PLASMIDS OF

AZOTOBACTER VINELANDII

THESIS

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Nineteen laboratory strains of Azotobacter vinelandii and two organisms of the same species isolated from water samples were screened for the presence of plasmid deoxyribonucleic acid (DNA).

Three laboratory strains and both organisms isolated from water samples contained one plasmid each. The migration distances of the plasmids in agarose gel electrophoresis were different indicating that the strains harbored plasmids of different molecular weights. The plasmids were cured by SDS or ethidium bromide treatment of the cultures.

Attempts were made to find a plasmid-correlated trait in organisms isolated from water samples. Wild-type and cured strains were compared by various criteria but no differences between them were observed.

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INTRODUCTION

The purpose of this work was to investigate the presence of plasmids in different strains of Azotobacter vinelandii, a free-living nitrogen fixing bacterium of the family Azotobacteraceae. These organisms are found in nature living in the water and in the soil. The investigation was considered important because of the role plasmids play in determining the genetic and physiological characteristics of many bacteria and because so little is known of the plasmids of A. vinelandii. A recent report states that A. vinelandii have no plasmids (37) while another says that they do (43). Part of the value of this research will lie in the fact that this confusion will be resolved.

A simple method of cell lysis and plasmid extraction was used in the screening of nineteen laboratory strains and two organisms of the same species isolated from water. Molecular weights of the plasmids were determined by electrophoresis migration in comparison to plasmids of known molecular weights.

Plasmids

Plasmids are defined as molecules of extrachromosomal deoxyribonucleic acid (DNA) capable of carrying hereditary determinants which are not essential for the host cells but which may be expressed changing, thereby, the phenotype of the organism.

Initially, proof of the occurrence of plasmids depended

on the loss of drug resistance at rates higher than normal mutation frequencies, and on the transfer of other easily recognized traits. The elimination of certain heritable characteristics by incubation in the presence of acridine orange (19), later shown to interfere with plasmid replication, was very useful for this purpose and still serves as a first indication of the presence of extrachromosomal DNA. In 1952, Lederberg and co-workers (23) showed that the sex factor, F, could be transfered among individuals in bacterial populations and seemed not to be part of the chromosomal DNA of E. coli. These studies made Lederberg believe in the existence of "plasmagenes" in those strains for which he suggested the denomination of plasmids.

It was not until 1961 that plasmid DNA was isolated for the first time by Marmur et al. (41). They detected the presence of an extra-chromosomal band of DNA in a sample of a crude lysate of Serratia marcescens which later proved to be the plasmid F of Escherichia coli.

In 1963, Watanabe (31) reviewed the available information on the existance of multiple antibiotic resistances in Shigella which could be transferred as a group to other bacteria. The presence of this multiple antibiotic resistance had been associated with genetic factors named R (resistance)-factors. They were compared to the sex factor F (fertility) of E. coli by Mitsuhashi et al.

(ref.).

In 1967, Roth and Helinski (38) developed a method for releasing the plasmids from host cells while avoiding steps that could cause the breakage of long DNA molecules. They showed by electron microscopy that plasmids were circular molecules of double stranded DNA.

The possibility of introducing purified plasmid DNA into cells by transformation, which is the process of uptake of DNA from the medium, made more rigorous the demonstration that a given plasmid was responsible for a determined genetic trait, and was first reported by Cohen et al. (11). This finding became an important part of the technology of genetic manipulation as a result of the work of Humphreys et al. (20) who introduced plasmids, mutagenized in vitro, into host cells.

Examples of independently isolated plasmids showing identical or very similar properties suggests that some plasmids may have had a common origin and diverged with time by means of genetic modifications (mutations) of their DNA sequences (40). It is assumed that by this means they can become more and more adapted to a specific host (18). On the other hand, many plasmids are able to exist in a wide range of different host cells (8, 26, 40).

From the point of view of evolution, the plasmid and the bacterium are symbionts (5, 34). Conjugative plasmids, for example, can be transferred from one cell to another of the

same species or to another of a different species. They are also able to insert themselves into the chromosome of their host. During excision from the host cell (the donor), they may carry a piece of the chromosomal DNA with them and transfer this material into another cell (the recipient). When inside the recipient, the chromosomal DNA from the donor may "recombine", or exchange nucleotide sequences, with the chromosome of the recipient cell. During recombination, genes coding for selective traits such as antibiotic resistance or resistance to heavy metals, may be transferred to the recipient. If the recipient is a cell that already carried different selective genes, it is probable that this new recombinant strain will compete successfully with other strains of the same species and with other microorganisms, should that microbial population be submitted to selective conditions of high concentrations of antibiotics and/or heavy metals.

Some bacteria are able to release into the medium substances known as bacteriocins. These are substances that kill other bacteria but usually do not affect cells of the producer strain. Bacteriocins produced by different species of both Gram-negative and Gram-positive organisms have been described (16, 35). The best studied class of bacteriocins, are the colicins. The colicins are produced by certain strains of E. coli. All the colicins are proteins coded for by plasmid genes (16). The same is true for all other

bacteriocins so far isolated from other organisms (35).

One of the most striking features about plasmids is that they have the ability to confer to the cells that harbor them the capacity to degrade complex organic compounds (42, 13). The smaller molecules resulting from degradation can enter metabolic pathways catalysed by constituitive cell enzymes. Cells harboring these plasmids are able to grow on substrates such as camphor, octane, xylene, toluene and other similar compounds. These plasmids, referred to as degradative plasmids, are commonly found in strains of Pseudomonas isolated from the soil (42). They may carry a whole array of functionally related genes. An example of the later is the plasmid TOL (toluene), which is responsible for the production of enzymes that break down benzoate and toluene into catechol. The selective advantage provided by such metabolic versatility is evident and probably contributes to the wide distribution of the genus Pseudomonas in nature.

Degradative plasmids belong in the larger group of the metabolic plasmids. These confer the ability to utilize substrates not specified by the cell's chromosome. This is the case in some strains of Salmonella that harbor plasmids that confer the Lac phenotype, that is, the ability to utilize lactose as a source of energy (14). Without plasmids, Salmonella lacks the ability to hydrolyze lactose. Some of the bacteria of the genera Proteus and Serratia, as

well as Streptococcus lactis have also been found to contain Lac plasmids.

Plasmids also play an important role in the biology of soil microorganisms. Bacteria of the genus Rhizobium fix molecular nitrogen in symbiotic association with root cells of specific leguminous plants. Genetic and physical evidence indicates that the majority of the symbiotic genes, including the genes for the nitrogenase enzyme system, are carried by plasmids (33). The plant utilizes the nitrogen reduced by the bacteria and in turn provides the bacterial cells with nutrients and leghemoglobin, an oxygen-binding protein that carries oxygen to the bacteria.

It has been proved that the capacity to induce crown gall tumors in dicotyledonous plants by strains of Agrobacterium tumefaciens, another organism in the family Rhizobiaceae, is controlled by genes found in plasmids referred to as Ti (tumor inducing) plasmids (10). Tumor inducing plasmids are transferred from the bacteria to plant cells, inducing their growth and altering their metabolism. They can also be transferred from one strain of A. tumefaciens to another by conjugation. The molecular weight of Ti plasmids so far isolated varies from 90 to 160 megadaltons.

The Organism

Using a different approach to Winogradsky's principle of enrichment culture developed in 1893, Beijerinck (6)

isolated for the first time, from garden soil and from water, two species of bacteria which he named Azotobacter chroococcum and A. agilis. The ability of these organisms to fix atmospheric nitrogen was clearly demonstrated eight years later (24).

The description of a number of other species followed Beijerinck's findings, and the original description of the species Azotobacter vinelandii was made by Lipman, in 1903 (25). In 1933, Pribram (1) grouped the free-living Gram-negative obligate aerobes capable of fixing atmospheric nitrogen in one family, the Azotobacteraceae. According to the most recent edition of the Bergey's Manual of Determination Bacteriology (2), this family consists of two well accepted genera: Azomonas and Azotobacter.

The genus Azotobacter is described as being made up of large pleomorphic Gram-negative cells which do not form spores but are able to form unique resistant forms refered to as cysts.

Studies concerning the biochemistry of nitrogen fixation by azotobacters have accounted for a good share of the literature on these organisms (4, 7, 32). Only very recently have some workers turned their attention to the search for plasmids in members of this family. In 1984, Robson et al (37) did a survey on the presence of plasmids in different laboratory strains and ten soil isolates of A. chroococcum. They found plasmids in six laboratory strains

and in five soil isolates. Most of the strains had more than one plasmid. One of them harbored six different plasmids with molecular weights ranging from 5.5 to 220 megadaltons. It is known that the average size of a gene is approximately 0.66 megadaltons. Thus, the genetic potential represented by the plasmids found by those authors in A. chroococcum is evident.

Robson and co-workers (37) cured one of the azotobacter strains of plasmids and compared the cured strain with the wild type strain. No differences could be observed between the two strains in their abilities to fix nitrogen and to utilize different compounds as carbon sources. The cured strain also showed the same level of resistance to different antibiotics, heavy metals and ultra-violet radiation as that of the wild-type.

So far, it appears that only one report on plasmids of A. vinelandii has been made. Yano and co-workers (43) recently described the presence of three indigenous plasmids in strain AV15. They also showed that the larger plasmid carried genes involved in benzoate utilization and that the base sequence in the DNA of this plasmid had homology with the base sequence of the Klebsiella pneumoniae genes that code for nitrogenase. Since nitrogenase, together with nitrogenase reductase, are the enzymes responsible for nitrogen fixation, that is, the reduction of gaseous nitrogen to ammonia, the confirmation of these findings

could represent an important step towards a better understanding of the genetics of nitrogen fixation in this species.

Perhaps the most important question that has arisen from the report of Yano et al. (43) is whether or not other strains of A. vinelandii also harbor plasmids. Could other plasmids carry different genetic information? Would such plasmids be found in soil or water isolates of this species? Could they provide important selective traits to their hosts?

The questions addressed by my investigation are: does A. vinelandii have plasmids as Yano et al. (43) say, or do they not as Robson et al. (37) claim; and do different strains of A. vinelandii have different plasmids? The objective of this work, therefore, was to answer these questions by doing a survey on strains received from other laboratories and with organisms isolated from water.

MATERIALS AND METHODS

Cultures, Growth Conditions and Media

In this work, 23 cultures of nineteen different strains of Azotobacter vinelandii were received from seventeen laboratories (Table I). The cultures were kept on agar slants of Burks modified medium (3). The non-nitrogen fixing mutant strain, UW-1 was grown on Burks medium supplemented with 8 g of ammonium acetate per liter of medium. The cultures were tested for purity and viability by streaking

TABLE I

STRAINS OF AZOTOBACTER VINELANDII
AND THEIR SOURCES

Strain	Source
BAHAA	B.T. Shawky, Nat. Res. Center, Cairo, Egypt
CA	P.E. Bishop, North Carolina St. Univ., NC
OP	E.M. Barnes, Baylor Coll. Med., Houston, TX
OP	S. Mulrooney, Univ. Calif., at Irvine, Irvine, CA
OP	D. Kleiner, Universitaet Bayreuth, Fed. Rep. Germany
OP	J. Meyer, Centre d'Etudes Nucl., Grenoble, France
PPD 15	B.E. Ferzaghi, Private Bag, New Zealand
SM52B	A.J. Anderson Univ. Hull, Hull England
UW	V. Shah, Univ. Wisc., Madison, WI
UW	H.K. Das, Jawaharlal Nehru Univ., New Delhi, India
478	H. Haaker, Agric. Univ. Dreijen, Nageningen, Netherlands

TABLE I---(Continued)

Strain	Source
9104	J.R. Sokatch, Univ. Oklahoma, Oklahoma City, OK
12837	G.R. Vela, North Texas St. Univ., Denton, TX
12837	W.D. Braymer, Louisiana St. Univ., Baton Rouge, LA
12837	L.E. Casida, Penn. St. Univ., University Park, PA
12837, UW	W.J. Page, Univ. Alberta, Edmonton, Canada
12837, UW-1	H.J. Sadoff, Mich. St. Univ., East Lansing, MI
12518, 9046, A, UW	P. Jurtshuk, Univ. Houston, Houston, TX
OP, 2489, PcT, PCM289, S-4, S-5	J. Moreno, Univ. Granada, Spain

out on nutrient agar plates and Burks agar plates immediately upon arrival.

Two strains of A. vinelandii isolated from water samples brought from Mexico were also used. They were also kept on slants of Burks agar medium.

Seven strains of Escherichia coli, kindly provided by Professor R. Zsigrey, University of New Hampshire, Durham, NH, were used as sources of plasmid deoxyribonucleic acid (DNA) of known molecular weight (Table II). They were kept on agar slants of tryptone salt yeast extract (TSY) agar. Antibiotics and other chemicals (Sigma Chemical Co., St. Louis, MO) were added, when appropriate, at the following concentrations: ampicillin, 15 mg/ml; carbenicillin, 15 mg/ml; streptomycin, 20 mg/ml; sulfonamide-trimethoprim, 250 mg/ml; and tetracycline, 15 mg/ml.

The other chemicals were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, MO).

For the isolation of A. vinelandii from water samples, sodium benzoate was added to Burks agar medium in order to inhibit the growth of other azotobacters (12). This medium was composed of Burks agar medium supplemented with 10 g of sodium benzoate per liter. The pH of the medium was adjusted to 7.0 before sterilizing.

Azotobacter cells used for lysis were grown on liquid Burks medium enriched with 10 g of tryptone and 5 g of yeast extract per liter. Cells of E. coli were grown on

TABLE II

ESCHERICHIA COLI PLASMIDS USED AS REFERENCE FOR MOLECULAR WEIGHT DETERMINATIONS

Plasmid	Host	Molecular Weight (Mdal)	Resistance*
R40a	1**	96	Ap, Km, Str
R1	1**	62	Ap, Km, Str, Cm, Su
RP4	1**	34	Ap, Km, Tc
RSF1030	2***	5.5	Ap

*Symbols used are: Ap, ampicillin; Km, kanamycin; Str, streptomycin; Cm, chloramphenicol; Su, sulfonamide; Tc, tetracycline.

**1, RC709= J-53F : metF63, pro-22(λ)

***2, W1485-1=K12, F^-, thy^-, nal

antibiotic-free TSY medium.

All strains of A. vinelandii were grown at 30°C while the E. coli were incubated at 37°C. Flasks containing the liquid cultures were incubated on a rotary shaker.

Isolation of Azotobacter vinelandii from Water

Thirty five samples of water brought from the state of Chihuahua in Mexico were used in this study. A volume of 15 ml of each sample was centrifuged at 10,000 x g for 15 min., using a Sorval centrifuge with an SS-35 rotor. The supernatant was discarded, and a sterile cotton swab was used to spread-inoculate the pellet on a plate of Burks modified agar medium. These plates were incubated at 37°C, a temperature that promotes selective growth of A. vinelandii. After incubation for 48 hours, the colonies that appeared on the plates were observed macroscopically. Those colonies morphologically similar to A. vinelandii were transferred to test tubes containing 1 ml of sterile water and the cells suspended. An inoculating loop was used to transfer the cells. The suspensions were streak-inoculated onto plates of Burks agar and Burks agar with 10 g of sodium benzoate per liter of medium. Cultural characteristics, cell morphology under phase-contrast microscopy and production of a diffusible greenish-yellow fluorescent pigment were used as initial evidence of A. vinelandii. The specific identification of the isolates was facilitated by observing:

i) the capacity to grow on nitrogen-free medium with sodium benzoate and; ii) the ability to form cysts when grown on Burks medium with butanol (4 g/l) in place of glucose of sucrose (2).

Cell Growth and Lysis

Azotobacter vinelandii and E. coli cultures were grown in liquid media until optical densities of 1.0 and 0.8, respectively, were reached. Growth of the cultures was measured at 600 nm using a Bausch and Lomb Spectronic 20 spectrophotometer. The cells were lysed as described by Kado and Liu (22). Accordingly, 3 ml of the culture were centrifuged at 6,000 x g for 10 min and the supernatant was discarded. The pellet was thoroughly resuspended in 1 ml of E-buffer. This buffer was composed of a 40 mM solution of Tris (THAM-tris hydroxymethyl aminomethane; Fisher Scientific Co., Fairlawn, NJ), and was 1.5 mM in relation to EDTA (ethylene diamine tetracetic acid; Fischer Scientific). The pH was adjusted to 7.9 with glacial acetic acid. A pH meter model 5993-10 (Horizon Ecology Co., Chicago, IL, U.S.A.) was used for this purpose.

Two ml of lysing buffer were then added. The lysing buffer was composed of an aqueous solution of 1.5% (v/v) sodium dodecyl sulfate (Fischer Scientific) in 40 mM of Tris buffer. The pH was adjusted to 12.6 with a 2 N solution of NaOH. A 1 N solution of NaOH (pH 12.88) was used as a pH standard. After gentle mixing, the suspension was incubated

in a water bath at 65°C for 60 min.

After incubation, 6 ml of a phenol-chloroform (1:1 v/v) solution were carefully added. After inverting the tube once for mixing, the suspension was centrifuged at 7,500 x g for 15 min. The aqueous phase containing the water-soluble molecules such as DNA and RNA was collected with a Drummnond plastic pipette (catalog #222, SAMCO, San Fernando, CA). The interphasic material, rich in chromosomal DNA was avoided. All samples were transferred to 5 ml test tubes and stored at 4°C until ready for use.

All glassware that would or might be in contact with the extracted DNA was sterilized in the autoclave before use in order to eliminate nuclease activity. Plastic gloves were routinely used in order to avoid contamination of the samples with skin nucleases.

Gel Electrophoresis

Horizontal agarose (Marine Colloids, FMC Corporation, Rockland, ME, U.S.A.) gel slabs were used to resolve plasmid bands. This technique was also used to determine the molecular weights of the plasmids (30). Gel slabs with a concentration of 0.7% agarose were used in the screening of the samples. A lower concentration of agarose (0.4%) was employed in the preparation of slabs used for molecular weight determinations.

The electrophoresis apparatus employed was purchased from LKB (model 2117 Multiphor, LKB, Bromma, Sweden). A

water-cooling system (Wilkens-Anderson Co., Chicago, IL) was used to avoid over-heating the gel slabs. A current of 40 mA and 300 V was supplied by power source model 3371 E power supply (LKB, Brommma, Sweden). For molecular weight determinations, 18 mA and 120 V were used, since it has been shown that such low currents provide good resolution of large DNA molecules (5). The previously described E-buffer was used to the dissolve the agarose. It also served as the electrophoresis buffer. Paper wicks (Whatman #3, Whatman Limited, England) bridged the buffer in the tanks and the edges of the gel. Two sets of slots were made in the gel with an eight-tooth comb (Marine Colloids, FMC Corporation) and with a six-tooth comb made in our laboratory. The wells were loaded with 30 µl of the cell extracts mixed with 20 µl of tracking dye. The tracking dye was composed of an aqueous solution with 20 mg of sodium dodecyl sulfate per ml, and was 7 M in relation to bromothymol blue. The samples were transferred into the slots with capillary pipets (Kimble, Division of Owens-Illinois, Toledo, OH, U.S.A.). During electrophoresis, the distance migrated by the tracking dye served as an indication of the distance migrated by the samples. The electrophoresis was allowed to proceed until the tracking dye had migrated 9 cm along the gel.

Staining and Photographing The Gels

The gels were stained in a solution (0.5 µg/ml) of

ethidium bromide (Sigma, St. Louis, MO, U.S.A.) in E-buffer for 30 min. After washing for 1 h in E-buffer, the gels were placed on a UV Transilluminator (model 3-3000, Fotodyne Inc., New Berlin, WI, U.S.A.) and photographed with a Polaroid model CU-5 Land camera (Polaroid Co., Cambridge, MA, U.S.A.). Polaroid type 665 film was used to photograph the gels. A Kodak #25 orange filter (Eastman Kodak Co., Rochester, NY, U.S.A.) was used to filter ultra-violet rays. The time of exposure was three minutes.

Plasmid Curing

All strains of A. vinelandii which harbored plasmids were grown in liquid Burks yeast tryptone medium made up to different concentrations of one of the following chemicals: ethidium bromide 0.125, 0.25, 0.5, 1.0, 1.5, and 2.0 mM; sodium dodecyl sulfate 0.625, 1.25, 2.5, 5.0, 10.0, and 15.0 g/l; or acridine orange 0.125, 0.25, 0.5, 1.0, 1.5, and 2.0 mM. These chemicals are commonly used to eliminate plasmid DNA from bacterial cells (37). The cultures were incubated at 37°C on a rotary shaker. After 48, 72 and 96 hours, nutrient agar plates were streak-inoculated and colonies arising on these plates were screened for the absence of plasmids by the same procedure used to establish their presence. In order to confirm the elimination of the plasmid, the cured organism was run simultaneously with the original for comparison.

Attempt to Find Plasmid Correlated Traits

Cured cells of the A. vinelandii isolated from water samples were spread on agar plates of Burks nitrogen-free medium lacking-glucose or sucrose. Bacto differentiation disks (Difco Laboratories) containing mannitol, sorbitol, dulcitol, galactose, sucrose and mannose were placed onto the surface of the inoculated plates. The plates were incubated at 30°C for 72 hours. Growth of colonies near the disks indicated that the organism was able to utilize that compound as a source of carbon and energy.

The cured organisms were grown in Burks liquid medium with butanol (4 g/l) in place of glucose or sucrose in order to test the production of cysts.

Also, Burks agar medium lacking iron was used in order to test the production of diffusible greenish-yellow fluorescent pigment by cured cultures of this organism.

RESULTS

Plasmids in Laboratory Strains

Three laboratory strains of Azotobacter vinelandii were found to harbor extrachromosomal deoxyribonucleic acid (DNA). Strains UW received from two different laboratories contained one plasmid each. Strain UW-1 and strain 2489 also harbored one plasmid each (Table III).

The following photographs (Figures 1, 3, 5, and 7) show the agarose gel electrophoresis of the DNA extracted from actively growing cells of A. vinelandii and from different

TABLE III

PLASMIDS OF THE DIFFERENT STRAINS OF
AZOTOBACTER VINELANDII

Strain	Plasmid (Mdal)
BAHAA	none
CA	none
OP (S.M. Mulrooney)	no growth
OP (P.E. Barnes)	no growth
OP (D. Kleiner)	no growth
OP (J. Meyer)	no growth
OP (J. Moreno)	none
PPD 15	none
SM52B	none
UW (V. Shah)	45
UW (H. K. Das)	none
UW (W.J. Page)	12
UW (P. Jurtshuk)	none
UW-1	52
478	none
9104	none
12837 (G.R. Vela)	none
12837 (W.D. Braymer)	none
12837 (L.E. Casida)	none
12837 (W.J. Page)	none

TABLE III (continued)

Strain	Plasmid (Mdal)
12837 (H.J. Sadoff)	none
12518	none
9046	none
A	none
2489	9
PCM289	none
S-4	none
S-5	none
organism isolated from water sample #3	11
organism isolated from water sample #16	10

Fig. 1--Agarose gel electrophoresis of DNA extracted from actively growing cells of Azotobacter vinelandii and Escherichia coli. Gels were stained with ethidium bromide (see text). Lanes A, B, D, E, and F contain samples of DNA from cells of E. coli K-12, each with a different plasmid of known molecular weight. (A), Plasmid RP4; (B), plasmid R1; (D), plasmids R1 and RSF1030; and (E), plasmid RP4. Lane C contains DNA obtained from cells of A. vinelandii strain UW, received from Dr. V. Shah. Lane F contains DNA obtained from wild-type E. coli, a strain that does not harbor plasmid DNA. Sizes of plasmids (in megadaltons) are indicated. The following phrases are used: Chrom., chromosomal DNA; CCC, covalently closed circular DNA.

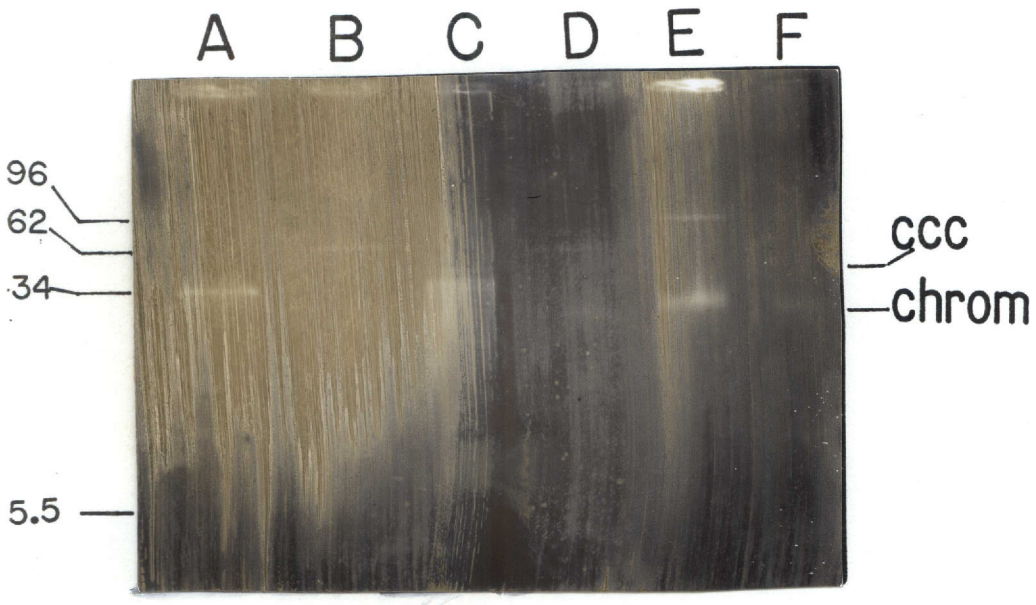

A
B
C
D
E
F
96
62
34
5.5
ccc
chrom

Fig. 2--Determination of the molecular weight of the plasmid found in A. vinelandii strain UW received from Dr. V. Shah. The natural logarithm of the molecular weight of standard plasmids extracted from cell of E. coli were plotted versus the migration distance of these plasmids. The following symbols are used: ●, plasmid R40a; o, plasmid R1; Δ, plasmid RP4; ▲, plasmid RSF1030; □, plasmid of A. vinelandii strain UW.

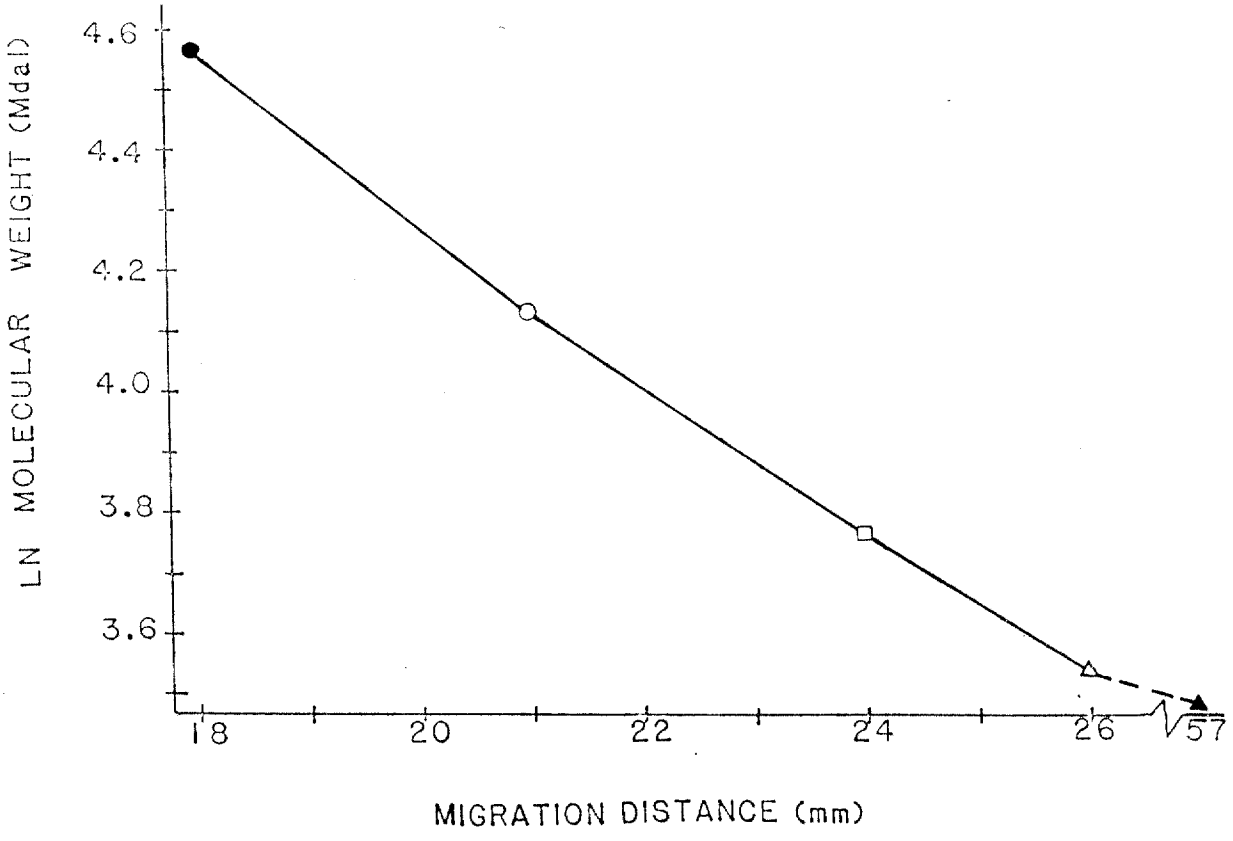

LN MOLECULAR WEIGHT (Mdal)
4.6
4.4
4.2
4.0
3.8
3.6
18
20
22
24
26
√57
MIGRATION DISTANCE (mm)

Fig. 3--Agarose gel electrophoresis of DNA extracted from actively growing cells of E. coli and A. vinelandii. Gels were stained with ethidium bromide (see text). Lanes A, C, D, G, and H contain DNA obtained from cells of E. coli K-12, each with a different plasmid of known molecular weight. (A), plasmid R1; (C), plasmid R40a; (D), plasmid RP4; (G), plasmid R1; (H), plasmid RSF1030. Lanes B, E, and F contain DNA extracted from cells of A. vinelandii. (A), Strain UW received from Dr. Page; (E), strain 2489; (F), organism isolated from water sample #3. Sizes of plasmids (in megadaltons) are indicated. The following phrases are used: Chrom, chromosomal DNA; CCC, covalently closed circular DNA.

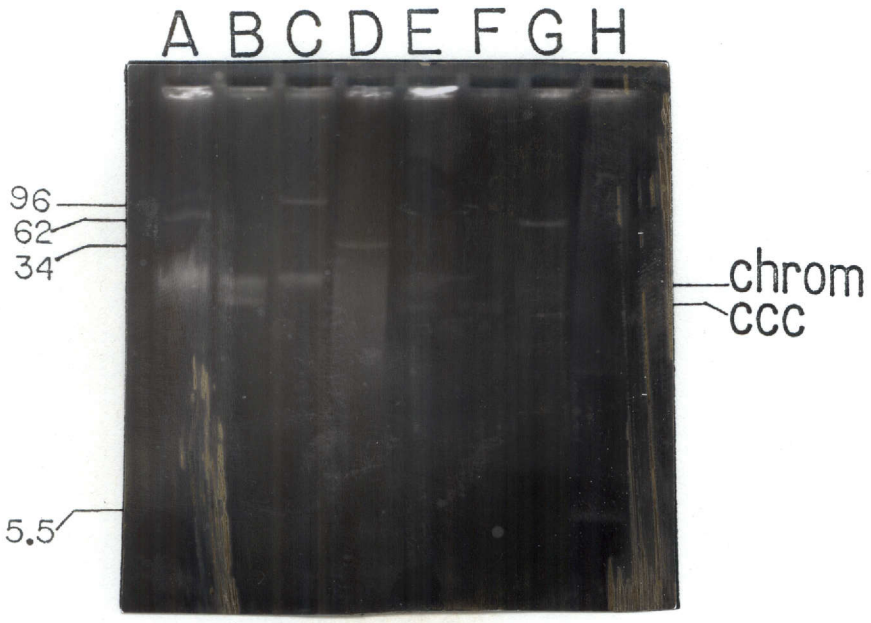

A B C D E F G H
96
62
34
5.5
chrom
ccc

Fig. 4--Determination of the molecular weight of plasmids found in Azotobacter vinelandii strain UW received from Dr. Page, in strain 2489, and in the wild-type strain isolated from water sample #3. The natural logarithm of the molecular weight of standard plasmids extracted from cells of E. coli was plotted versus the migration distance of the plasmids. The following symbols are used: ●, plasmid R40a; o, plasmid R1; □, plasmid RP4; ▲, plasmid of RSF1030; △, plasmid of A. vinelandii strain UW received from Dr. Page; ■, plasmid of A. vinelandii isolated from water sample #3; and *, plasmid of A. vinelandii strain 2489.

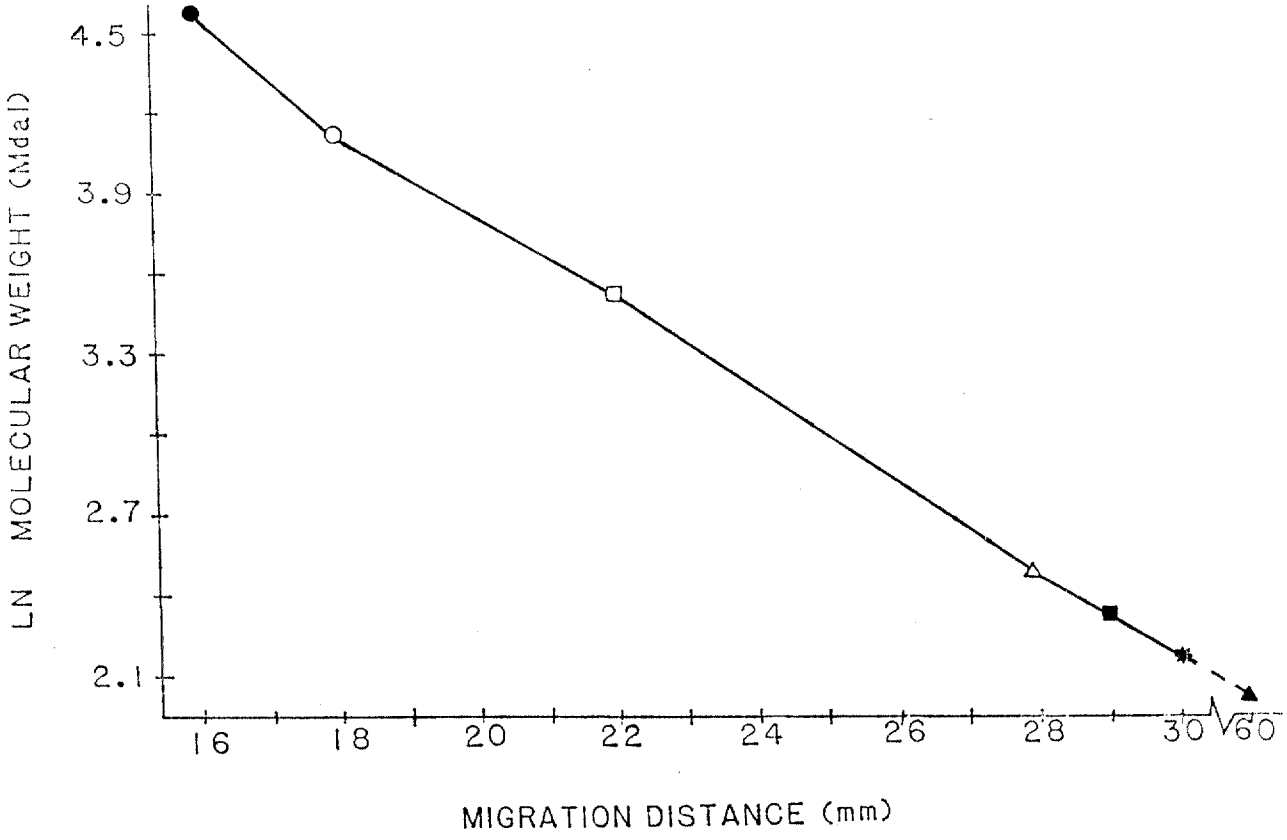

LN MOLECULAR WEIGHT (Mdal)
4.5
3.9
3.3
2.7
2.1
16
18
20
22
24
26
28
30
60
MIGRATION DISTANCE (mm)

Fig. 5--Agarose gel electrophoresis of the DNA extracted from actively growing cell of A. vinelandii and E. coli. Gels were stained with ethidium bromide (see text). Lanes A, B, E, G, and H contain samples of the DNA from cells of E. coli, each with a different plasmid of known molecular weight. (A), Plasmid RP4; (B), plasmid R1; (E), plasmids R1 and RSF1030; (G) and (H), plasmid R40a. Lanes C and F contain DNA obtained from cells of A. vinelandii. (C), Strain UW-1; (F), strain UW received from Dr. Shah. Sizes of plasmids (in megadaltons) are indicated. The following phrases are used: Chrom, chromosomal DNA; CCC, covalently closed circular DNA.

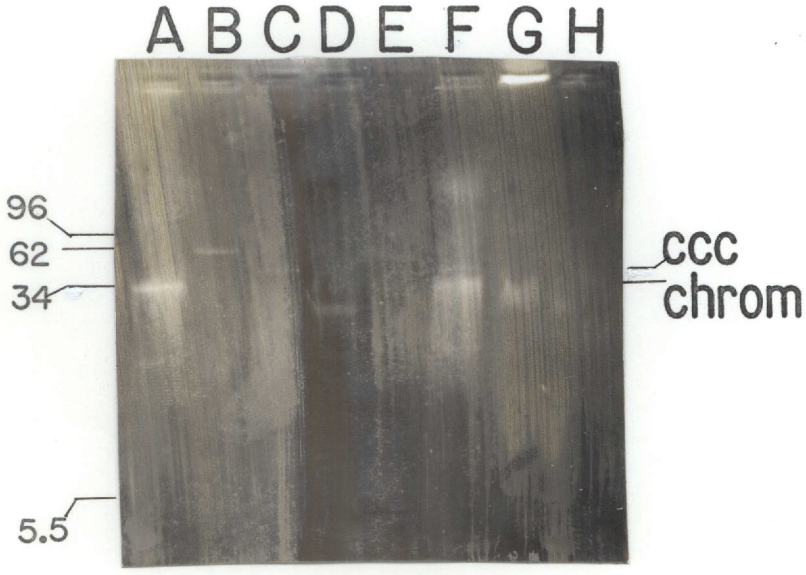

A B C D E F G H
96
62
34
5.5
ccc
chrom

Fig. 6--Determination of the molecular weight of the plasmid found in A. vinelandii strain UW-1. The natural logarithm of the molecular weight of standard plasmids extracted from cells of E. coli was plotted versus the migration distance. The following symbols are used: ●, plasmid R40a; o, plasmid R1; ■, plasmid RP4; ▲, plasmid RSF1030; ◘, plasmid found in A. vinelandii strain UW-1; Δ, plasmid of strain UW received from Dr. Shah.

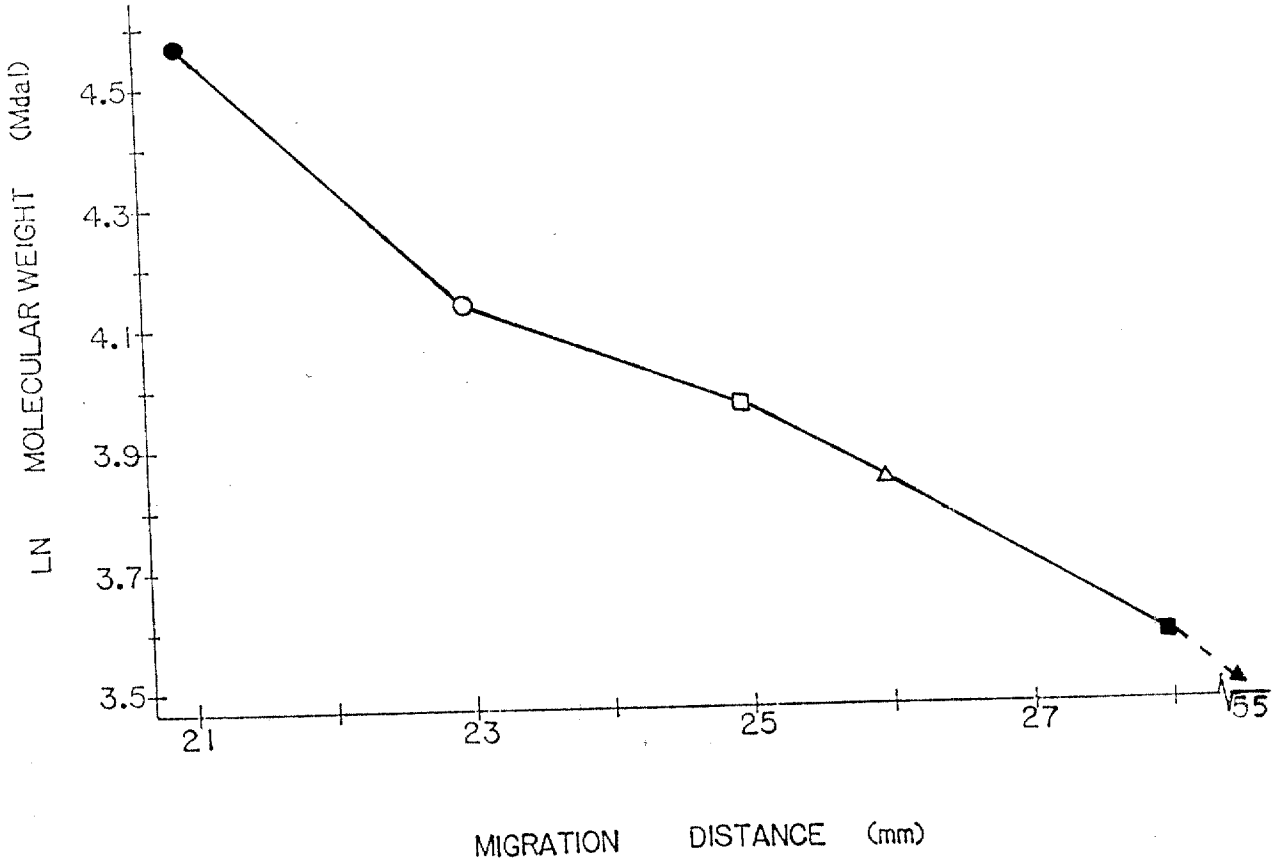
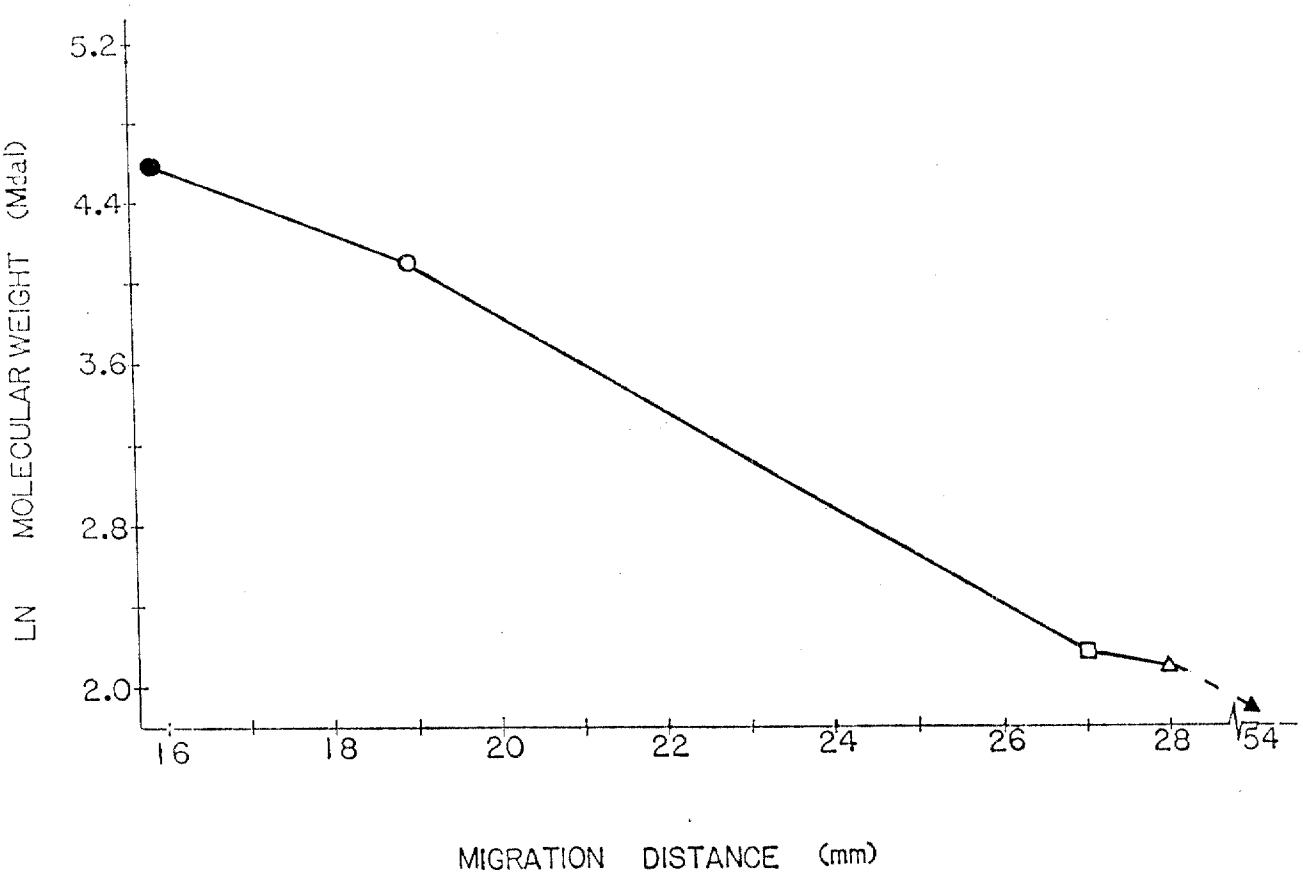

LN MOLECULAR WEIGHT (Mdal)
4.5
4.3
4.1
3.9
3.7
3.5
21
23
25
27
55
MIGRATION DISTANCE (mm)

Fig. 7--Agarose gel electrophoresis of DNA extracted from actively growing cells of Azotobacter vinelandii and Escherichia coli. Samples of the DNA extracted from cured cells of A. vinelandii isolated from water sample #16 and strain UW received from Dr. Page were compared to DNA samples extracted from original organisms. Gels were stained with ethidium bromide (see text). Lanes A, B, and C contain samples of the DNA extracted from cells of E. coli K-12, each with a different plasmid of known molecular weight. (A), Plasmid RSF1030; (B), plasmid R40a; (C), plasmid R1. Lanes E, F, G, and H contain DNA obtained from cells of A. vinelandii. (E), organism isolated from water sample #16 (cured); (F), strain UW (cured); (G) original organism isolated from water sample #16; (H) original strain UW. Sizes of plasmids (in megadaltons) are indicated. The following phrases are used: Chrom, Chromosomal DNA; CCC, covalently closed circular DNA.


A B C D E F G H
96
62
5.5
chrom
ccc

Fig. 8--Determination of the molecular weight of the plasmid found in A. vinelandii isolated from water sample #16. The natural logarithm of the molecular weight of standard plasmids obtained from cells of E. coli was plotted versus the migration distance of these plasmids. The following symbols are used: ●, plasmid R40a; o, plasmid R1; ▲, plasmid RSF1030; □, plasmid of A. vinelandii strain UW received from Dr. Page; △, plasmid of organism isolated from water sample #16.


LN MOLECULAR WEIGHT (Mdal)
5.2
4.4
3.6
2.8
2.0
16
18
20
22
24
26
28
54
MIGRATION DISTANCE (mm)

sublines of E. coli K-12, each harboring one different plasmid of known molecular weight. These plasmids were used for comparison with the plasmids of A. vinelandii. The fragments of the chromosomal DNA of the cells resolved into one band on each lane.

The ribonucleic acid (RNA) molecules migrated faster than the molecules of plasmid and chromosomal DNA and formed a sheared band on each lane of the gel. The RNA bands do not appear in the photographs.

Figures 2, 4, 6, and 8 show the plots of molecular weight versus the natural logarithm of the migration distance of standard plasmids extracted from cells of E. coli. Migration distances of plasmids of A. vinelandii were plotted on the graphs and their molecular weights were thereby obtained.

Plasmids of Azotobacter vinelandii Isolated from Water Samples

Azotobacter vinelandii was isolated from two of the thirty water samples analysed. Both organisms harbored one plasmid each (Figures 3 and 7).

The plasmid found in the organism isolated from water sample #3 was cured by growth in tryptone yeast extract medium with 1.25 g of sodium dodecyl sulfate per liter of medium. The organism isolated from water sample #16 was cured by growth in the same medium with 2.5 g of sodium dodecyl sulfate per liter.

Attempts were made to find a plasmid-correlated trait by comparing cured strains with the original organisms by a number of different characteristics It was observed that cells cured of their plasmids were still able to utilize sorbitol, dulcitol, galactose, sucrose, mannitol, and mannose as sources of carbon and energy. They were able to grow on nitrogen-free Burks medium and produce diffusible greenish-yellow fluorescent pigment. Also, the loss of the plasmid did not alter their ability to grow on Burks medium supplemented with 1% (w/v) sodium benzoate. The cured organisms were still able to form cysts when grown on Burks medium with butanol.

DISCUSSION

Deoxyribonucleic acid (DNA) molecules can be resolved by agarose gel electrophoresis according their molecular sizes (30). However, plasmids which occur in the cells as covalently closed circular DNA may be nicked during cell disruption in one or both strands to become open circular or linear molecules. Linear forms migrate faster than covalently closed circular DNA which in turn migrate faster than molecules of open circular DNA. The method of cell lysis employed in this work, however, minimized the chance of plasmid nicking upon breaking the cells open by avoiding manipulations which could submit the plasmid molecules to shearing forces, such as vigorous shaking and frequent transfers of the crude lysates from one container to

another.

The fragments of chromosomal DNA molecules present in crude lysates of bacterial cells form a band of sheared strands of DNA in agarose gel slabs after appropriate electrophoresis. The presence of plasmid DNA in the crude lysates can therefore be detected by the appearance of extrachromosomal bands in the gel. Those bands may either migrate more or less than the fragments of the chromosomal DNA, depending on the size of the molecule. It also depends on the concentration of agarose used, which determines the mean size of the pores in the gel. In this study, a gel concentration of 0.4 percent agarose was used in the determination of the molecular weight of the plasmids found in Azotobacter vinelandii, since it has been shown (5) that a resolution of large plasmid molecules is accomplished when low concentrations of agarose are used.

Several workers have reported the difficulty in obtaining stable mutants of Azotobacter vinelandii other than mutants deficient in nitrogen fixation (7). The isolation of antibiotic and antimetabolite resistant mutants has also been reported (36). On the other hand, amino acid auxotrophs and purine or pyrimidine mutants have not been encountered as yet, despite concerted efforts with a variety of mutagenic agents and the use of several different procedures (36).

Sadoff and co-workers (39) have suggested that the basis

for this difficulty might be the genetic redundancy of A. vinelandii since it has been estimated that this organism contains at least forty chromosomal copies per cell.

Roberts and Brill (36) have suggested that in A. vinelandii amino acid biosynthetic enzymes are coded for by genes that are present in at least 40 copies per cell, whereas purine, pyrimidine and nif genes are present in only a small number of copies. Das and co-workers (28) proposed that those genes which are easily mutated are present on a unique chromosome, or on a single-copy plasmid.

However, this work has shown that only three of the nineteen different strains of A. vinelandii studied harbor plasmid DNA. It is not the fact that these plasmids differ in their molecular weight that disproves the assumption made by Das et al. (28), but rather that it was shown that the majority of the strains herein studied do not harbor plasmid DNA.

This work has also shown that the plasmids found in two A. vinelandii isolated from water samples are not required for benzoate utilization or nitrogen fixation by those organisms, as opposed to the plasmid isolated from A. vinelandii strain AVY5 by Yano et al. (43)

In conclusion, this investigation represents the first survey on the presence of plasmids in A. vinelandii. It is regretable that the strain AVY5 of Yano and co-workers (43) could not be obtained and included in this survey. However,

it was found that different plasmids were present in some laboratory strains of this organism and that two wild-type strains isolated from water samples also harbored plasmid DNA. From this, we feel that the Robson-Yano *et al.* controversy is resolved and that, in accord with Yano *et al.*, *A. vinelanii* harbor plasmids and these are of different molecular weights.

Further studies aimed at the genetic characterization of the plasmids herein described should provide a better understanding of the molecular biology, the genetics, and the physiology of this microorganism.

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