corresponds to the temperature at 50 per cent change in A_{260} on the curve which is 46.5 for the B. mycoides DNA. The T_m obtained for B. subtilis DNA was 50.5 which compares to a value of 52.0 reported by Szybalski and Mennigmann (1962) (See Figure 5.).

The melting temperature of DNA is greatly influenced by the ionic strength of the solution in which the DNA is dissolved; the lower the ionic strength, the lower the melting temperature. The B. mycoides DNA was dissolved in a 10^{-3}M NaCl-10^{-2}M versene solution (pH 9.0). The ionic strength of this solution enables a recording thermospectrophotometer to record melting temperatures within a range of 20 to 60 C.

The dependence of T_m on DNA composition in 51 per cent methanol containing 10^{-3}M NaCl and 10^{-3}M Tris buffer at pH 7 (similar in composition and ionic strength to the methanol-versene-saline mixture) is 0.35 C/mole per cent guanine + cytosine (G-C) (Geiduschek, 1962). The recorded G-C content (mole per cent) of B. subtilis DNA is 43 (Marmur and Doty, 1962).

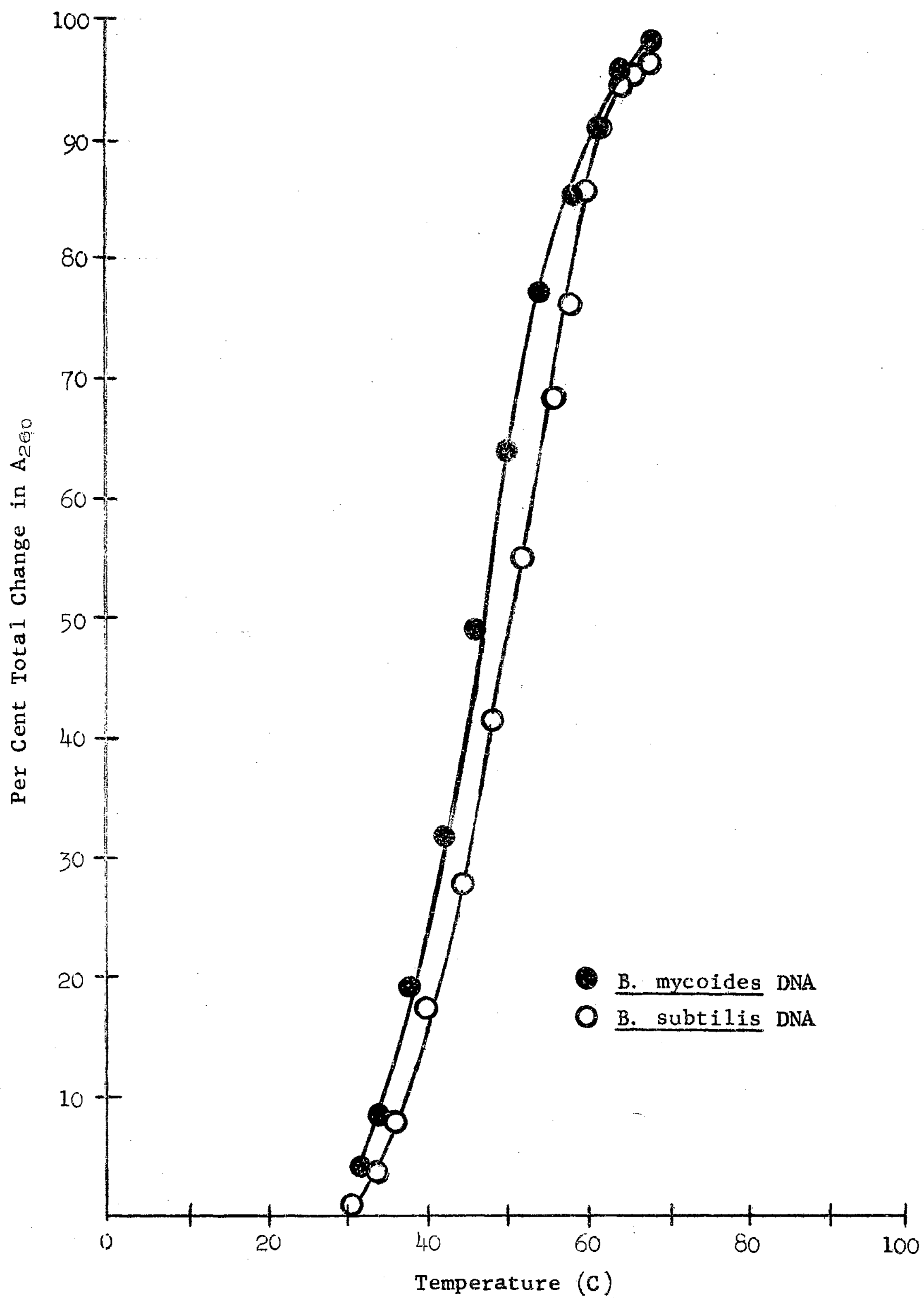

Figure 5: Thermal denaturation curve for isolated deoxyribonucleic acids

Therefore, by using the difference in the experimentally determined T_m's for B. subtilis and B. mycoides, 4 C, the G-C content of B. mycoides can be calculated to be 31.6 mole per cent. This compares to a value of 34 to 36 mole per cent reported by Marmur, Falkow and Mandel (1963).

CHAPTER IV

SUMMARY AND CONCLUSIONS

The smooth dissociant of a dextral strain of *B. mycoides*, BmD2, could not be transformed to its original rhizoid state, nor could a sinistral strain, BmS6, be transformed from streptomycin sensitivity to streptomycin resistance.

This is the first attempt at transforming a morphologic or biochemical characteristic in *B. mycoides*. This necessitated attempts to define the optimum conditions necessary for such a transformation (i.e. development of competency in the recipient cell, preparation of an active donor DNA, and means for assaying for transformation).

Procedures attempted in an effort to achieve transformation of *B. mycoides* include: 1) the use of different culture media; 2) variations in the age of the recipient cells; 3) time and temperature of incubation of the recipient cells and donor DNA; 4) the amount of donor DNA used; and 5) the addition of known competent cells to transformation systems in an attempt to make the recipient cells competent by transferring a reported "competency factor." None of these factors caused transformation in *B. mycoides*.

Although not all strains of a species can be transformed, this does not mean that transformation in *B. mycoides* cannot be accomplished. Transformability is a genetically determined property and to date there are only a few known species which have a consistently high degree of

transformability.

Additional studies will have to be conducted to discover those very critical cultural conditions which are necessary to make the *B. mycoides* cell competent. Should these be found, a useful tool for studying the relationship between the dextral and sinistral colonial forms of *B. mycoides* will have been developed.

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APPENDIX

TEST ORGANISMS

Organism	Source
Bacillus cereus 569	Dr. O. Wyss, Department of Microbiology, University of Texas
Bacillus cereus $569S^r$	Dr. O. Wyss, Department of Microbiology, University of Texas
Bacillus cereus var. *mycoides* BmD2 (Dextral strain)	Isolated from soil at Stillwater, Oklahoma, by Dr. E. C. Noller, Department of Microbiology, Oklahoma State University
Bacillus cereus var. *mycoides* BmS6 (Sinistral strain)	Isolated from soil at Stillwater, Oklahoma, by Dr. E. C. Noller, Department of Microbiology, Oklahoma State University
Bacillus subtilis W23	Dr. N. N. Durham, Department of Microbiology, Oklahoma State University
Bacillus subtilis 168	Dr. N. N. Durham, Department of Microbiology, Oklahoma State University

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