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Transfection in *Agrobacterium tumefaciens*

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Intact cells of *Agrobacterium tumefaciens* were examined for ability to take up biologically active LR-4 phage deoxyribonucleic acid (DNA) from the surrounding medium. DNA incorporation as measured by subsequent plaque formation (transfection) failed to occur when the bacteria were grown in defined minimal salts media, and was restricted to a 4-hr period in the early log phase of growth in enriched media. In the latter case, maximal transfection frequencies were obtained after a 25- to 30-min incubation with 22.5 μg of phage DNA/ml. Higher DNA concentrations or longer incubation times were inhibitory. Transfection was completely inhibited by deoxyribonuclease but not by ribonuclease, trypsin, or phage-specific antisera.

Agrobacterium tumefaciens (Smith and Townsend) Conn induces autonomous crown gall tumors in a variety of dicotyledonous plants. There is considerable evidence to suggest that deoxyribonucleic acid (DNA) from the bacterium or one of its temperate phages plays a passive, if not active, role in the induction process (2, 11, 12, 19). Nevertheless, the number and nature of the cistron products responsible for conversion of normal cells to tumor cells has yet to be resolved.

In light of the above, it is surprising that so little attention has been given to the development of a well-defined system for genetic analysis in *A. tumefaciens*. Conjugation and transduction, unfortunately, have yet to be demonstrated in this genus. Early studies by Klein and Klein (8, 9) and more recently by Kern (6, 7) would suggest, however, that *A. tumefaciens* is capable of undergoing genetic transformation, although no reproducible system was defined.

The present study was undertaken in an effort to confirm "competence" in *A. tumefaciens* and to characterize some of the parameters providing for maximal DNA uptake. A temperate phage of *A. tumefaciens* was used as the source of biologically active DNA.

MATERIALS AND METHODS

Bacteria and phage. *A. tumefaciens* (Smith and Townsend) Conn strain B66 and the virulent phage LR-4 were obtained through the courtesy of Robert Manasse of Boyce Thompson Institute for Plant Research, Yonkers, N.Y. B66 was selected for use in this study because of its sensitivity to LR-4 phage, and its failure to yield plaque-forming units after treatment with mitomycin C and ultraviolet light.

Media and buffers. The following eight media were utilized for culturing the bacteria. Basic nutrient broth (BNB) contained 8 g of Difco nutrient broth, 5 g of sucrose, and 1 g of Difco yeast extract/liter of distilled water; basic nutrient agar (BNA) was BNB plus 1.5% Difco agar. Peptone broth (PTB) consisted of 1% Difco peptone/liter of distilled water; peptone agar (PTA) was PTB plus 1.5% Difco agar. Minimal medium broth (MMB) contained 5 g of glucose, 1 g of KNO_3, 1.5 g of NaCl, 2 g of NaH_2PO_4, 250 mg of $MgSO_4 \cdot 7H_2O$, and 25 mg of $CaCl_2$/liter of distilled water (*p*H 7.0); minimal medium agar (MMA) was MMB plus 1.5% Difco agar. Sucrose salts broth (SSB) contained 5 g of sucrose, 1 g of KNO_3, 1.5 g of NaCl, 2 g of NaH_2PO_4, 250 mg of $MgSO_4 \cdot 7H_2O$, and 25 mg of $CaCl_2$/liter of distilled water (*p*H 7.0); sucrose salts agar (SSA) was SSB plus 1.5% Difco agar.

Two media were used to assay for plaque-forming units: Fisher phage overlay agar (3 g of beef extract, 5 g of peptone, 5 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4$, 0.15 g of $CaCl_2$, and 7.0 g of agar/liter of distilled water) and Fisher phage base agar (phage overlay plus 8.0 g of agar/liter).

The following two buffers were employed during the course of this investigation: 0.067 M phosphate buffer, *p*H 7.0, and 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer (Mann Chemical Co.), *p*H 7.5.

Preparation of phage. A 5-ml amount of LR-4 phage stock was added to a 24-hr BNB culture of B66. This preparation was incubated for 12 hr at 27 C on a gyratory shaker (ca. 120 rev/min) to allow for phage replication and bacterial lysis. This lysed culture was centrifuged at 12,100 × *g* for 20 min at 4 C in a Sorvall RC 2-B Superspeed centrifuge (rotor SS-34). The supernatant fluid was filtered through several membrane filters (0.22 μm; Millipore Corp.) to avoid clogging. The sterile filtrate was centrifuged in a Beckman L2-65B ultracentrifuge (rotor 50.1) at 29,473 × *g* for 90 min at 4 C to sediment the phage

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particles. The phage pellets were resuspended in 0.1 M Tris buffer, *p*H 7.5, and were maintained at 4 C until needed.

Extraction of phage DNA. An equal volume of freshly distilled phenol, neutralized to *p*H 7.0 with NaOH, was added to the phage stock. This mixture was mildly agitated for 30 min. The above preparation was then centrifuged at 480 × *g* for 20 min at 4 C, in a Sorvall RC 2-B superspeed centrifuge (rotor SS-34). The aqueous supernatant fluid containing the phage DNA was removed and mixed with fresh phenol. This preparation was again slowly agitated for 30 min and centrifuged. The aqueous layer containing the phage DNA was removed, and dialyzed at 4 C overnight against a solution of 0.067 M phosphate buffer, *p*H 7.0. A few drops of chloroform were added to the dialyzed DNA solution to prevent bacterial contamination.

All DNA preparations were tested for sterility by plating on BNA prior to use. The DNA concentration was determined by the diphenylamine method of Burton (3).

Transfection procedure. Flasks containing 50 ml of desired media were inoculated with a loopful of *A. tumefaciens* B66 that had been cultured on a BNA slant. These cultures were incubated for 24 hr at 27 C on a gyratory shaker (ca. 120 rev/min). Side-arm flasks were then inoculated with an adequate sample of each respective culture to give an optical density of 0.14 with a Klett Summerson photoelectric colorimeter (green filter no. 54). Samples were removed from these flasks at various times and placed in sterile test tubes. An appropriate concentration of LR-4 phage DNA was then added to each tube. Sterile preparations of deoxyribonuclease (10 μg/ml, Worthington Biochemical Corp.), ribonuclease (10 μg/ml, Worthington Biochemical Corp.), trypsin (10 μg/ml, Sigma Chemical Co.), and LR-4 phage antisera (0.1 ml) were also added to control tubes at the time of DNA addition. All tubes were incubated in a tilted position, on a gyratory shaker (ca. 120 rev/min) at 27 C. Samples were removed at various times, and were tested for plaque-forming ability by overlay plating on a defined medium with 0.5 ml of log-phase B66 cells. Plates were incubated overnight at 27 C. A second plating was accomplished by first adding 2 ml of 0.1 M Tris buffer (*p*H 7.5) to each plate, and scraping the overlay agar into a sterile test tube. An additional 10 ml of Tris buffer was added, and the mixture was vortexed prior to centrifugation at 12,100 × *g* at 4 C for 20 min in a sterile tube. The supernatant fluid was passed through a membrane filter (0.22 μm) and plated on desired media. Plates were examined for plaques after overnight incubation at 27 C.

RESULTS

Attempts to characterize DNA uptake (competence) in *A. tumefaciens* B66 by means of transfection were at first unsuccessful. Plaques failed to develop when cultures of the bacterium were plated on different media and incubated at various temperatures after exposure to LR-4 phage DNA (1 to 45 μg/ml), for periods up to 3 hr. This was true regardless of culture age at the time of DNA addition, or the nature of the media used to culture the bacteria (Table 1). Further investigation revealed, however, that some of the assay plates contained "intact" infectious phage particles after incubation, though plaques were not apparent. As a consequence, a second plating was introduced into the transfection assay in all subsequent experiments (see Materials and Methods), so as to allow adequate time for virus expression in the form of plaques. As shown in Table 1, only the bacteria originally cultured in SSB and MMB failed to undergo transfection, as indicated by the absence of plaques after a second plating.

Transfection as observed above was completely inhibited by deoxyribonuclease, whereas ribonuclease, trypsin, and LR-4 phage antiserum caused only minor inhibition (Table 2).

Electron microscopy of the parent LR-4 phage (from which transfecting DNA was isolated), and of the transfecting progeny phage, revealed that these phage particles were morphologically indistinguishable. Furthermore, the progeny phage and parent phage were found to be equally susceptible to neutralization by antisera prepared against LR-4 parent phage.

TABLE 1. *Conditions leading to plaque-forming activity after incubation of A. tumefaciens B66 with 22.5 μg of LR-4 DNA/ml*

Growth and incubation medium	First plating[a]		Second plating[a]	
	Conditions	Results	Conditions	Results
BNA	BNA, 4 C	No plaques	BNA, 27 C	Plaques
BNB	BNA, 15 C	No plaques	BNA, 27 C	Plaques
BNB	BNA, 27 C	No plaques	BNA, 27 C	Plaques
PTB	PTA, 27 C	No plaques	BNA, 27 C	Plaques
PTB	PTA, 27 C	No plaques	PTA, 27 C	Plaques
SSB	SSA, 27 C	No plaques	BNA, 27 C	No plaques
MMB	MMA, 27 C	No plaques	BNA, 27 C	No plaques
MMB	MMA, 27 C	No plaques	MMA, 27 C	No plaques

[a] See Materials and Methods.

Cultures of *A. tumefaciens* B66 were examined for competence throughout the complete growth cycle. As shown in Fig. 1 and 2, the ability of this bacterium to incorporate biologically active phage DNA (22.5 μg/ml) and replicate intact infectious phage particles was restricted to a period of 2 to 7 hr in the early log phase of growth in BNB medium. The data indicate that the bacteria were most competent after 4 hr of incubation in the above media. At incubation times shorter than 4 hr, competence was found to the maximum. A noticeable degree of clumping of cells was observed throughout the competent fluctuate; however, it always remained less than period. At maximal competence, optical density and cell concentration were 0.164 and 4.3 × 10^5 cells/ml, respectively.

Table 3 shows the results of an experiment in which different concentrations of phage DNA

TABLE 2. *Effect of deoxyribonuclease, ribonuclease, trypsin, and LR-4 phage antiserum on transfection in A. tumefaciens B66 by 22.5 μg of LR-4 phage DNA per ml*

Substance added	Plaque-forming units/ml
Control	270
Deoxyribonuclease, 10 μg/ml	0
Ribonuclease, 10 μg/ml	174
Trypsin, 10 μg/ml	164
LR-4 phage antiserum, 1:52 dilution[a]	178

[a] A 1:52 dilution neutralized 3.3 × 10^8 plaque-forming units of LR-4 phage per ml.

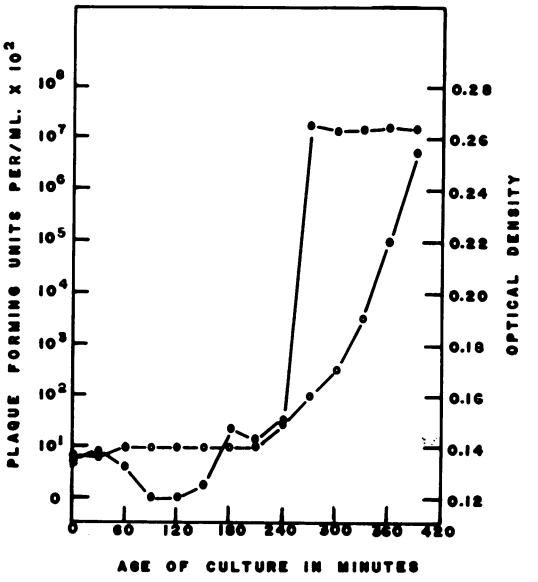

FIG. 2. *Transfection competence in A. tumefaciens B66 with LR-4 phage DNA during the lag and early log phase of growth.* (○) *Optical density of the culture at time of phage addition.* (●) *Log of plaque-forming units per milliliter on second plating.*

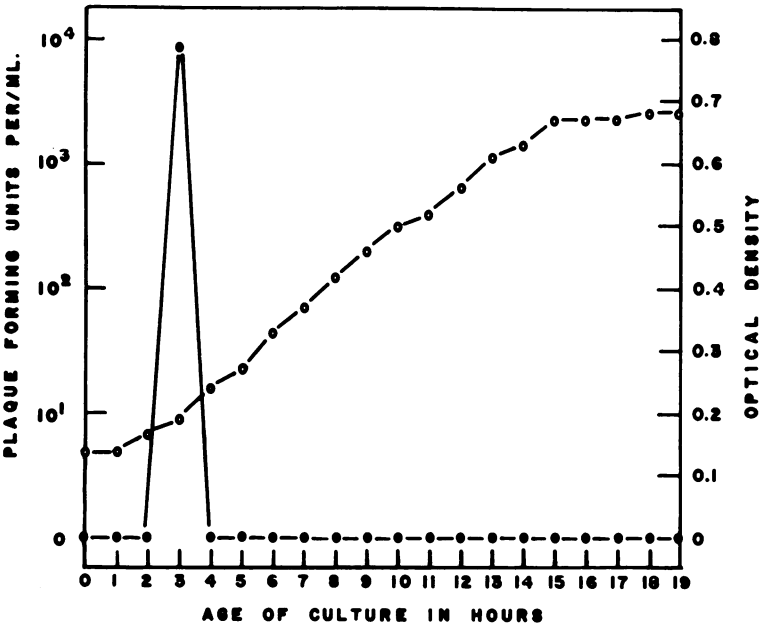

FIG. 1. *Transfection competence in A. tumefaciens B66 with LR-4 phage DNA as a function of culture age in BNB medium.* (○) *Optical density of culture at time of DNA addition.* (●) *Log of plaque-forming units per milliliter on second plating.*

were added to bacterial cultures at various periods of competence. A concentration of 22.5 μg of phage DNA/ml gave optimal transfection frequencies. This was true regardless of the particular level of competence of the bacterial cells.

The effect of incubation time on transfection in *A. tumefaciens* B66 with LR-4 viral DNA is shown in Fig. 3. Samples were removed from the experimental culture at 30, 240, and 360 min, and were allowed to incubate with phage DNA for various lengths of time. Transfection frequencies were found to be optimal with incubation times between 25 and 30 min, thereafter showing a decrease in plaque-forming units. In a few cases, however, transfection frequencies were observed to increase again after 90 min of incubation.

DISCUSSION

The data presented in this paper clearly demonstrate that extracellular biologically active phage DNA can penetrate viable cells of *A. tumefaciens* B66. The extent of DNA penetration varies with the growth medium, and is restricted to a limited period in the early log phase of growth. The extent of DNA uptake is also a function of DNA concentration and incubation time.

Competence, as stated by Ravin (14), is a normal transitory state in the growth of a culture. Zelazna (21) reported that in *Rhizobium trifolii*, a close relative of *A. tumefaciens* (5), the acquisition of a specific cell density is essential for attaining competence for transformation. In the present study, *A. tumefaciens* competence was maximal after cultures reached an optical density of 0.164, from a starting optical density of 0.14. Attempts to correlate accurately optical density at the time of maximal competence with mean number of cells per milliliter were complicated by extensive clumping in the cultures. Clumping, in fact, was found to be a characteristic property of competence in this system.

Possibly some diffusible substance ("transfection principle") may be released by cells at a specific cell density, which allows the population to become competent. Cell competence factors have been demonstrated in *Bacillus subtilis* (1), *B. sterothermophilus* (18), and pneumococci (20), and appear at least in some cases to be proteins.

Wide variations in the concentration of bacteriophage DNA required for optimal transfec-

TABLE 3. *Relation between LR-4 DNA concentration and transfection in A. tumefaciens B66 at various stages of competence*

Time sample was removed from culture (min)	DNA concn (μg/ml)	Plaque-forming units/ml
30	10	0
180	10	0
240	10	0
300	10	0
360	10	0
30	22.5	8.80×10^5
180	22.5	1.34×10^6
240	22.5	1.28×10^7
300	22.5	1.72×10^9
360	22.5	1.65×10^9
30	45	0
180	45	0
240	45	5.64×10^3
300	45	322
360	45	242

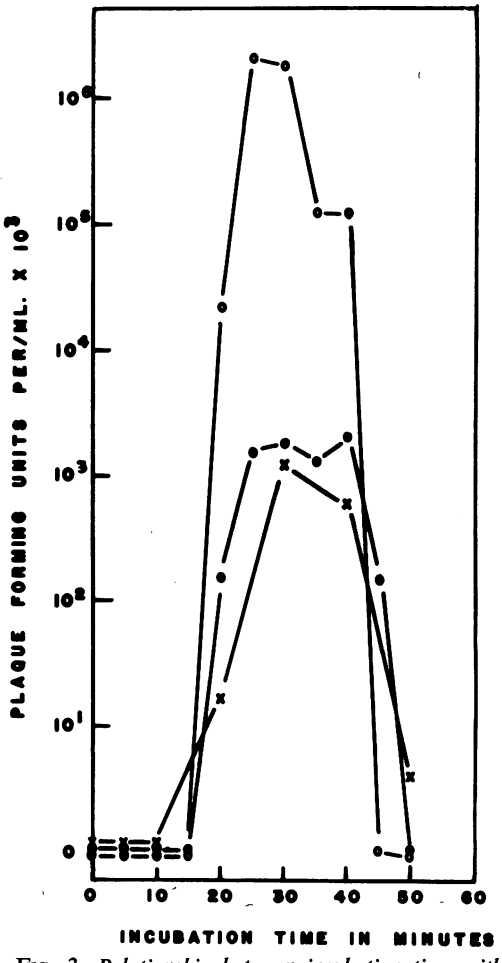

FIG. 3. *Relationship between incubation time with LR-4 DNA and transfection during three stages of A. tumefaciens B66 competence.* (×) *A 0.5-hr culture;* (●) *a 4-hr culture;* (○) *and a 6-hr culture.*

tion frequencies have been reported. Romig (16) observed maximal transfection frequencies when cells of *B. subtilis* were infected with DNA preparations of SP3 in a concentration of 100 μg/ml. Meyer et al. (13) found transfection to be optimal when DNA in a concentration of 35 μg/ml was used to infect protoplasts of *Escherichia coli*. Transfection frequencies in our study were optimal with a concentration of 22.5 μg of phenol-extracted LR-4 phage DNA/ml.

Low DNA concentrations (1 and 10 μg/ml) were not sufficient to promote transfection, and a higher concentration (45 μg/ml) was inhibitory. The reason for this latter inhibition is not known; however, it is conceivable that high DNA concentrations stimulate deoxyribonuclease production or excretion, or both.

Transfection was completely inhibited by deoxyribonuclease, whereas ribonuclease, trypsin, and LR-4 phage antiserum resulted in only minor inhibition (Table 2). The inhibition observed with ribonuclease and trypsin may well have been due to residual deoxyribonuclease in these purchased preparations. Some of the DNA strands in our stock may have had adhering antigenic proteins, in which case the antisera might have prevented DNA from penetrating the competent cells. The possibility, however, that foreign proteins (antibodies, enzymes, etc.) in themselves act as inhibitory agents cannot be excluded.

The results from this study indicate that a 30-min incubation period yielded maximal transfection frequencies. The data also suggest (Fig. 3) that phage DNA may require at least 10 to 15 min of incubation time to penetrate or adhere to the bacterial cell. In a few experiments, lesser peaks in transfection frequency occurred after prolonged incubation with DNA. This phenomenon might be due to physiological changes within the bacterial cell population, to modifications of the transfecting DNA due to the accumulation of extracellular enzymes, or to both.

The necessity for the second, or "indirect," plating suggests that during the first plating the original transfected viral genome either replicates at a slower than normal rate or results in the production of only a few phage.

Attempts to encourage plaque formation in the first plating, by reducing the rate of bacterial growth relative to the rate of viral replication by plating on suboptimal media and incubating at suboptimal temperatures, proved unsuccessful (Table 1). Strauss (17) showed that energy is required for the entry of transforming DNA into *B. subtilis*. Transforming DNA may remain in the extramembrane space beneath the cell wall, until energy is made available for it to be transported across the cell membrane. Epstein (4) suggested that, to incorporate DNA, bacterial cells must make room for the incoming DNA. They accomplish this by halting DNA synthesis for one cell division. The virus DNA that enters the bacterial cell at this time may then have to wait for bacterial DNA synthesis to begin, or for some other specific stage of growth to occur. It is also conceivable that the viral DNA may have to attach to some specific site in the cell in order to replicate; such seems to be the case with φx 174 (10). If the number of replication sites is assumed to be limited, it would not be surprising that several bacterial replication cycles are necessary to insure attachment to the appropriate site. Riggs and Rosenblum (15) reported that only a single viral progeny is obtained from each cell as a result of transfection in *Staphylococcus aureus*. If the burst size in this transfection system is also one, this would account for the delay in viral expression.

The LR-4 phage stocks have a titer of approximately 4.2×10^{11} infectious particles/ml. Optimal transfection frequencies are achieved with a concentration of 22.5 μg of LR-4 DNA/ml. This is equivalent to 3.4×10^{10} viral genomes per ml. The concentration of bacteria at the period of optimal competence was 4.3×10^{5} cells/ml. Thus, 7.9×10^{4} viral genomes were required to infect a single bacterium.

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LITERATURE CITED

1. Akrigg, A., and S. R. Ayad. 1970. Studies on the competence inducing factor of *Bacillus subtilis*. Biochem. J. 117:397–403.
2. Beltra, R., and J. Rodriguez. 1971. Aseptic induction of the crown gall tumors by the nucleic acid fraction from *Agrobacterium tumefaciens*. Phytopathology 70:357–358.
3. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Med. Res. Counc. (Gt. Brit.) Dept. Biochem. 62:315–322.
4. Epstein, H. T. 1968. Factors affecting bacterial competence for transfection and transfection enhancement. Bacteriol. Rev. 32:313–319.
5. Heberlein, G. T., J. DeLey, and R. Tijtgat. 1967. Deoxyribonucleic acid homology and taxonomy of *Agrobacterium*, *Rhizobium*, and *Chromobacterium*. J. Bacteriol. 94:116–124.
6. Kern, H. 1965. Untersuchungen zur genetischen Transformation zwischen *A. tumefaciens* und *Rhizobium* sp. Arch. Mikrobiol. 51:140–155.
7. Kern, H. 1969. Interspezifische Transformationen zwischen *A. tumefaciens* und *Rhizobium leguminosarum*. Arch. Mikrobiol. 66:63–68.
8. Klein, D. T., and R. M. Klein. 1953. Transmittance of tumor-

inducing ability to avirulent crown-gall and related bacteria. J. Bacteriol. 66:220–228.
9. Klein, D. T., and R. M. Klein. 1956. Quantitative aspects of transformation of virulence in *Agrobacterium tumefaciens*. J. Bacteriol. 72:308–313.
10. Knippers, R., and R. L. Sinsheimer. 1968. The process of infection with bacteriophage ϕX 174. Attachment of the parental DNA of bacteriophage to a fast sedimenting cell component. J. Mol. Biol. 34:17–29.
11. Kovoor, A. 1967. Sur la transformation de tissus normaux de *Scorsonere provoquee* in vitro par l'acide desoxyribonucleique d' *Agrobacterium tumefaciens*. C.R.H. Acad. Sci. 265:1623–1626.
12. Leff, J., and R. Beardsley. 1970. Action tumorigne de l'acide nucleique d'un bacteriophage present dans les cultures de tissu tumoral de tournesol (*Helianthus annus*). C.R.H. Acad. Sci. 270:2505–2507.
13. Meyer, F., R. P. Mackal, M. Tao, and E. A. Evans. 1961. Infectious DNA from lambda bacteriophage. J. Biol. Chem. 236:1141–1143.
14. Ravin, A. W. 1961. The genetics of transformation. Advan. Genet. 10:61–163.
15. Riggs, M. G., and E. D. Rosenblum. 1969. Transfection of lysostaphin treated cells of *Staphlococcus aureus*. J. Virol. 3:33–37.
16. Romig, W. R. 1962. Infection of *Bacillus subtilis* with phenol extracted bacteriophages. Virology 16:452–459.
17. Strauss, N. 1970. Early energy-dependent step in the entry of transforming DNA. J. Bacteriol. 101:35–37.
18. Streips, U. N., and N. E. Welker. 1971. Competence-inducing factor of *Bacillus sterothermophilus*. J. Bacteriol 106:955–959.
19. Stroun, M., P. Anker, and G. Auderset. 1970. Natural release of nucleic acids from bacteria into plant cells. Nature (London) 227:607–608.
20. Tomasz, A. 1970. Cellular metabolism in genetic transformation of pneumococci: requirement for protein synthesis during induction of competence. J. Bacteriol. 101:860-871.
21. Zelazna, I. 1964. Transformation in *Rhizobium trifolii*. Microbiol. Polonica 13:283–289.