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# Genetic Transformation Among *Azotobacter* Species

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AN ABSTRACT OF THE THESIS of Wayne H. Voth for the Master of Science in Biology presented 14 December 1977.

TITLE: Genetic Transformation Among *Azotobacter* Species.

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Previous methods for genetic transformation in *Azotobacter vinelandii* have employed poorly defined genetic markers or crude DNA extracts. An improved transformation technique has been developed for use in *Azotobacter*. The technique was used to transform several strains of *Azotobacter* with DNA carrying a defined genetic marker. A method

for isolating pure, high molecular weight, biologically active DNA from *Azotobacter* is also presented. Purity of the extracted DNA was determined by standard chemical assays. The molecular weight was determined by boundary sedimentation techniques to be 18.2 megadaltons. DNA was obtained from several mutant strains of *Azotobacter*. Biological activity of these samples was demonstrated by using them to accomplish both intra- and interstrain transformation. Thermal denaturation profiles of several DNA samples are presented, from which guanine plus cytosine content was determined. Among the *Azotobacter* species examined, GC content ranged from 65.1 to 67.8%. The use of the new transformation and DNA isolation methods in taxonomic and mapping studies is discussed.

GENETIC TRANSFORMATION AMONG  
*Azotobacter* SPECIES

by  
WAYNE H. VOTH

A thesis submitted in partial fulfillment of the  
requirements for the degree of

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in  
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TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

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## TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENTS.....	iii
LIST OF TABLES.....	v
LIST OF FIGURES.....	vi
INTRODUCTION.....	1
MATERIALS AND METHODS.....	7
Bacterial Strains.....	7
Media and Culture Conditions.....	7
Isolation of Mutants.....	8
Verification of Strain Purity.....	9
Electron Microscopy.....	10
Extraction and Purification of Deoxyribonucleic Acid.....	11
Analytical Methods.....	13
Transformation Procedures.....	16
RESULTS.....	18
Development of Improved DNA Extraction Method....	18
Characterization of Extracted DNA.....	21
Determination of DNA Molecular Weight .....	23
Determination of $T_m$ and GC Content.....	26
Transformation.....	34
DISCUSSION.....	40
BIBLIOGRAPHY.....	43

## LIST OF TABLES

TABLE		PAGE
I	Recovery of DNA Extracted from Azc2.....	22
II	T <sub>m</sub> and Percent GC Values of DNA Isolated from <i>Azotobacter</i> Strains.....	33
III	Detection of Transformation Among <i>Azotobacter</i> Strains.....	37



## LIST OF FIGURES

FIGURE		PAGE
1	Sedimentation Boundaries of Az17 DNA.....	24
2	Thermal Denaturation Profile of <i>E. coli</i> DNA....	28
3	Comparison of Thermal Denaturation Profiles of Azv1 and <i>E. coli</i> .....	29
4	Comparison of Thermal Denaturation Profiles of Azc2 and <i>E. coli</i> .....	30
5	Comparison of Thermal Denaturation Profiles of Az3 and <i>E. coli</i> .....	31
6	Comparison of Thermal Denaturation Profiles of Az17 and <i>E. coli</i> .....	32
7	Factors Influencing Transformation Frequency in <i>A. vinelandii</i> (Azv1).....	36

## INTRODUCTION

Members of the genus *Azotobacter* are large, pleomorphic, Gram-negative rods. All are obligate aerobes and elaborate a heavy, extracellular slime. Two rather special, if not unique, properties of these organisms have brought them under intense study during recent years. One of these is the ability to form thick-walled, metabolically dormant exocysts when grown on simple organic acids and alcohols (Winogradsky, 1938). The processes of encystment and cyst germination form a morphogenetic pattern which has been used as a model system of simple cell differentiation. Research in this area was reviewed by H. L. Sadoff (1975).

He states:

Differentiation in *A. vinelandii*, as in all biological systems, entails the selective expression of its genetic potential in a temporal sequence whose primary control must occur at the transcriptional level.

Understanding this and other simple systems of transcriptional control (or the loss thereof) could form the basis of future knowledge of embryological development, differentiation of antibody-forming cells, and of cancer development.

The second noteworthy feature of *Azotobacter* is the ability to fix molecular nitrogen. While other microorganisms are capable of nitrogen fixation, *Azotobacter* is

among the few that carry out the process while growing under aerobic conditions. Investigations of the fixation process in bacteria have been led by W. J. Brill at the University of Wisconsin using *Azotobacter vinelandii* and *Klebsiella pneumoniae* (Brill, 1975), and by a team of scientists at the Agricultural Research Council's Unit of Nitrogen Fixation at the University of Sussex, England, studying *Azotobacter chroococcum* and *K. pneumoniae*.

The enzyme responsible for dinitrogen ( $N_2$ ) fixation is nitrogenase (Eady and Postgate, 1974). Its action, the reduction of  $N_2$  to ammonia without detectable intermediates, seems similar in all free-living organisms which synthesize the enzyme. The nitrogenase system is comprised of two different protein complexes called components I and II (Bulen and LeCompte, 1966). Both components are required for activity, as are  $Mg^{2+}$  and large amounts of ATP. Nitrogen fixation is inhibited by ADP, which inhibits nitrogenase activity, or by ammonia, which represses nitrogenase synthesis.

Component II of nitrogenase is an iron-containing protein. Component I contains iron and molybdenum. An iron-molybdenum cofactor (FeMoCo) has been isolated from component I by Shah and Brill (1977). "FeMoCo" is required for activity of component I. While component I from one organism does not necessarily complement the component II of another organism to form an active system, "FeMoCo" from

different sources may be used interchangeably in *in vitro* systems.

The sensitivity of the nitrogenase system to irreversible inactivation by oxygen is a major limitation of biological nitrogen fixation. "FeMoCo" may be the most oxygen sensitive portion, but is protected somewhat by the protein "core" of component I. Understanding the mechanism of protection from  $O_2$  in aerobic organisms such as *Azotobacter* may lead to the ability to implant the necessary genetic information required for nitrogen fixation into some higher plant. Such a plant could be a valuable food source and could be grown in poor soils without the need of nitrogenous fertilizers. With projected fossil fuel shortages, manufacture of such soil additives may soon become unfeasible due to prohibitive costs. In this situation, a plant capable of fixing its own nitrogen would be a valuable asset.

Study of encystment and of nitrogen fixation in *Azotobacter* would be greatly facilitated by genetic analysis and manipulation. One prerequisite to genetic analysis in bacteria is the availability of mutant strains. These make possible the detection of successful gene transfer through selection between altered and unaltered survivors. Various reports point out the difficulty of obtaining mutants in *Azotobacter* using standard methods (Page and Sadoff, 1976a, Sorger and Trofimenkoff, 1970, Sadoff, *et al.*, 1971, Sadoff, *et al.*, 1975, and Mishra and Wyss, 1969). Most known mut-

ants of *A. vinelandii* have been isolated following N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis. Sorger and Trofimenkoff (1970) used this powerful mutagen to isolate only nine nitrogenaseless ( $\text{Nif}^-$ ) mutants in a total of twelve mutant hunts. Of the nine, six were found to be leaky. Shah, *et al.* (1973) also used NTG to isolate a number of  $\text{Nif}^-$  mutants. Mishra and Wyss (1969) mutagenized *A. vinelandii* with NTG, and reported isolation of an adenine auxotroph. Page and Sadoff (1976a) reported having used NTG to obtain auxotrophic mutations of *A. vinelandii* for adenine ( $\text{Ade}^-$ ), uracil ( $\text{Ura}^-$ ), and hypoxanthine ( $\text{Hyp}^-$ ), as well as  $\text{Nif}^-$  and rifamycin-resistant mutants ( $\text{Rif}^r$ ). While the list may appear to be extensive, it is very small when compared to the many hundreds of mutants known in *Escherichia* and *Salmonella*.

Genetic transfer is a second prerequisite to genetic analysis in bacteria. Transfer systems fall into three main classes (Hayes, 1968). *Conjugation*, often termed a sexual process, requires cell-to-cell contact. The "female" or recipient cell is a normal bacterium. The "male" or donor cell carries a dispensable DNA element which may be present as an autonomously replicating plasmid, or as an integrated episome. In either case, the dispensable element directs the host cell to synthesize pili. These hollow protein rods comprise the means by which cell-to-cell contact is established and by which DNA is transferred from donor to recip-

ient cells.

In *transduction*, DNA transfer is mediated by a bacteriophage vector. During assembly of the viral particle, a portion of the host genome may occasionally be incorporated in place of phage genome. Gene transfer is accomplished by the infection of a further host.

The third class of transfer mechanism is *transformation*. Soluble DNA is directly responsible for the phenomenon. The transforming DNA can be taken up by competent recipient cells and integrated into the recipient genome.

Transformation is the only gene transfer mechanism to have been reported in *Azotobacter*. Methods used for interspecies transformation in *Azotobacter* (Sen and Sen, 1966) and for intergeneric transformation between *Rhizobium* and *Azotobacter* (Sen, *et al.*, 1969) have not been successfully employed by other researchers (Page and Sadoff, 1976a). The reason for this is not clear. Recently, Page and Sadoff (1976a,b) reported using a modification of the "plate method" (Gwinn and Thorne, 1964) to transform *A. vinelandii*. The method was used to transfer rifamycin resistance, and to restore prototrophy to their  $Nif^-$  and auxotrophic mutants. All markers used appeared to be linked to the  $Rif^r$  locus, which was used as the standard marker. Bishop and Brill (1977) used the methods of Page and Sadoff to determine the relative positions of eight separate  $Nif^-$  lesions in *A. vinelandii*. Bishop, *et al.* (1977) used the same methods to re-



store prototrophy to Nif<sup>-</sup> *A. vinelandii* using crude lysates of Nif<sup>+</sup> *Rhizobium*. They were also able to transfer specific *Rhizobium* surface antigens to *Azotobacter*.

Results obtained using the plate method provide the first mapping of *A. vinelandii*. But the results which can be obtained with the technique are limited because of two features of the method: First, a crude cell lysate was used as a source of DNA because normal methods for purification would not yield DNA capable of effecting transformation (Sadoff, 1975, and Page and Sadoff, 1976a). The disadvantage of using crude extract is that the molecular weight of the DNA is not known, nor is the effect of contaminants. Second, the plate method does not allow determination of absolute transformation frequencies which are necessary for efficient linkage studies (Spizizen, *et al.*, 1966).

The purpose of this research was two-fold: One, to develop a method for purification of high molecular weight, biologically active *Azotobacter* DNA, and two, to develop a quantitative means of detecting transformation among *Azotobacter* species. An efficient DNA purification method was developed. It was used to obtain mutant DNA from several *Azotobacter* strains. The DNA was then employed in transformation studies. An improved transformation method was also developed which can provide at least semi-quantitative transformation frequencies in *A. vinelandii*.

## MATERIALS AND METHODS

### Bacterial Strains

*Azotobacter vinelandii* (ATCC 12837) and *Azotobacter chroococcum* (ATCC 9043) were obtained as lyophils from the American Type Culture Collection. Once activated, these were renamed as laboratory strains Azv1 and Azc2, respectively. The other strains used in the project were isolated by Dr. J. W. Myers from sand, soil, and water samples collected from various parts of Oregon and California. These were arbitrarily labelled Az3, Az6, Az8, and Az17.

### Media and Culture Conditions

Two media were used, both of which were modifications of Burk's nitrogen-free medium. B medium (Newton, *et al.*, 1953) was used for routine growth of the organisms in liquid cultures. T medium (Page and Sadoff, 1976b) was used in transformation experiments. Solid forms of each were prepared by the addition of 15 grams of agar (Difco) to a liter of liquid medium. Unless otherwise stated, media were supplemented with 1% glucose (w/v) as the carbon source. Incubation during growth was at 30°C. Liquid cultures were incubated in a gyratory incubator shaker (New Brunswick Scientific) set at 200 rpm.

Standard inocula for liquid cultures were prepared by



harvesting exponentially growing cells by centrifugation. After washing and resuspending the cells in an equal volume of B medium without carbon source, they were stored at room temperature. The suspensions remained viable for several weeks, and would reliably provide log-phase cultures when 1:100 dilutions in B medium were incubated for 18 hr.

Small liquid cultures were routinely grown in 10 or 20 ml B medium in a 125 ml Erlenmeyer or Nephelo culture flask. For large batch cultures, as used in DNA extraction, dilutions of exponentially growing small cultures were used to inoculate 400 ml of fresh medium in 1-liter Erlenmeyer flasks.

Stock cultures of the various strains were maintained in screw-cap tubes on B agar slants supplemented with 0.3% sodium benzoate (w/v). Viability of these stocks was approximately one year when kept at room temperature. They could be activated at any time during this period by plating on B agar.

#### Isolation of Mutants

Spontaneously occurring rifamycin-resistant mutants of each strain were selected as follows: Exponentially growing cells in liquid culture were harvested by centrifugation, resuspended in 0.5 volumes B medium, and plated on B agar containing 100  $\mu\text{g/ml}$  rifamycin (Sigma or Calbiochem). Resistant cells produced visible colonies after four days of

growth.

### Verification of Strain Purity

In order to ensure that DNA extracts contained only the DNA of the desired organism and that studies involved the use of pure strains, culture purity was continuously monitored. Cultures were routinely streaked for isolated colonies on B agar. The degree of purity at each subculture was assessed by examination of wet-mounts with the phase contrast microscope. If contaminants were detected, one or more of the following methods was employed to repurify the strain.

All *Azotobacters* used in the study were motile and produced an extracellular slime. These properties made it difficult to remove contaminants. The restreaking of isolated colonies was employed, but often was not effective. If non-motile contaminants were detected in wet mounts, colonies were subcultured to 0.3% agar which allowed the separation of motile from non-motile organisms. (This was prepared by carefully layering 5 ml of plain 0.3% agar over a B glucose plate.) A tiny inoculum was placed on the center of the agar, and the plate was incubated for 24 hr. Subculture was accomplished by picking inocula from the leading edge of the swarming colony with a sterile needle, then touching the needle on the center of a fresh plate.

Another method for eliminating contaminants was growth

of *Azotobacter* in B medium containing 1% n-butanol. Since this concentration of butanol inhibits the growth of most other micro-organisms, subculture of *Azotobacter* to liquid cultures containing this carbon source allowed them to out-grow the contaminants.

Final determinations of culture purity and of the flagellation pattern of some strains were made by electron microscopy.

### Electron Microscopy

Grid Preparation. A plastic base film (1% Formvar in chloroform) was floated from a glass slide onto a water surface. Copper grids (Pelco, 200-mesh) which had been washed with toluene were placed on the film. The film/grid layer was then adsorbed to a strip of Parafilm and air dried. The plastic covered side of the grids was coated with carbon in a Denton DV-515 shadowing apparatus.

Sample Preparation. Samples from isolated colonies or liquid cultures in log-phase were first washed with unsupplemented B medium. Suspensions were then made using the same medium. A drop of suspension was placed on a prepared grid. Excess liquid was drawn off with a micropipette after 20-30 min, during which the cells were allowed to settle onto the grid. While moist, the cells were simultaneously fixed and negatively stained by the application and immediate removal of a drop of 0.5% phosphotungstic acid titrated to pH 7.2

with KOH. After complete drying in air, the grids were examined with a Zeiss EM9S electron microscope.

#### Extraction and Purification of Deoxyribonucleic Acid

DNA was isolated from *Escherichia coli* according to the method of Marmur (1961). Extraction of pure, high molecular weight DNA from *Azotobacter* strains required the development of a special technique:

1. Batch cultures were prepared according to Methods.
2. Harvest fresh cells (log-phase) by centrifugation and wash once with 0.1 M tris(hydroxymethyl)-aminomethane (TRIS) containing 0.05 M ethylenediaminetetraacetic acid (EDTA), pH 8.0 (TRIS-EDTA).
3. Resuspend pellet in TRIS-EDTA at 4-5 ml per gram wet cells.
4. Heat cell suspension to 70°C in a water bath.
5. Add 0.25 volumes protease VI (Sigma, 5 mg/ml), which was preheated to 70°C. Swirl.
6. Add an equal volume of 4% sodium dodecyl sulfate (SDS), also preheated to 70°C.
7. Incubate 10-15 min, or until maximum viscosity is reached. Swirl occasionally.
8. Cool the mixture rapidly to room temperature (21°C).
9. Add 0.25 volumes cold 5 M sodium perchlorate.
10. In order to denature and precipitate proteins, add an equal volume of cold chloroform:isoamyl alcohol

(24:1, v/v), and swirl vigorously by hand for 20-30 min at room temperature.

11. Centrifuge at 13,000 x g for 10 min at 0°C.
12. Remove aqueous layer containing the DNA. Repeat deproteinization (step 10) until little or no precipitate is found at the phase interface following centrifugation.
13. Add 0.01 volumes each: Ribonuclease T<sub>1</sub> (Sigma, 3000 U/ml), and ribonuclease A (Sigma, 5 mg/ml). RNase T<sub>1</sub> was pretreated in a boiling water bath for 10 min and RNase A at 80°C for 15 min.
14. Incubate 45 min in a 40°C bath, swirling occasionally.
15. Repeat deproteinization (step 10).
16. Remove the aqueous layer to a beaker and precipitate DNA by swirling the beaker while slowly adding 0.35 volumes of isopropyl alcohol, dropwise.
17. Wind out the DNA with a glass rod and wash in 70% ethanol.
18. Redissolve the DNA in 0.5 volumes of dilute saline citrate: 15 mM sodium chloride, 1.5 mM trisodium citrate, pH 7.2 (0.1 X SSC).
19. Adjust to standard saline citrate concentration: 0.15 M sodium chloride, 15 mM trisodium citrate, pH 7.2 (SSC), by adding 0.1 volumes concentrated saline citrate: 1.5 M sodium chloride, 0.15 M trisodium citrate, pH 7.2 (10 X SSC).

20. Repeat deproteinization (step 10).
21. Remove aqueous layer to a beaker and add 0.1 volumes 3 M sodium acetate, containing 10 mM EDTA, pH 7.0 (acetate-EDTA).
22. Precipitate DNA by the slow, dropwise addition of 0.50-0.55 volumes of isopropanol while swirling the beaker by hand.
23. Wind out the DNA with a glass rod and wash in 70% ethanol, then wash again in 95% ethanol.
24. Redissolve the DNA in 9.0 ml 0.1 X SSC.
25. Adjust to SSC concentration by adding 1 ml 10 X SSC. Store at 4°C over chloroform.

### Analytical Methods

DNA concentrations of extracts and purified DNA samples were obtained by the method of Burton (1956) using the diphenylamine reagent. Standards were known concentrations of salmon-sperm DNA (Sigma). Unknowns and standards were read using a Klett-Summerson photoelectric colorimeter with a #54 filter.

Ribonucleic acid (RNA) content was determined according to Schneider (1957) using the orcinol reaction. Yeast RNA and salmon-sperm DNA were used as standards. Samples and standards were read with a Klett-Summerson photoelectric colorimeter using a #66 filter.

Protein content was assessed by the method of Lowry,

*et al.* (1951) using bovine serum albumin (BSA:Sigma) solutions as standards. Samples and standards were read with a Klett-Summerson photoelectric colorimeter using a #66 filter.

Carbohydrate content was determined by the anthrone test using procedural modifications of Ashwell (1957) and glucose standards. Samples and standards were read with a Klett-Summerson photoelectric colorimeter using a #62 filter. Correction for DNA concentration was made using the data presented by Herbert, *et al.* (1971).

Total cellular DNA was estimated according to Herbert, Phipps and Strange (1971), except that fresh cells were extracted for 30 min at 90°C rather than the recommended 15 min intervals at 70°C.

Molecular weight determination of DNA was performed according to standard boundary sedimentation techniques outlined by Eigner (1968). DNA samples (2 ml) were dialyzed for 36 hr against SSC as follows: Three changes of the buffer, 500 volumes each, were made at 12 hr intervals. The temperature was 4°C.

Samples were diluted to desired concentrations with SSC. The DNA was sedimented at 34,000 rpm in a Spinco model E analytical ultracentrifuge (Beckman) using a 12 mm Kel-F centerpiece in an AnD rotor maintained at 20°C. Photographs were taken with ultraviolet optics at 4 min intervals. Negatives were analyzed with a Chromoscan MkII



recording densitometer (Joyce, Loebel and Co, Ltd.).

Melting Profiles of DNA. Thermal denaturation profiles of DNA samples were obtained by monitoring the increase of absorbance at 260 nm according to standard procedures outlined by Mandel and Marmur (1968).

The spectrophotometer used was a Gilford 2000, fitted to a Beckman DUR monochromator. Between these two components, a cuvette chamber section was constructed using a Beckman sideless carrier (four position) and two water jackets separated by bakelite spacers. The inner jacket was heated by circulating water from a Haake model F water bath. Protective cooling of the outer jacket was provided by circulating water from a Heto four-liter bath without heating. An insulated lid was fabricated from 2 cm thick styrofoam to cover the entire chamber-jacket section.

Quartz cuvettes with a 1 cm light path were used. These were fitted with tapered teflon stoppers which provided an excellent seal because they expanded with increased temperature to prevent vapor loss.

Internal cuvette temperature was monitored by copper-constantan thermocouples attached to a Fluke digital multimeter which read millivolts electro-motive force. These EMF values were converted to temperature readings using published values (Lange, 1967). The reference junction of each thermocouple was placed in an ice bath. The test junction was placed in the DNA solution within the cuvette as



follows: The wire was inserted through a hole drilled through the center of the teflon stopper and bonded in place with a slow-drying, two-part epoxy. Care was taken so that the junction did not interfere with the light path when the cuvette was in place.

DNA samples (2ml) were dialysed for 36 hr against 0.1 X SSC as follows: Three changes of the buffer were made, 100 volumes each, at 12 hr intervals. Dialysis was carried out at 4°C.

#### Transformation Procedures

Attempts were made to transform *Azotobacter* by the liquid transformation procedures developed for *Bacillus* (Anagnostopoulos and Spizizen, 1961), *Methylobacterium* (O'Connor, *et al.*, 1977) and *Azotobacter* (Sen and Sen, 1965 and Sen, *et al.*, 1969). The plate method of transformation developed for *Bacillus* (Gwinn and Thorne, 1964), and modifications thereof for *Acinetobacter* (Juni and Janik, 1969), for *Moraxella* (Juni, 1973) and for *Azotobacter* (Page and Sadoff, 1976a,b) were each used.

Further, a method of plate transformation which yields semi-quantitative results was developed during this study. Plates were prepared using 12.5 ml T agar. A desired amount of DNA (200 µg) in 1.0 ml SSC was mixed with  $1-2 \times 10^7$  recipient cells (early log-phase) in 1.0 ml T medium in a test tube. The mixture was poured into 2.0 ml of melted and

cooled (45°C) 1.5% agar and gently dispersed, then poured over the base agar in the plate. When this layer solidified, it was covered with a plain soft agar blank (4.0 ml, 0.8% agar). Care was taken so that each layer was level.

When transformants selected for were other than prototrophic (eg. Rif<sup>r</sup>), the selective agent was applied after a suitable period of growth (18-21 hr) in another soft agar overlay. Transformant colonies were counted after 4-5 days incubation.

## RESULTS

### Development of Improved DNA Extraction Method

DNA isolated from *Azotobacter* by the method of Marmur (1961) was of low apparent molecular weight, and yields were less than expected. Development of a better method was based upon stepwise improvement of both viscosity and yield. Viscosity was used as a qualitative indicator of molecular weight.

It was observed that lysates of fresh cells appeared more viscous than did lysates of frozen cells. Only freshly harvested cells were used thereafter. It was then discovered that viscosity increased after lysis if the temperature during lysis was increased from 60°C to 70°C. This temperature is well below that necessary to melt *Azotobacter* DNA. By preheating the detergent solution (SDS) to 70°C and by using a large volume of it, relative to the volume of the cell suspension, a rapid and more complete mixing could be achieved. Lysis suspensions resulting from the addition of an equal volume of preheated 4% SDS were more viscous than were those obtained with Marmur's procedure. Further increase of the final SDS concentration to as much as 6% did not improve lysis, but interfered with subsequent steps.

Microscopic examination of lysates revealed that many cells remained intact with the above treatment, regardless

of the time of incubation. In order to improve cell disruption and to inhibit nuclease activity, protease type VI (Sigma, 0.5 mg/ml final concentration) was added for its proteolytic action. Preliminary tests were made to determine whether the protease would be active under conditions of 70°C and 2% SDS. This was shown by hydrolysis of BSA under these conditions.

To improve the effectiveness of the buffer, TRIS-EDTA at pH 8.0 was employed. The killing effect of this combination on *Azotobacter* was established by Goldschmidt and Wyss (1966), and the effectiveness of its chelating power is well known. Lysis using various ratios of buffer to cell mass indicated that 4-5 ml:1 gm of wet cells was most effective. Lysis with this combination of conditions was complete within 15 min. This time coincided with the appearance of maximum viscosity.

Having established optimal lysis conditions (see Methods), it was important to maintain viscosity of the suspension through the balance of the procedure. The lysis suspension was quickly cooled to room temperature under running water. Cold sodium perchlorate was added to a final concentration of 1M in order to increase the ionic strength and for the deproteinizing effect of the perchlorate. A series of deproteinizations was then begun by adding an equal volume of chloroform:isoamyl alcohol. It was found that the amount of long-stranded DNA which could be precipitated fol-

lowing deproteinization was enhanced if, instead of shaking, the emulsion was rapidly swirled by hand in a large Erlenmeyer flask (5 volume capacity:1 volume emulsion). Viscosity of the emulsion during deproteinization also remained high with the more gentle treatment. After swirling for 20-30 min at room temperature, the emulsion was centrifuged at 13,000 X  $g$  for 10 min at 4°C. The aqueous phase was then removed to a clean flask and deproteinization was repeated until little or no denatured protein was found at the phase interface after centrifugation.

In order to consolidate steps and minimize excessive handling, the nucleic acid solution was treated with RNase prior to any precipitation steps. Most effective digestion of RNA was accomplished using two enzymes (RNase T<sub>1</sub> at 30 units per ml, and RNase A at 50  $\mu$ g/ml, final concentrations) similar to the method of Schilperoot (Patt, *et al.*, 1974). RNase treatment was continued for 45 min at 40°C with occasional swirling, after which the ribonucleases were removed by another deproteinization. Following this, the aqueous phase was removed to a small beaker. (The wide mouth of the beaker allowed easy removal of the precipitate later.)

Preliminary experiments had shown that DNA could be effectively precipitated using either isopropyl or ethyl alcohol. Because isopropanol should selectively precipitate DNA from a mixed nucleic acid solution (Marmur, 1961), the DNA was precipitated by the slow, dropwise addition of 0.3-

0.35 volume of isopropanol while swirling the beaker by hand. The resulting precipitate could be wound out of the solution with a glass rod, whereupon it was simultaneously sterilized and dehydrated by washing in 70% ethanol. After draining, the precipitate was suspended in a flask containing 0.5 volumes (relative to the initial volume) of 0.1 X SSC. The DNA was dissolved by gentle swirling, after which the solution was adjusted to SSC concentration by the addition of 0.1 volumes of 10 X SSC. The solution was again deproteinized. After centrifugation, the aqueous phase was again transferred to a beaker and was subjected to DNA precipitation according to Marmur (1961), including the addition of 0.1 volume acetate-EDTA prior to precipitation with 0.5-0.55 volumes isopropanol. Mixing, however, was effected as before: gentle swirling rather than mechanical agitation or shaking.

#### Characterization of Extracted DNA

In order to test the effectiveness of the purification procedure, a series of analytical experiments was performed on a representative sample. Recovery analysis is summarized in Table I. Purity of the final solution was assayed according to standard procedures (see Methods). The preparation contained 2.6% carbohydrate. Analysis of this and several other samples for RNA and for protein indicated that neither could be detected in any quantity.

TABLE I

## RECOVERY OF DNA EXTRACTED FROM Azc2

<u>Purification Step*</u>	<u>DNA</u>	<u>Recovery</u>
(2) Cell pellet (2.61 gm, wet wt.)	10.96 mg	100%
(12) After three chloroform deproteinizations	9.00 mg	82.1%
(19) After first isopropanol precipitation	7.44 mg	67.9%
(25) Final Solution	4.70 mg	42.9%

\* Numbers in parentheses refer to steps in the extraction protocol (see Methods).

### Determination of DNA Molecular Weight

As a further assessment of the DNA extraction procedure, boundary sedimentation techniques were employed (see Methods) in order to estimate the molecular weight of a DNA isolate.

Tracings of the analytical ultracentrifuge negatives made by the recording densitometer are reproduced in Figure 1. These were measured to obtain the 50% boundary sedimentation rate (Crothers and Zimm, 1965). The data were plotted to produce a series of lines whose slopes were used to derive values of observed sedimentation coefficients according to the equation of Svedberg:

$$s_{\text{obs}} = \frac{1}{\omega^2} \frac{d \ln \bar{x}}{dt}$$

where  $\omega$  is the angular velocity in radians per second of the ultracentrifuge rotor,  $\bar{x}$  is distance in cm of boundary migration from the center of rotation during  $t$ , the time in seconds, and  $s_{\text{obs}}$  is given in units of Svedbergs ( $S=10^{-13}$  seconds). Observed values were corrected to standard conditions using

$$s_{20,W} = \left[ \left( \frac{\eta_t}{\eta_{20}} \frac{\eta}{\eta_0} \right) \right] \left( \frac{1 - \bar{v} \rho_{20,W}}{1 - \bar{v} \rho} \right)$$

where  $\eta_t$  and  $\eta_{20}$  are viscosities of water at the temperature of the run and at 20°C;  $\eta/\eta_0$  is the viscosity of the buffer (SSC) relative to that of water;  $\bar{v}$  is the partial specific volume of the solute (DNA);  $\rho_{20,W}$  is the density of



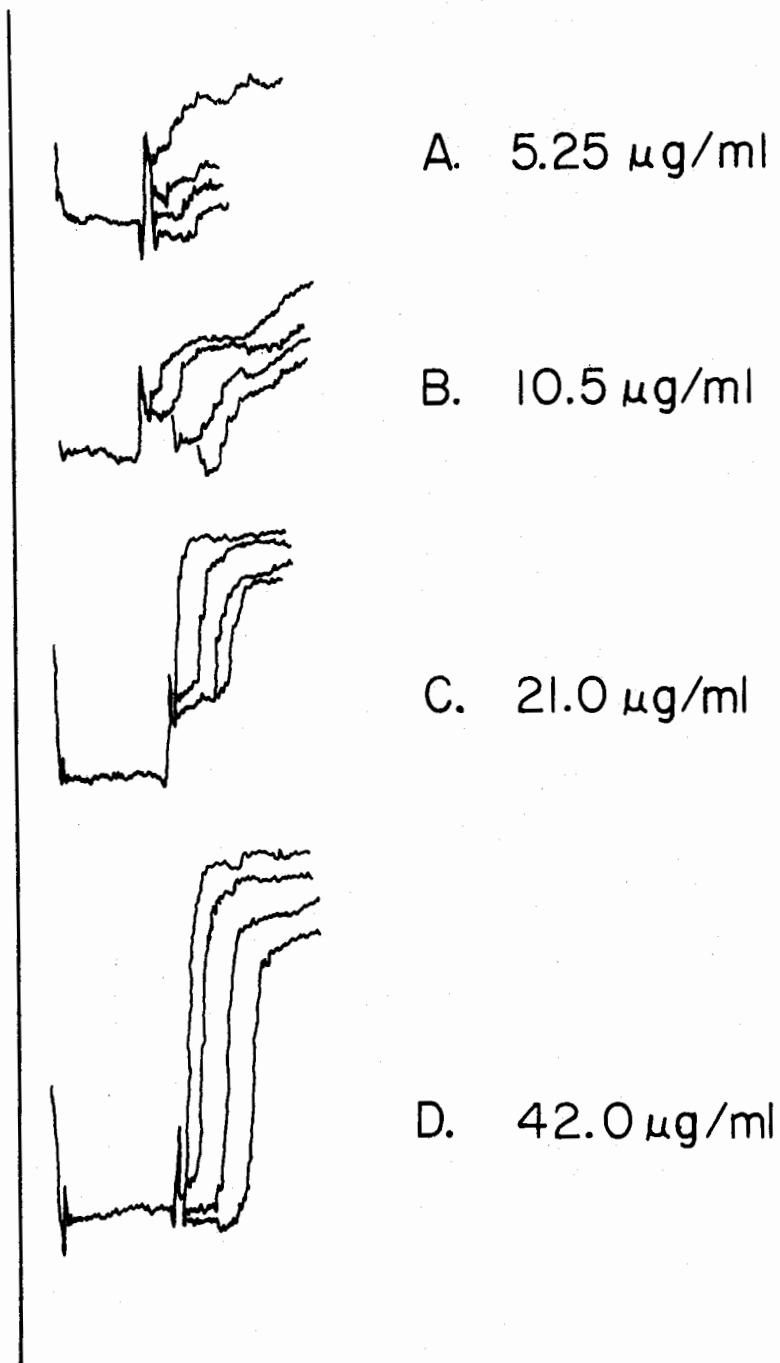


Figure 1. Sedimentation boundaries of Az17 DNA. A through D are composites of Chromoscan traces made at a ratio of 1:3. The original negatives were 1:2 reproductions due to optics employed. The line at the left indicates the reference which is 5.7 cm from the axis of rotation.

water at 20°C; and  $\rho$  the density of the solvent at running temperature (Schachman, 1957). Standard conditions are those which would be obtained if water were the solvent and runs were made at 20°C. This is not practical due to the instability of DNA in solvents which do not contain monovalent cations. After correcting for the effects of the buffer system,  $s_{20,w}$  values were corrected for the effect of DNA concentration by plotting  $1/s_{20,w}$  against DNA concentration according to Eigner, *et al.*, (1962). By extrapolating this curve to the ordinate, the inverse of  $s_{20,w}$  at zero DNA concentration was obtained. Finally, the molecular weight was obtained using this value in the expression

$$s_{20,w}^2 = 0.034 \text{ MW}^{0.405}.$$

This equation describes a curve which relates a large amount of empirical data ( $s_{20,w}$ ) to the molecular weight values which had been derived using a combination of sedimentation and diffusion data (Eigner and Doty, 1965).

As may be seen in Figure 1, the tracings made at 5.25  $\mu\text{g/ml}$  were extremely difficult to analyze. This was due to the use of a 12 mm Kel-F centerpiece which, because the light path is short, produced negatives which had insufficient contrast to detect the boundary with accuracy. As a result, it was deemed justifiable to disregard the 5.25  $\mu\text{g/ml}$  point in extrapolating  $s_{20,w}^0$ . The accepted values produce a sedimentation coefficient of 29.6 S. Applying the above equation,

a molecular weight of 18.2 megadaltons was calculated. Since the minimum molecular weight required for transforming DNA is generally accepted to be 1 megadalton (Spizizen, *et al.*, 1966), this DNA should be capable of effecting transformation.

The steepness of boundaries in composites C and D, and the sharpness of the shoulders of these traces can also be seen in Figure 1. Taken together, these two factors indicate that the preparation was homogeneous as to DNA fragment size.

#### Determination of $T_m$ and GC content

$T_m$  is defined as that temperature at which the hyperchromicity of a given sample of DNA (ie. the increase in ultraviolet absorbance due to denaturation of the double helix as a result of the thermal breaking of hydrogen bonds between the bases) reaches its half-maximal value. The guanine plus cytosine content of any class of DNA has a direct influence on the  $T_m$  value. Bacteria are thought to contain a single class of DNA. But it has been reported (Sadoff, 1975) that *A. vinelandii*, ATCC 12837, exhibits a bimodal melting profile, with 30-40% of its DNA comprising a component whose  $T_m$  is five degrees lower than the remainder. This suggests that the bacterium contains two classes of DNA, each with a different GC content. Having obtained pure, high molecular weight DNA from several strains of *Azotobacter*, in-

cluding ATCC 12837, it was of interest to substantiate this finding, if possible, and to extend it to species and strains other than *A. vinelandii*.

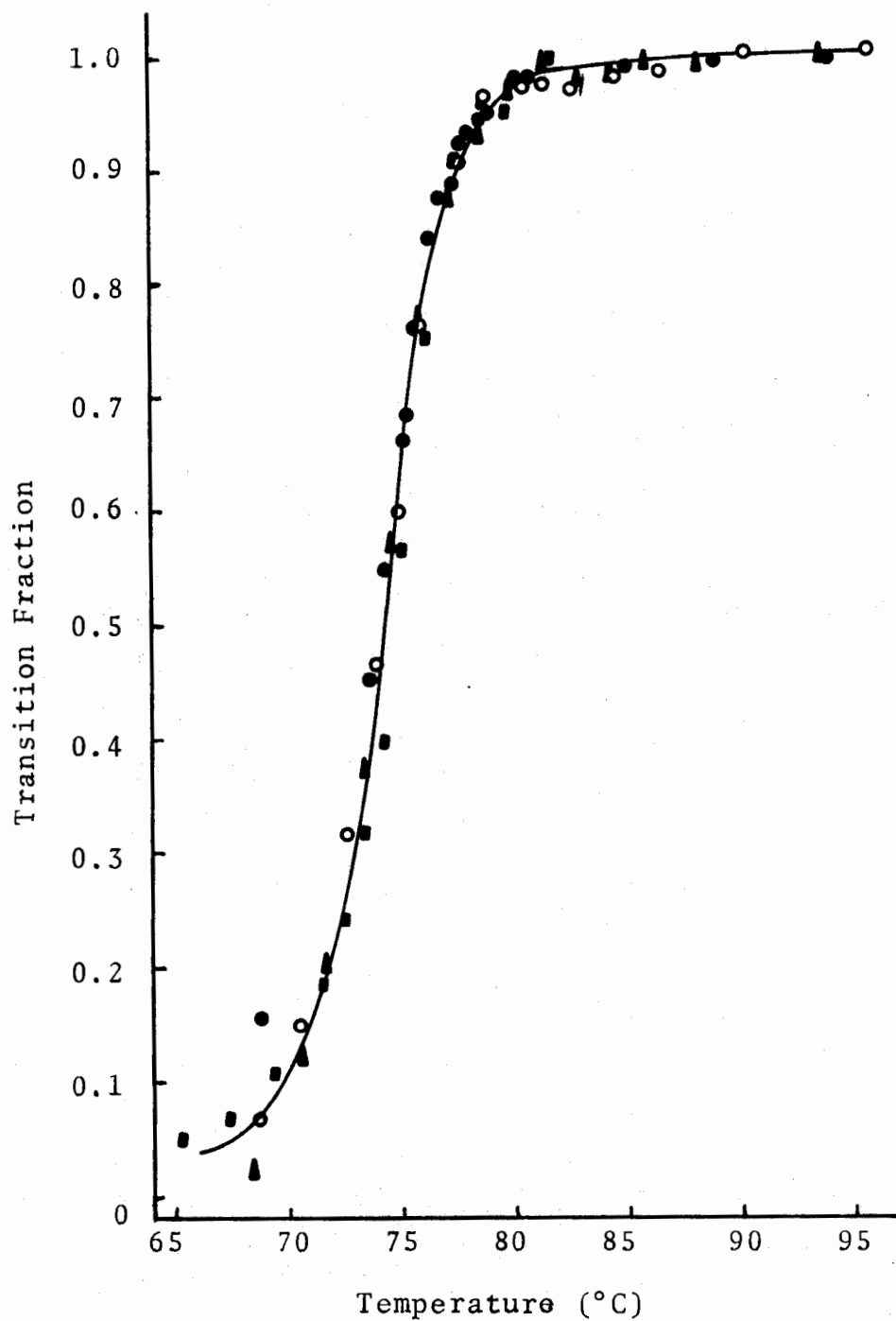
Using the apparatus described in Methods, each denaturation run included four cuvettes: one contained 0.1 X SSC as a blank, another contained *E. coli* DNA as a standard, and two contained samples of *Azotobacter* or "Az" DNA. As the internal cuvette temperature equilibrated with each increase, the OD at 260 nm was measured. These values were then corrected for the expansion of water in the buffer, relative to 25°C. The ratio of the absorbance of each DNA sample, relative to its absorbance at 25°C, was then calculated. Normalized values of the ratios were then calculated as

$$\text{Transition Fraction} = \frac{\text{Observed increase in OD}}{\text{Total increase in OD}}$$

according to Mahler and Cordes (1971), and the values plotted against temperature as shown in Figures 2 through 6. The  $T_m$  values obtained from these curves are tabulated in Table II.

Dilute saline-citrate was selected as the buffer because  $T_m$  values in it are lower than in SSC (thus lessening problems of evaporation and heating). The GC content was therefore estimated using the equation

$$\text{percent GC} = (T_m - 53.9) 2.44$$



**Figure 2.** Thermal denaturation profile of *E. coli* DNA. (o, experiment 1; ●, experiment 2; ▲, experiment 3; ■, experiment 4)

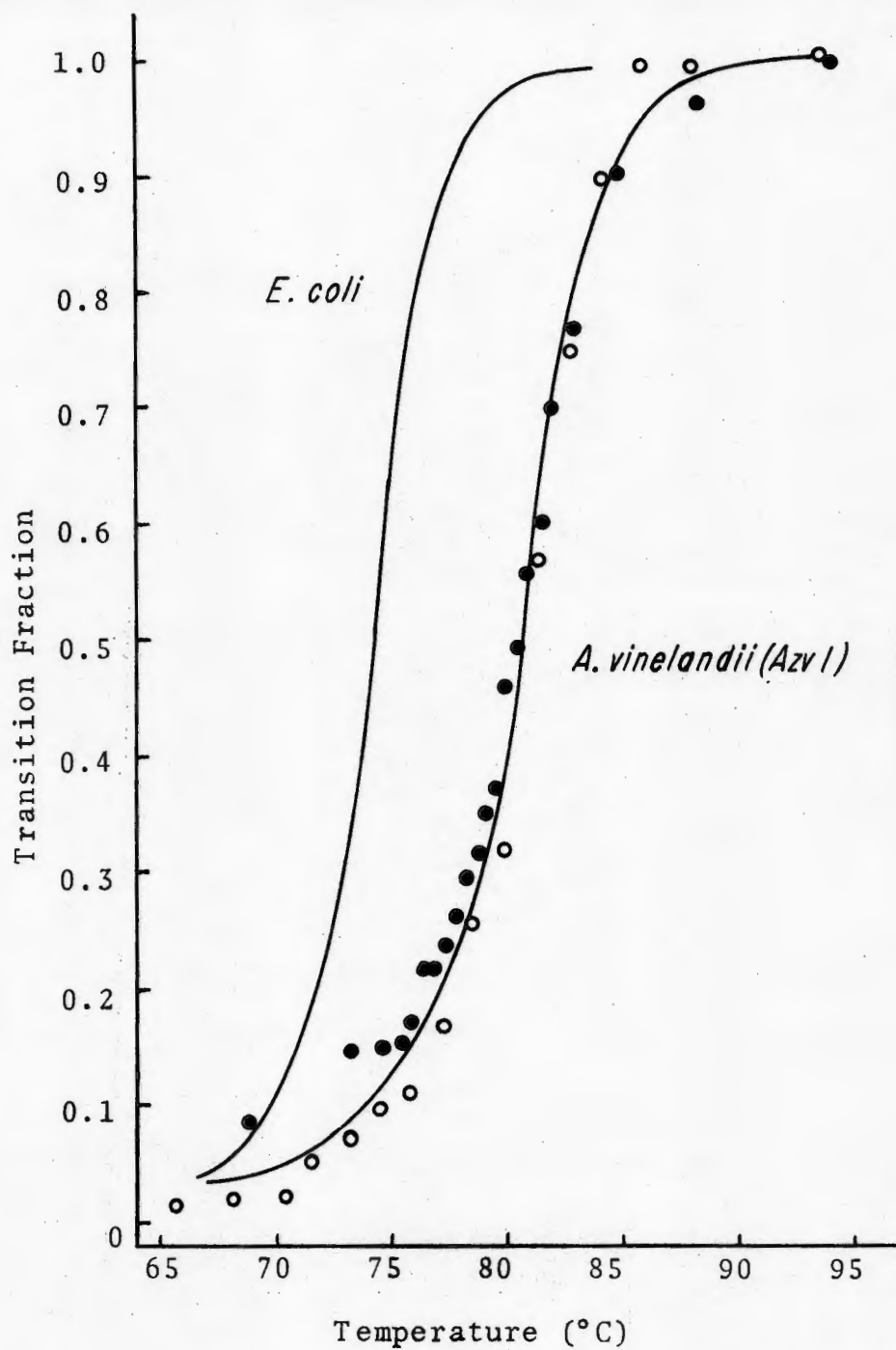


Figure 3. Comparison of thermal denaturation profiles Azv1 and *E. coli*. (o, experiment 1; ●, experiment 2)

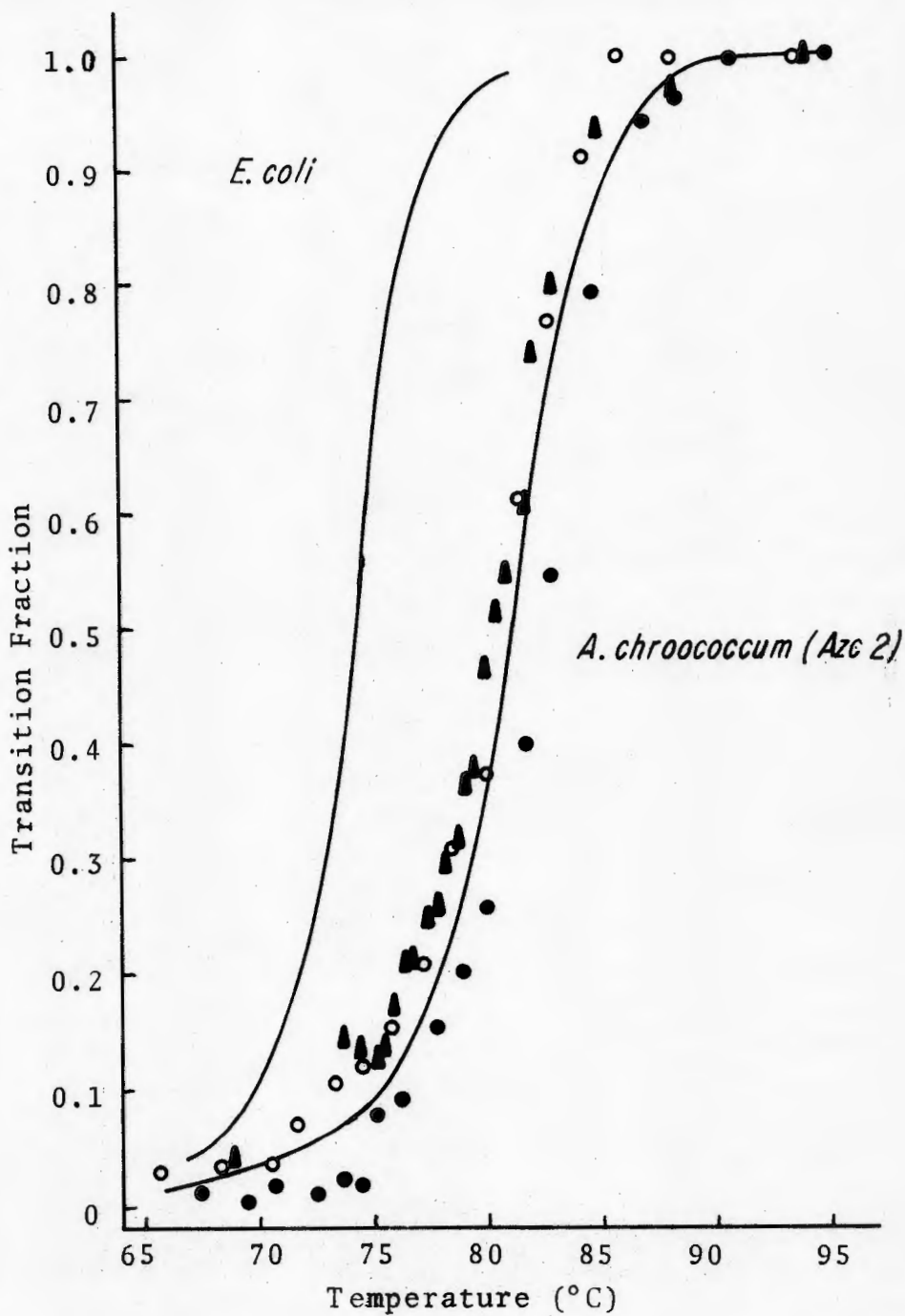


Figure 4. Comparison of thermal denaturation profile for Azc2 and *E. coli*. (o, experiment 1; •, experiment 2; ▲, experiment 3)

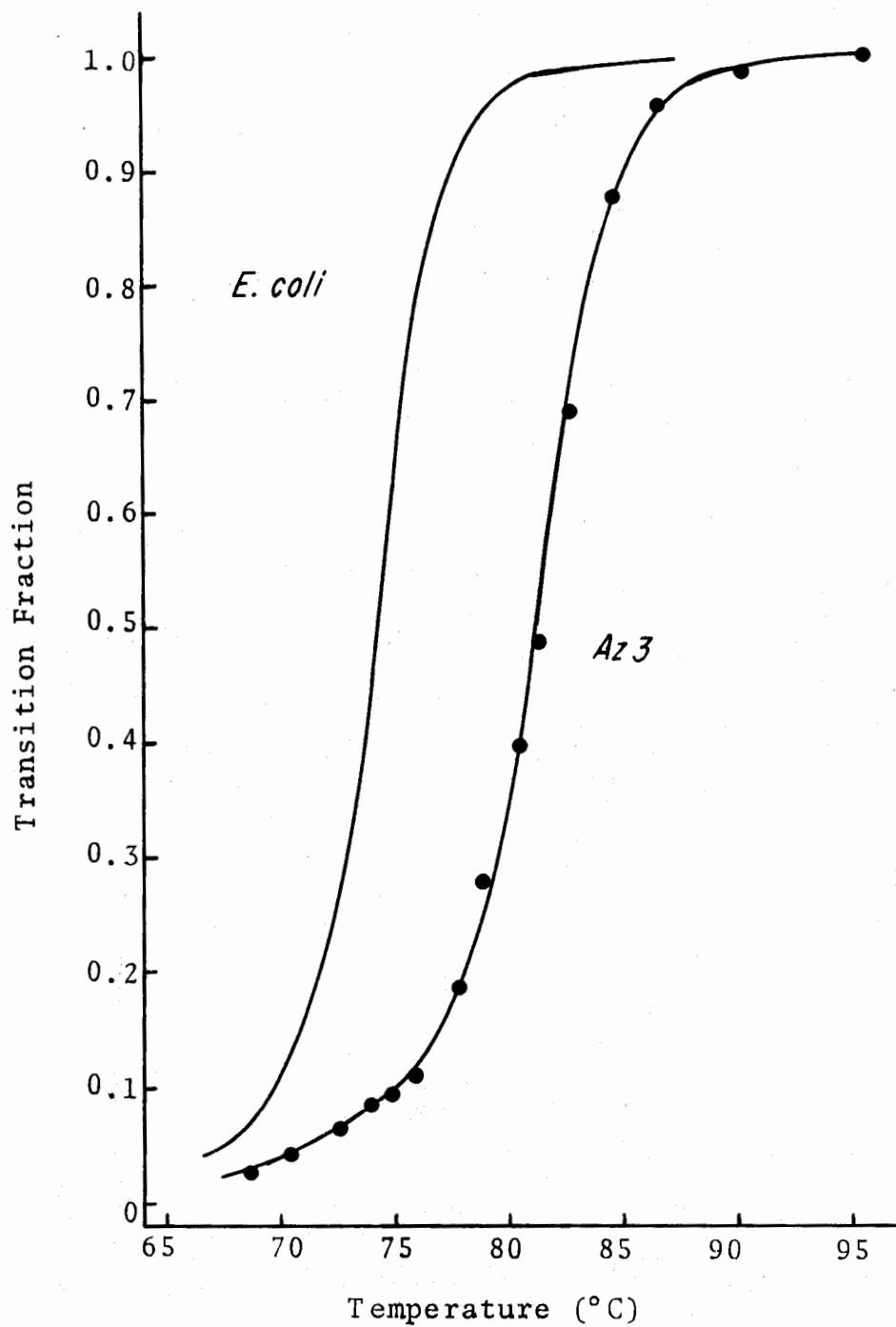


Figure 5. Comparison of thermal denaturation profiles of Az3 and *E. coli*.



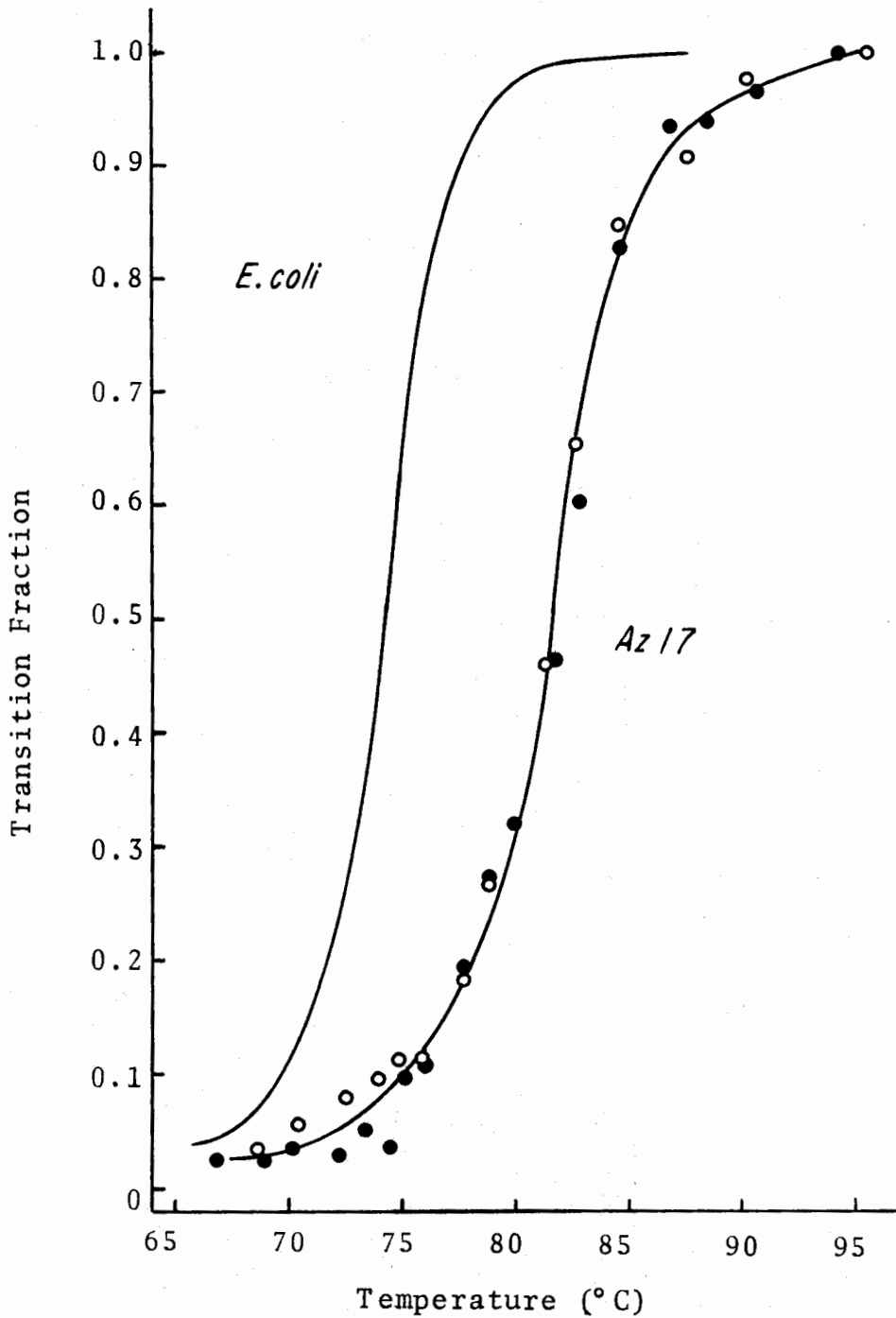


Figure 6. Comparison of thermal denaturation profiles of Az17 and *E. coli*. (o, experiment 1; ●, experiment 2)

TABLE II

$T_m$  AND PERCENT GC VALUES OF DNA ISOLATED  
FROM *Azotobacter* STRAINS\*

<u>Strain</u>	<u><math>T_m</math></u>	<u>%GC</u>
<i>E. coli</i> (standard)	74.4 $\pm$ 0.3	50.0 $\pm$ 1.0
<i>A. vinelandii</i> (Azv1)	80.6 $\pm$ 0.3	65.1 $\pm$ 0.8
<i>A. chroococcum</i> (Azc2)	81.2 $\pm$ 1.0	66.6 $\pm$ 2.5
Az3	81.1	66.4
Az17	81.7 $\pm$ 0.1	67.8 $\pm$ 0.3

\* Ranges listed are the results of multiple runs.

according to Mandel and Marmur (1968). Results are listed in Table II. Since the values for *E. coli* DNA coincide with published values, the values obtained for "Az" DNA can be used without correction.

Attention should be drawn to the melting profiles, for they provide valuable information. First, the slopes of the mid-portions are steep and the shoulders are relatively sharp, indicating the homogeneity and high molecular weight of the samples. Second, the curves provide no evidence for bimodal melting profiles in any of the *Azotobacter* strains tested, including ATCC 12837 (Azv1). It is not possible, therefore, to substantiate the findings of Sadoff mentioned earlier.

### Transformation

Marker selection. Donor DNA for all transformation experiments was extracted from rifamycin-resistant mutants. Mutants resistant to naladixic acid, streptomycin, or ampicillin were available for some strains, but these markers proved to be unsuitable because of their leakiness or variable character. Strains resistant to 100 µg/ml rifamycin were stable.

Procedure. Liquid transformation procedures were attempted without success, indicating that competence cannot be easily specified for *Azotobacter*. This finding substantiates those of Page and Sadoff (1976b) with *A. vine-*

*landii*. They concluded that optimal transformation can be accomplished by incubating recipient cells through a period of growth in the presence of transforming DNA prior to selection. In order to quantify the results, however, it was decided to plate the mixture in soft agar so that each immobilized cell which was transformed would give rise to a single colony after selection (see Methods).

Initial transformation frequencies using this technique ranged from  $1 \times 10^{-7}$  to  $1 \times 10^{-6}$ . Figure 7 presents data collected in attempts to enhance transformation frequencies with *A. vinelandii*. Figure 7A shows the effect of varying the amount of transforming DNA while the number of recipient cells was held between 1 and  $2 \times 10^7$  per plate and the time of rifamycin addition was 21 hr. Pretreatment of the DNA with deoxyribonuclease resulted in no transformation. The time of rifamycin addition was varied in Figure 7B, while 200  $\mu$ g of DNA was mixed with  $1 \times 10^7$  cells per plate. The optimum time for addition was found to be at least 18 hr. Finally, Figure 7C indicates that the minimum number of recipients required to obtain maximum rate of transformation was  $1-2 \times 10^7$  per plate with 200  $\mu$ g DNA, when rifamycin was added at 18 hr. After optimal conditions were established, transformation frequencies of  $1 \times 10^{-4}$  were obtained.

Detection of Transformation. Table III is a compilation of qualitative transformation data. Each entry is the

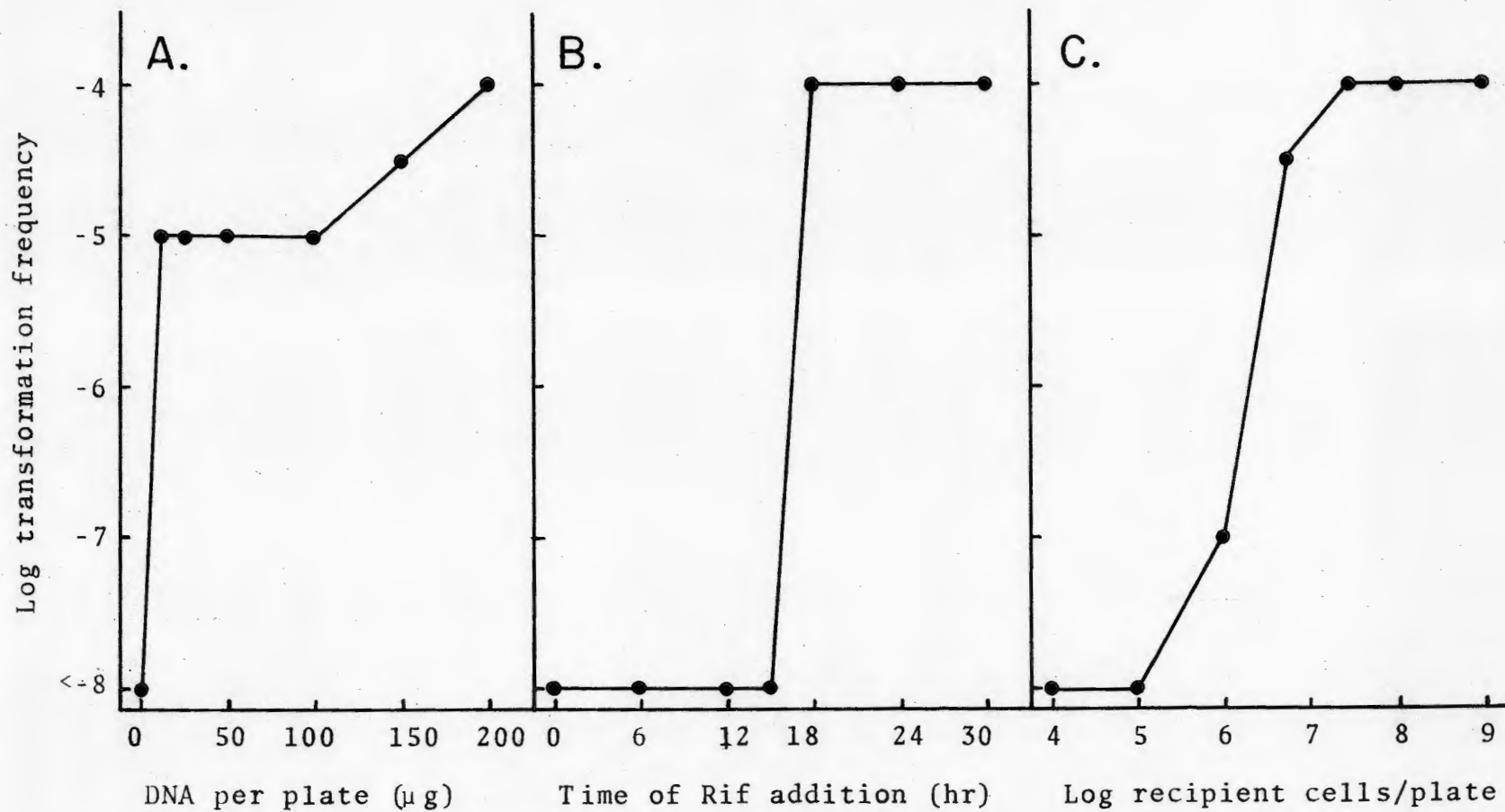


Figure 7. Factors influencing transformation frequency in *A. vinelandii*. For explanation of conditions, see text. Frequencies reported as  $<10^{-8}$  indicate that transformation could not be detected in excess of the mutation frequency.

TABLE III

DETECTION OF TRANSFORMATION AMONG *Azotobacter* STRAINS\*

Receipient (Rif <sup>S</sup> )	Source of Donor DNA					
	Azv1 Rif <sup>r</sup>	Azc2 Rif <sup>r</sup>	Az3 Rif <sup>r</sup>	Az6 Rif <sup>r</sup>	Az8 Rif <sup>r</sup>	Az17 Rif <sup>r</sup>
<i>A. vinelandii</i> (Azv1)	+	-	-	+	+	-
<i>A. chroococcum</i> (Azc2)	-	-	-	NT	NT	-
Az3	-	-	(+)	NT	NT	-
Az6	+	NT	NT	+	+	NT
Az8	-	NT	NT	-	-	NT
Az17	-	-	-	NT	NT	-

\*

+ = Transformation detected by the ability of transformed cells to grow in the presence of rifamycin (50  $\mu$ g/ml)

- = Transformation not detected

NT = Not tested

result of at least two separate experiments which were in agreement. Among those strains within which transformation was detected, the quantitation of frequencies was attempted using the new method. Highest frequencies of  $1 \times 10^{-4}$  were obtained with Azv1 as the recipient using Rif<sup>r</sup> DNA from either Azv1, Az6, or Az8. These two strains, Az6 and Az8, have been tentatively identified as *A. vinelandii*, except that Az8 does not utilize rhamnose as a carbon source and does not produce the characteristic fluorescent pigment of the species after prolonged cultivation in the laboratory. When used as recipients, Az6 behaved as did Azv1, being transformed at a frequency of  $5 \times 10^{-5}$  by Rif<sup>r</sup> DNA from Az6, Az8, or Azv1. While its DNA was capable of effecting transformation in Azv1 and Az6, Az8 was not transformable.

*A. chroococcum*, Azc2, was not transformable, nor could its DNA transform other strains.

Az3 and Az17 seemed identical to each other in characterization tests, but different from *A. vinelandii* and *A. chroococcum*. Az17 was not found active as donor or recipient in transformation experiments. Az3, however, was found to be transformable in intrastrain experiments. These results are subject to interpretation: The "frequency" determined by the Page-Sadoff method (1976a) was low ( $1.6 \times 10^{-7}$ ), and no transformation was detected using the quantitative method. This points out another problem of the standard plate transformation method. It may be that the colo-

nies detected with Az3 arose from progeny of a very few spontaneous mutations. Az3 was not found active in inter-strain transformation using either method.



## DISCUSSION

The fact that transformation of *Azotobacter* using highly purified DNA has not been reported previously suggests that normal extraction methods induce excessive shearing of the DNA, or fail to sufficiently inhibit nuclease activity. Making this assumption, the method for extraction and purification of DNA described in this thesis should be useful in further studies of *Azotobacter* genetics.

Several lines of research might be followed to further develop a genetic system for *Azotobacter*. Of paramount importance is research in mutagenesis. Reports of attempts to isolate mutants in the genus have pointed out that its members are very resistant to mutagenesis using standard techniques. This may be due to one of several reasons. The most obvious reason is that *Azotobacter* may lack an error-prone repair system. If this is the case, lesions in DNA would be repaired without errors. Auxotrophs are especially difficult to isolate, indicating that the necessary growth factor transport systems may not be present. It has been suggested (Sadoff, 1975) that the genome of *A. vinelandii* may contain several repeated segments. If this is the case, or if the life cycle of *Azotobacter* has no stage at which the genome of the organisms is present as a single copy, only dominant mutations would be expressed while recessive

mutations in the multiple regions may not segregate.

It should be noted that while the transformation method developed in this study is a definite improvement over previous techniques, it may not provide absolute transformation frequencies. The uncertainty arises because it cannot be determined at which point during the necessary preselection incubation of DNA with growing cells the transformation actually takes place. The question of competence requires further study. Results may provide improved plate transformation or better, may allow liquid transformation methods to be developed.

The inability to transform strains other than *A. vine-landii* at high frequency may be explained in several ways: (1) The possibility exists, especially in light of the difficulties of DNA isolation, that most strains produce an efficient nuclease or restriction enzyme for which *A. vine-landii* is naturally deficient. When used as recipients, these strains may not be transformed because their nucleases attack the transforming DNA. (2) It may be that *A. vine-landii* is the only strain whose cell wall allows efficient uptake of DNA. (3) Non-competent strains may lack the enzymes required to recombine transforming DNA into the recipient genome. The answer to this problem might be provided by a study of the fate of transforming DNA as has been performed in *Pneumococcus* (Lacks, 1962), but this would require the isolation of a suitable auxotrophic mutant so that DNA

could be efficiently labelled with radioactivity. All attempts to isolate a thymineless mutant have failed.

Once made available, a genetic system in *Azotobacter* could be used in studies mentioned earlier, as well as others such as identification of a conserved genetic core among *Azotobacter* species (see Dubnau, *et al.*, 1965). It would also be interesting to investigate the use of the system in a taxonomic study of the genus (Jones and Sneath, 1970).

The availability of purified DNA might hasten molecular studies of *Azotobacter*, especially the still uncharacterized strains in our laboratory. The use of DNA hybridization may provide resolution not obtained in GC analysis in order to distinguish between them. Experiments on renaturation kinetics would also be useful in this work.

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