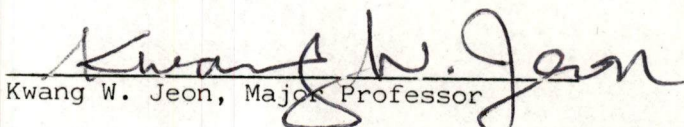
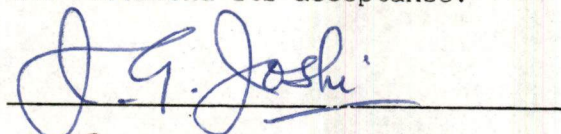


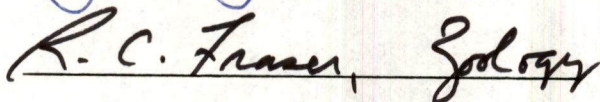
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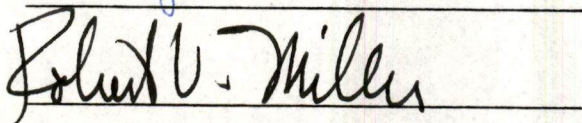

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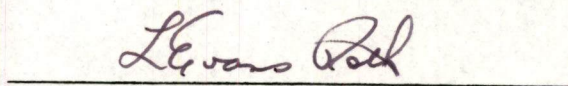



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ISOLATION AND CHARACTERIZATION OF TWO PLASMID DNAs
FROM ENDOSYMBIOTIC BACTERIA FROM AMOEBAS PROTEUS

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Jang H. Han
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ABSTRACT

Two plasmid DNAs have been detected, isolated, and characterized from endosymbiotic bacteria that have established an obligatory symbiosis with a strain of Amoeba proteus. The two plasmid DNAs were detected by electrophoresis in 0.7% agarose gels after removing chromosomal DNA either by density-equilibrium centrifugation in a cesium chloride-ethidium bromide medium or by the sodium dodecyl sulfate-sodium chloride cleared lysate method.

The two plasmid DNAs, designated as pHJ11 and pHJ12, exist as covalently-closed circles. These plasmid DNAs have molecular weights of 39×10^6 and 14×10^6 respectively, as determined by their relative mobilities in agarose gels compared with those of known molecular weight plasmids, and by measuring their contour lengths under the electron microscope. The two plasmid DNAs were not amplifiable by the treatment with chloramphenicol and were curable by either ethidium bromide or acridine orange. pHJ11 molecule was cleaved into several fragments by the restriction endonuclease Bam HI and pHJ12 was cleaved into several fragments by Eco RI.

The roles played by these plasmid DNAs in the symbiotic x-bacteria within amoebae are not understood, but indirect evidence suggests that they are involved in the establishment of endosymbiosis during the early stage of infection of the x-bacteria into the amoeba. The presence of plasmid DNAs within the x-bacteria together with other known properties such as inability of x-bacteria to grow in vitro and at temperatures above 27° C might render these symbionts useful as host for recombinant DNA studies.

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CHAPTER I

INTRODUCTION

Historical Sketch

An endosymbiotic relationship has been established between an unidentified Gram-negative rod-shaped bacterium and a strain of Amoeba proteus (called xD strain). In 1966, the x-bactrium infected D strain of amoeba by accident and started as a harmful intracellular parasite in the surviving amoebae. However, within a few years, the x-bacteria gradually became harmless among the progeny of infected amoebae, and finally about five years after the original infection, they showed mutual dependence for survival both for host (xD) and symbionts (x-bactria) (Jeon and Lorch, 1967; Jeon, 1972; Jeon and Jeon, 1976). At present, each symbiotic xD amoeba carries 40,000 to 50,000 x-bactria; this corresponds to a density of more than 1.5×10^{10} bacteria per ml of amoeba cytoplasm, or put in other way 10% of the cytoplasmic volume of the amoeba is occupied by the x-bacteria. Within the amoeba's cytoplasm, all x-bacteria are found in membrane-bounded organelles called bacterial vesicles (Jeon and Jeon, 1976).

Many attempts to identify the x-bacteria have failed mainly due to the fact that these organisms do not grow outside the amoeba under all possible culture conditions tested so far (J. Freesh and J. Tullock, unpublished results). Consequently, the endosymbiotic x-bacteria are not well characterized either physiologically or genetically. The known characteristics of x-bacteria other than their inability to grow

in vitro include their cell wall rigidity, their resistance to digestion either by the amoeba or other enzymes such as amylases, cellulase, lipase, and trypsin (Han 1979)⁷, and their inability to grow very well at temperatures above 27°C (Jeon and Ahn, 1978). The infectivity of x-bacteria is still highly retained as evidenced by infection of nonsymbiotic D amoeba by isolated x-bacteria when amoebae are fed with or without food organism¹. After such infection, the D strain of amoeba is quickly converted into the xD strain and x-bacteria can not be removed from the amoeba without reducing viability of the host cells (Jeon and Ahn, 1978).

One prerequisite for the x-bacteria to establish endosymbiosis with the amoebae is to avoid digestion by the amoeba. Since x-bacteria are found in bacterial vesicles after the stable establishment of endosymbiosis, no protection seems[?] to be required after infection. However, amoebae are phagocytosing animals which are very rich in lysosomal enzymes (Chapman-Andresen, 1973) and destroy most exogenous material, including microorganisms (Casley-Smith and Savanat, 1966; Savanat and Pavillard, 1964). Therefore, x-bacteria should require a protective device during the initial stage of infection when the x-bacteria are directly exposed to the amoeba's enzymes. In a test in vitro, the x-bacteria were found resistant both to several digestive enzymes and to a concentrated supernatant from amoebae homogenate suggesting that x-bacteria seem to have a protective device within their cell wall. Thus, the resistance property of the x-bacterial cell wall is a quite unique characteristic of x-bacteria.

Amoebae are large, free-living protozoa which grow attached to the bottom of a dish, capturing and digesting food organisms. The

culture dish usually contains a thick film of different kinds of microorganisms growing on the organic material produced by the amoebae and other food organisms. Some of these microorganisms are continuously captured and digested by the amoeba by phagocytosis. Considering this situation, it is not difficult to conceive that the present x-bacteria might have originated from a mutant strain of culture dish bacteria which became resistant to the amoeba's digestive enzymes and required some growth factors which could be found in the amoeba's cytoplasm. After this selection by the amoeba, it is believed that the bacteria started to lose their initial parasitic effect on the host and thus became harmless endosymbionts which are now fully dependent on their host. During this process, the amoeba seems to have overcome the initial adverse effect created by the bacteria and acquired the ability to use, in turn, a bacterial factor(s), which resulted in establishment of the present relationship.

Plasmid DNA

A plasmid is an extrachromosomal genetic element which is physically separated from the chromosome of the cell and is able to perpetuate itself stably within the cell. This element is nonessential for the growth and survival of normal host cells, so that under most conditions it may be gained or lost without inducing a lethal effect. Those plasmids which are able to undergo integration into a chromosomal site are referred to as episomes (Clowes, 1972; Meynell, 1971; Falkow, 1975; Mitsuhashi, 1977, Bukhari et al, 1977).

The size of most plasmids falls in the range of one to a hundred megadaltons. Plasmids usually exist as a supercoil of covalently-closed circles (CCC or form I) within the cell. This CCC molecule is thermodynamically unstable and tends to be converted into an open circle (OC, relaxed circle, or form II) by introducing a single strand break, or into a linear duplex (form III) by a double-strand break. Because of the sealed ends and the three dimensional structure characteristic of CCC molecules, plasmid DNAs have distinct physical and chemical properties different from those of OC or linear duplex DNAs.

Although the content of plasmid DNAs within cells is usually low, the physicochemical properties of the plasmid molecule allow their separation from the linear duplex fragments of bulk chromosomal DNA. These physicochemical properties include a higher sedimentation rate for plasmid DNA than of OC or linear duplex. As an example, after centrifugation for 2 hours at 100,000 g in a 5-20% linear sucrose gradient, CCC sediments about 1.5 times faster than the corresponding linear DNA, while OC sediments at about 1.14 times the rate of linear duplex (Studier, 1965). This sedimentation rate is due to the compact three dimensional structure of the CCC in solvent. The CCC is also known to be resistant to shearing and DNA-denaturing agents such as alkali or heat (Jansz et al., 1967). If the pH is raised above 12.5, OC and linear duplex undergo complete strand separation while CCC collapses into a tightly compact structure whose sedimentation rate through an alkaline sucrose gradient increases about 3.8 times faster than a corresponding denatured DNA. The difference in

sedimentation rate can further be increased by shearing the single stranded DNA (Freifelder, 1971). If the temperature is raised to the melting point of the DNA followed by rapid cooling, the CCC molecule remains as coiled duplex while OC and linear duplex are converted into single stranded DNA (Vinograd et al., 1968) which can be removed by such techniques as cellulose nitrate absorption (Cohen, 1969; Wohlhieter, 1966).

The CCC molecule also has a different binding capacity to intercalative dye such as ethidium bromide (EtBr) or its analogues (Bauer et al., 1968; Hudson et al., 1977). Due to the inability of the CCC to unwind as the dye molecules intercalate between the stacked base, binding of the dye is limited while OC and linear duplex permit more dye to bind due to their unsealed ends. In a saturating concentration of EtBr (200 $\mu\text{g/ml}$) this results in a density decrease of about 0.125 g/ml for OC or linear duplex and a decrease of 0.085 g/ml for the CCC, thus, creating a net density difference by 0.04 g/ml between OC or linear and CCC DNA which can be resolved into two separate bands in CsCl density equilibrium centrifugation (Radloff et al., 1967; Bauer and Vinograd, 1968).

By virtue of its three dimensional structural configuration in solvent, the migration of CCC DNAs through an agarose gel when exposed to an electrical field is also unique; the CCC DNAs form a satellite band above or below the corresponding linear duplex under these conditions. The relative mobility of each form of DNA is a function of the molecular weight. For a small plasmid whose size is smaller than 5 megadaltons, the mobility, in increasing order, is

known to be OC, linear, and CCC. In larger plasmids the order is CCC and then coincident OC and linear (Grinter et al., 1976).

In nature, bacterial plasmids are found in many forms and are usually recognized by the functions they determine. This may be the ability to transfer genes by conjugation as is characteristic of the F-factor; synthesis of bacteriocin inhibiting the growth of strains related to themselves as with the col-factors; synthesis of a protein coat which enables the plasmid to be packed and transmitted from host-to-host in extracellular particles such as those of temperate phage; or it may manifest itself by conferring resistance to the host against single or, more frequently, multiple antibiotics as with R factors; or other functions that are not yet clearly understood (Meynell, 1972; Falkow, 1975).

Recently, R-factors have received much attention mainly due to their clinical importance and their versatile utility in gene recombination experiments. Historically, their existence in nature was undiscovered until World War II when antibiotics came into common use to treat diseases caused by pathogenic bacteria. At first the appearance of a strain resistant to the antimicrobial drugs was regarded as a result of spontaneous mutation, but this view soon failed to explain the resistance acquired, at the same time, to multiple drugs and a sudden gain or loss of such phenotypic traits. Since the existence of R-factors was first demonstrated in Japan in 1959 (Akiba, 1960), a large number of R-factors have been discovered throughout the world and two reasons have been given for this worldwide prevalence of R-factor; their efficient transmissibility even to

unrelated strains and their multiple resistance. Common use of antibiotics is believed to have provided selective pressure favoring drug-resistant bacteria which were not as widespread as they are today. Although the presence and prevalence of R-factors is troublesome in medicine, they can be used as good cloning vehicles in recombinant DNA work since both selection and amplification of R-factor are extremely simple and easy to carry out (Cohen et al., 1977).

R-factors have at least two genetic determinants; one for antibiotic resistance (r) which codes for enzymes able to decompose or modify drugs or sometimes to detoxify heavy metal such as mercury, nickel or cobalt. The other genetic determinant (resistant transfer factor, RTF) codes for protein necessary for the conjugal transfer of the R factor. These two determinants can exist within a single plasmid molecule as a composite or can reversibly dissociate into one or more nontransmissible r plasmids and a nonresistant RTF (Anderson and Lewis, 1965).

The transition of R factors has been well studied in R222 (also called R_{NRI} and R_{100}). R222 in E. coli exists as a single composite plasmid, but it undergoes disassociation into several plasmid molecules when transferred into Proteus mirabilis. These plasmids can undergo amplification when the concentration of drugs present in the medium is increased. The R222 in E. coli is sometimes referred to as a plasmid cointegrate and R222 in P. mirabilis as a plasmid aggregates (Clowes, 1972; Helinsky and Clewell, 1971).

The reason for the existence of plasmid DNA such as R-factors is often explained in terms of the control of gene-pool synthesis within a

population (Clowes, 1972). Under most circumstances, certain cellular functions are frequently required for the maintenance of cell activities while others are required very infrequently. In this case, the continual replication of the latter type of genes may impose more of an evolutionary burden than would be compensated for by the rare need for the functions (Chakrabarty and Gunsalus, 1969). However, if these types of genes were carried on a plasmid which might be present in only a few cells of a population, they would need to replicate only in those rare cells. Any time there arose a need for the function of these genes they could be spread quickly to all the cells of a progeny population if the plasmid carries a transfer factor as the plasmid cointegrate or if other rare cells contain transfer factors which could interact with other nontransmissible plasmid to form transmissible plasmid (Anderson, 1968b). In this manner, a bacterial population would economically and enormously increase the total gene pool which is necessary for the growth and multiplication of the bacterial population. This concept of genetic regulation represents a "species-wide control of gene pool synthesis" (Clowes, 1972).

Bacterial plasmids are often regarded as more successful symbionts at the genetic level than bacterial viruses in that they never kill their host, but rather render their host more defensive against adverse environments (Hayes, 1969). Still retaining some of the properties of viruses, the plasmid carries out an efficient conjugal transfer of its genes by coding for the production of pili protein on the surface of the host cell wall (Anderson, 1968a; Brinton, 1971).

Plasmid DNA in x-Bacteria

On the basis of known properties of x-bacteria described above, it appeared possible that these symbiotic bacteria contained extra-chromosomal DNA. If present, such DNA could be involved in the establishment of endosymbiosis either by encoding some protein component of bacterial cell wall or specifying nutritional or other factors necessary for the survival of host cell. In July 1978, two distinct groups of plasmid DNAs were found in x-bacteria, that were detectable by gel electrophoresis or by CsCl-ethidium bromide density-equilibrium centrifugation. In the following chapters, the methods of detection, characterization and known properties of these plasmid DNAs are discussed.

CHAPTER II

MATERIALS AND METHODS

Materials

Lysozyme, 3x crystallized, was purchased from Calbiochem Ltd., DNase I (bovine pancreas) was from Sigma Chemical Co., Proteinase K, from Beckman Instrument Inc., sodium dodecyl sulfate was electrophoresis grade from Bio-Rad Laboratory Ltd., sarkosyl NL30 (sodium dodecyl sarcosinate) from Geigy Chemical Co., Brij-58 and sodium deoxycholate from Sigma Chemical Co., EDTA, disodium salt was from Fisher Chemical Co., nuclease-free sucrose was from Schwarz/Mann, CsCl (optical grade) from Fisher Chemical Co., ethidium bromide from Calbiochem Ltd., agarose for gel electrophoresis from Bio-Rad Laboratory Ltd., cytochrome C was from Nutritional Biochemical Co., Parlodion Mallinkrodt Co., ammonium acetate was analytical grade from Baker Chemical Co., Diamino-benzoic acid (DABA) of the high purity was from Eastman Organic Chemical Co. (Cat. No. 3904), calf-thymus DNA (sodium salt) was from Sigma Chemical Co., ³H-thymidine (specific activity of 20 Ci/m mole) was from New England Nuclear and uridine was from Calbiochem. Ltd., Scintiverse for radioactivity counting was from Fisher Chemical Co., Restriction enzymes, T₄ DNA-ligase, and λDNA were purchased from Bethesda Research Laboratories, Inc.

Maintenance and Handling of the Cells

Culturing of Cells

Since x-bacteria do not grow in vitro, the bacteria were obtained only through host cell (xD) cultivation. Amoebae were cultured at room temperature (20°C) in 9 x 14 inch Pyrex dishes containing about 1 liter of Chalkley's medium. Washed Tetrahymena, grown axenically in enriched medium (Goldstein and Ko, 1976), were added as food organisms. Amoebae were transferred to clean dishes and fresh medium every two days. Dishes containing only healthy amoebae which doubled every 3 days were used in all experiments. After amoebae in the dish attained a maximum growth, they were starved for 3 days with daily washing with fresh medium to eliminate undigested food materials. The starved cells were washed 5 times with fresh medium by centrifugation for 7 seconds at 150 g to eliminate contaminating bacteria and once with 0.9% NaCl to remove divalent cations from the medium.

Each dish yielded about 3 ml of packed cells containing about 1.14×10^6 amoebae or 5.7×10^{10} x-bacteria. The packed amoebae were quickly frozen in dry-ice/acetone and stored at -100°C.

Counting of Cells

Amoebae. The counting of amoebae was done by measuring the volume of packed cell pellet after centrifugation for 1 min. at 150 g. The volume of cell pellet was calculated into cell number using a conversion factor of 1 ml pellet = 3.8×10^5 cells. When small numbers of amoebae were to be counted, clean amoebae were collected in 10 µl microcapillary pipets whose one end was sealed, these pipettes

were centrifuged at 150 g x 1 min., the volumes of cell pellets were read under a dissecting microscope, and converted into cell numbers using above conversion factor.

X-Bacteria. After the isolation and purification of x-bacteria (see below), the number of x-bacteria was counted by measuring the turbidity of an x-bacterial suspension at 600 nm in Beckman-DU Spectrophotometer. A calibration curve for x-bacterial counting was constructed by plotting optical density (A_{600}) against number of x-bacteria for known numbers which had previously been determined using a Petroff-Hausser bacterial counting chamber under a phase-contrast microscope.

Labeling of Cells

A preliminary study showed that x-bacteria did not incorporate free exogenous ^3H -thymidine (or ^3H -thymine) supplemented to amoeba culture medium. However, this problem was particularly solved both by feeding the amoebae with labeled Tetrahymena and subsequently treating the cells with uridine to lower intracellular thymidine pool (Freifelder, 1971). Uridine is known as a competitive inhibitor of thymidine phosphorylase in the thymidylate synthetic pathway. For the labeling of Tetrahymena, they were grown in a medium supplemented with 50 μC of ^3H -thymidine per ml. The labeled Tetrahymena were fed to amoebae which were pretreated with 200 μg of uridine per ml for 2 days prior to feeding. The amoebae which were larger than usual size were allowed to grow 2 more days after feeding and then harvested for isolation of labeled x-bacteria.

Isolation of X-Bacteria

The routine procedure used to isolate X-bacteria from the collected amoebae is outlined in Figure 1. About 10 ml of frozen amoeba pellet was taken from the freezer and 10 volumes of ice-cold isolation buffer (50 mM Tris/50mM EDTA/100mM NaCl, pH 8.0) containing 0.2% Triton X-100 was added, and the cells were allowed to thaw at 4°C. Three cycles of freezing-thawing followed by shearing with a syringe attached with 14 guage needle were introduced. By this technique, the rupturing of cells as well as the cell organelles such as the mitochondria, the DNA body or the bacterial vesicles was satisfactorily done. The x-bacteria were resistant to these treatments. The lysate was then centrifuged for 5 minutes at 1,500 g at 4°C. The initial supernatant contained the majority of x-bacteria in free form along with other contaminating debris. Some x-bacteria were coprecipitated with other heavy cell debris in the initial pellet. The initial supernatant was sonicated for 1 min. at the 60% maximum intensity on a Fisher Sonic Dismembrator (Model 300) to break up contaminating cell debris. The sonicated supernatant was centrifuged for 10 minutes at 10,000 g, and the pellet containing x-bacteria was washed 3 times with fresh ice-cold isolation buffer by centrifuging for 10 minutes at 10,000 g. The final pellet of x-bacteria which contained only a very small amount of cell debris was further purified by Ficoll gradient centrifugation. The pellet was suspended in 2 ml of 50 mM Tris/150 mM NaCl buffer (pH 8.0), laid over Ficoll 400 (Pharmacia) gradients (5 to 30% w/w), and centrifuged in a Spinco SW 50.1 rotor for 30 min. at 35,000 g. The visible layer near the middle of the tube containing

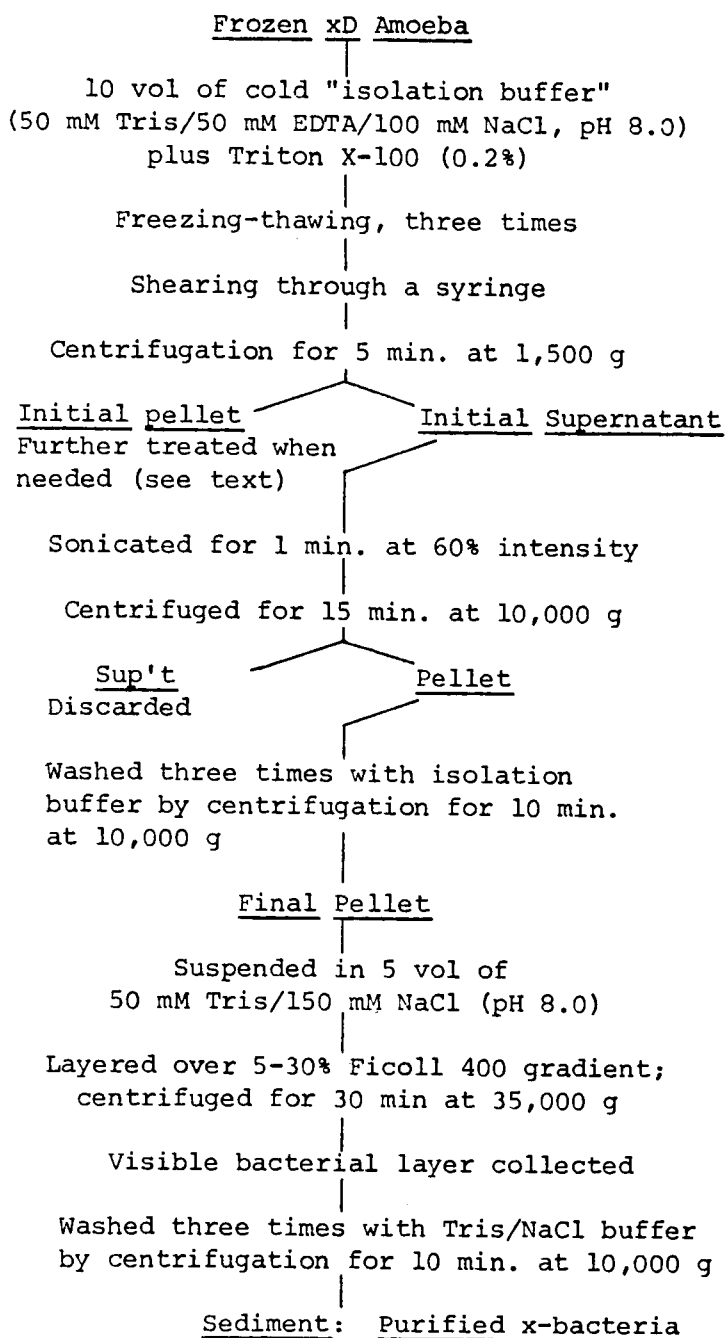


Figure 1. Isolation of x-bacteria

the x-bacteria was collected and washed with Tris/NaCl to remove Ficoll. Additional recovery of x-bacteria was made from the initial pellet as follows: the pellet was resuspended in isolation buffer, sonicated for 20 sec. at 30% intensity and centrifuged for 5 min. at 1500 g. This process further destroyed large cell debris and prevented the x-bacteria from co-precipitation with unbroken cell debris during centrifugation. This process was repeated about 5 times. The supernatant fractions were collected from each centrifugation, and pooled with the initial supernatant fluid. The washed x-bacteria were frozen at -20°C and used for DNA isolation.

Other Bacterial Strains

The strains of bacteria used in this study are listed in Table 1. Two strains of E. coli, strain GM8(pBR322) and strain GM8(RSF2124) harboring pBR322 and RSF2124, respectively, were obtained from Dr. D.H. Hamer, National Institute of Child Health and Human Development. Four strains of P. aeruginosa strain PU21(R2), strain PU21(PIP64), strain PA0303(pMG7), and strain PU21(R1162), bearing R2 pIP64, pMG7, and R1162, respectively, were kindly provided by Dr. R.V. Miller, Department of Microbiology, The University of Tennessee. These bacteria were grown in Luria broth containing antibiotics, harvested in their logarithmic growth phase and washed with cold 0.9% NaCl before lysis.

DNA Determination in x-Bacteria

To determine the total DNA content of x-bacteria, DABA (diaminobenzoic acid) flucrometry was carried out according to the procedure

TABLE 1

Other Strains of Bacteria Used in this Study

Strain	Plasmid Carried	Molecular Weight of Plasmid	Resistance Carried by Plasmid	Reference
<u>E. coli</u>				
GM8 (pBR322)	pBR322	2.6×10^6	ApTc	Bolivar <u>et al.</u> , 1977
GM8 (RSF2124)	RSF2124	7.3×10^6	ApTc	So <u>et al.</u> , 1966
<u>P. aeruginosa</u>				
PU21 (R2)	R2	45×10^6	SmSuTc	Holloway <u>et al.</u> , 1975
PU21 (pIP64)	pIP64	78×10^6	CmGmSuTmHg	Holloway <u>et al.</u> ,
PAO303 (pMG7)	pMG7	28×10^6	GmKnTmSuHg	Dr. R.V. Miller* Personal Communication
PU21 (R1162)	R1162	5×10^6	SmSu	Barth and Grinter, 1974

Note: Abbreviations used: Ap: Ampicillin; Tc: Tetracycline; Sm: Streptomycin; Su: Sulfonamide; Cm: Chloramphenicol; Gm: Gentamycin; Tm: Trimethoprim; Kn: Kanamycin; Hg: Mercury.

*Department of Microbiology, The University of Tennessee, Knoxville, Tennessee.

originally described by Kissane and Robins (1958), and modified by Setaro and Morley (1976). This technique was also used when accurate quantitation of DNA in a sample was necessary.

Diaminobenzoic acid (dichloride form) of the highest purity was used without further purification. For maximum sensitivity, a fresh solution of DABA (300 mg/ml H_2O) was prepared. To construct a standard curve, stock solutions of calf-thymus DNA (1 mg/ml) were diluted to contain 0.125, 0.25, 0.5, 0.75, 1.0 μg DNA per 25 μl in 1N perchloric acid (PCA). The blank tube contained 25 μl of 1N PCA only.

To extract lipid which interferes with the DABA reaction, a known number of purified x-bacteria was first extracted with 100 μl of alcoholic potassium acetate (9.8 g/liter of ethanol) for 15 min. at room temperature, and then further extracted with ethanol for 15 min. at 60°C. The DNA was extracted from cells by sonicating them in 1 N PCA for 2 min. at the 60% maximum intensity and incubating the residue for an additional 30 min. at room temperature. Varying amounts of the lysate were transferred to test tubes and the final volumes were adjusted to 25 μl each.

The DABA reaction was started by the addition of 100 μl of DABA (300 mg/ml) to the standard and sample tubes. The tubes were incubated for 45 min. at 60°C and the reaction was stopped by adding 1.9 ml of 0.6 N PCA at 0°C. The amount of fluorescence produced was measured in a Turner Spectro Fluorometer (Model 430) using Pyrex cuvettes. The excitation wavelength was 405 nm and the emission was read at 520 nm. Using 1.0 μg standard DNA, the sensitivity of the fluorometer was adjusted to 30 fluorescence emission units.

Lysis of Cells

Lysis of x-Bacteria

Total lysis: Sarkosyl (or SDS) proteinase K/Osmotic shock/Sonication method. Due to the x-bacteria's resistance to conventional methods used for bacterial lysis (see Results), a new procedure was designed to isolate the maximum amount of plasmid DNA possible as described below. Lysis was usually started with 5×10^{10} x-bacteria suspended in 1 ml of "lysing buffer" (25% Sucrose, 50 mM Tris, pH 8.0). All procedures were carried out at 4°C except where noted. First, 0.25 ml of 0.25 M Na_2EDTA and 0.3 ml of Proteinase K (5 mg/ml in lysing buffer) (Hansen, 1974) were added to the bacterial suspension and incubated for 5 min. at 37°C. Thereupon, 0.4 ml of sodium dodecyl sulfate (SDS) (10% in lysing buffer) was then added and incubated for 30 min. at 37°C. The mixture was re-incubated for 10 min. at 60°C and immediately cooled at 4°C. The mixture showing increased viscosity was centrifuged for 15 min. at 10,000 x g and the DNA-containing supernatant fluid was collected.

The pellet also contained a considerable amount of DNA. To introduce osmotic shock, the pellet was suspended in 1 ml of 0.1 x SSC (15 mM NaCl/0.15 mM sodium citrate), shaken for 15 min. at 4°C, and again treated with Proteinase K and detergent as before. After a 15 min. centrifugation at 10,000 x g, the second supernatant fluid was added to the first supernatant fraction.

The x-bacteria after such treatments still contained some plasmid DNA, but became sensitive to brief sonication. The bacterial pellet was suspended in 1 ml of ice-cold 0.1 x SSC and sonicated for

2 min. at 30% intensity. The sonicated suspension was again centrifuged for 30 min. at 10,000 x g and the third supernatant was added to the initial supernatant fraction. The procedure used is outlined in Figure 2.

Lysis of E. Coli and P. aeruginosa

Brij-DOC, cleared lysate method. The lysis procedure was that of Godstein and Sinsheimer (1967). The harvested cells of 5×10^9 were first resuspended in 1 ml of cold lysing buffer (25% sucrose/50 mM Tris, pH 8.0). Then, 0.2 ml of lysozyme (5 mg/ml in lysing buffer) was added and incubated for 5 min. at 0°C , after which 0.4 ml of 0.25 M Na_2EDTA was added. The suspension was kept at 0°C for another 5 min. after which lysis was brought about by the addition of 1.6 ml of a detergent mixture consisting of 1% Brij-58, 0.4% sodium deoxycholate, 0.0625 M Na_2EDTA , and 0.05 M Tris, pH 8.0. After 5-10 min., the sample became relatively clear and viscous. The sample was then centrifuged at 4°C for 25 min. at 48,000 x g, a step which normally pelleted the chromosomal DNA while leaving plasmids in the supernatant.

Total cell lysate. The method was taken from the procedure of Marmur (1960). All procedures were exactly the same as above with the cleared lysate method except that 1.6 ml of 2% SDS (or Sarkosyl) was added instead of Brij-DOC mixture. After the SDS addition, the mixture was incubated for 10 min. at 37°C during which time clearing was completed.

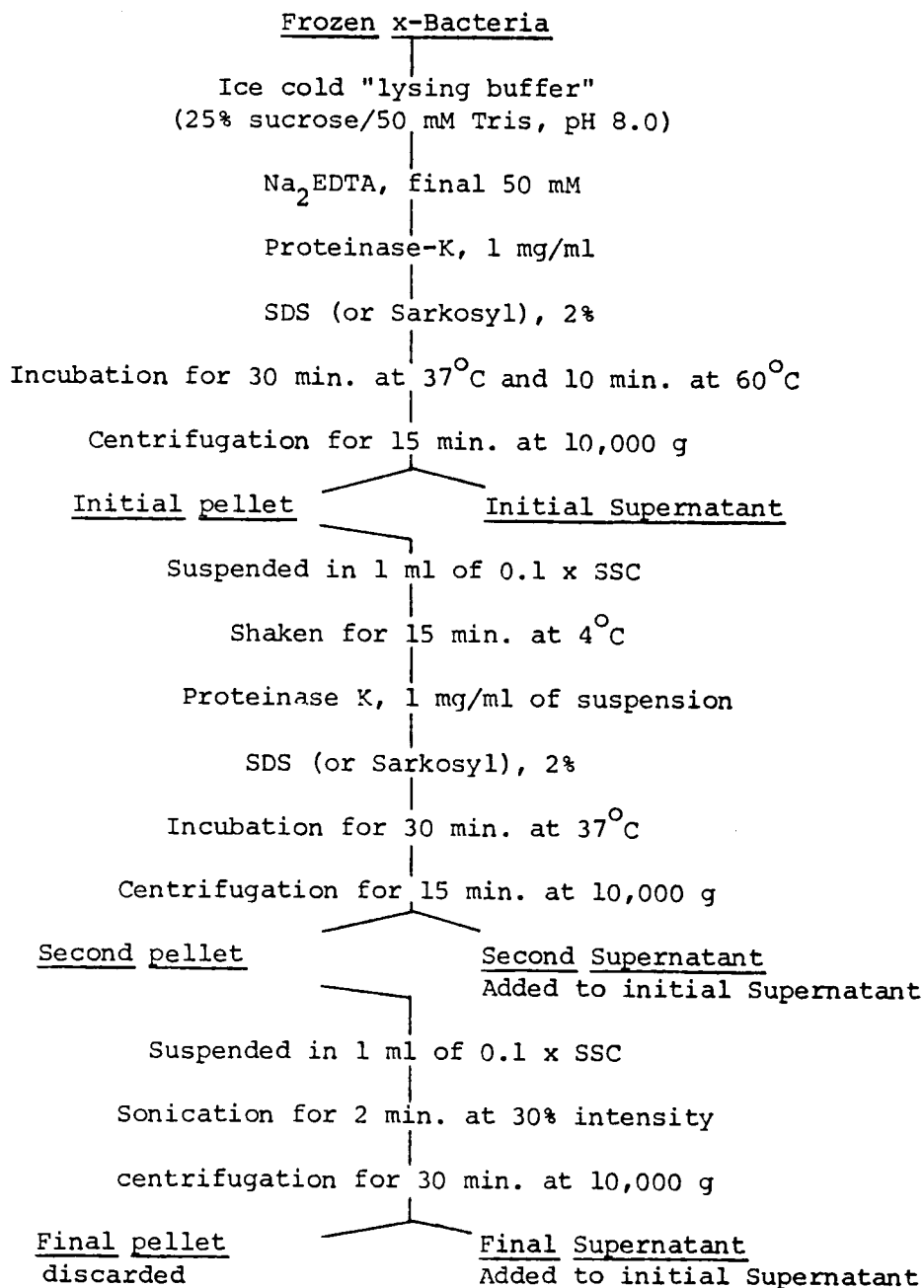


Figure 2. Preparation of x-bacterial lysate

Clearing of Chromosomal DNA

To increase the plasmid to chromosomal DNA ratio in the total lysate of x-bacteria, E. coli and P. aeruginosa, chromosomal DNA had to be removed. This was done by the method of Guerry et al. (1973). To the lysate, 0.1 volume of 10% SDS plus 0.2 volume of 5M NaCl was added and the mixture of 1% SDS, 1M NaCl was kept overnight at 4°C after which time it was centrifuges for 30 min. at 17,000 g. This step pelleted about 90% of chromosomal DNA and left the plasmid DNA in the supernatant fluid.

Methods of DNA Purification

Phenol Extraction

The cell lysate from the total lysis, cleared lysis, or SDS-salt precipitation method was subjected to deproteinization by the combined methods of Mamur (1960) and Kirby (1957). To the lysate, 1 volume of redistilled phenol saturated with Tris (100 mM, pH 9.0) was added and gently shaken for 30 min. at room temperature. The mixture was then centrifuged for 30 min. at 10,000 x g and the aqueous phase was collected. The interphase or protein gel was added to fresh Tris (100 mM, pH 9.0) and re-extracted with fresh phenol. The aqueous phases from the two extracts were pooled and further extracted with chloroform-isoamyl alcohol mixture (24:1) by gentle shaking for 20 min. at room temperature. The aqueous phase was collected by centrifugation for 20 min. at 10,000 x g. Using 3 M sodium acetate (pH 5.5), the aqueous phase was brought to 0.3 M sodium acetate, and 2.5 volume of -20°C ethanol was added and kept overnight at -20°C. To collect DNA,

the mixture was centrifuged for 30 min. at 12,000 x g. Ethanol was aspirated off and remaining ethanol was evaporated at room temperature.

The DNA could also be precipitated by polyethylene glycol (PEG) treatment, the DNA solution was adjusted to 10% PEG 6000 and 1 M NaCl, and stored overnight at 4°C after which time the tube was centrifuged. The DNA pellet was usually dissolved to desired concentration in a buffer containing 10 mM Tris (pH 8.0) and 1 mM EDTA. The solution was stored at -20°C until use.

Dye-buoyant Density-equilibrium Centrifugation

To separate plasmid DNA from chromosomal DNA, centrifugation was performed using the method of Radloff et al. (1967), modified by Clewell and Hellinsky (1969). To 1 ml of cell lysate, either from the total lysis or cleared lysis, 6 ml of TES buffer (50 mM Tris/5 mM Na₂EDTA/50 mM NaCl, pH 8.0) added. In above 7 ml mixture, 6.546g CsCl and 2.7 mg EtBr were dissolved to give a solution having a density of 1.60 and a EtBr concentration of 300 µg/ml. This solution of approximately 9 ml was then transferred to a polyallometer centrifuge tube and overlaid with light mineral oil. The centrifuge tube had been boiled for 10 min. in 1 mM EDTA, soaked for 1 hour in TES buffer containing 5 mg of BSA/ml and air-dried (Pemberton and Clark, 1973). After the addition of EtBr, all procedures were done in the dark to prevent a dye-mediated nicking of plasmid DNA by light. Centrifugation was carried out in a Spinco Model L2-65B Ultracentrifuge in a type 50-Ti fixed-angle rotor at 150,000 x g for 48-60 hours at 15°C. At the end of the run, DNA bands were photographed and the contents were fractionated by bottom puncture. When the lysate from x-bacteria was used, a plasmid DNA

band below the thick chromosomal DNA band was not visible after the initial centrifugation. In such a case, the regions containing plasmid DNA were pooled from about ten tubes, concentrated, and rebanded by a second centrifugation under similar conditions as described above. The plasmid DNA band, now visible under UV-illumination, was collected and then extracted with butanol or isopropanol (Cozzarelli et al., 1968) to remove ethidium bromide. The DNA solution was successively dialysed against SSC (150 mM NaCl, 15 mM Na-citrate) 0.1 x SSC, and Tris buffer (10 mM, pH 8.0) containing 1 mM EDTA. After the final dialysis, DNA was stored at -20°C .

During the sample preparation, the precise density of the solution accurate to $\pm 0.001 \text{ g/cm}^3$ was obtained with a refractometer (Bausch and Lomb, Model ABBE-3L) from which refractive index ($n_D^{25^{\circ}\text{C}}$) of the mixture was read. The density ($\rho^{25^{\circ}\text{C}}$) of the mixture was calculated from the equation described by Vinograd and Hearst (1962):

$$\rho^{25^{\circ}\text{C}} = 10.8601 \times (n_D^{25^{\circ}\text{C}}) - 13.4974$$

When adjustments of density were necessary, the following equation (Flamm et al., 1969) was used to determine the amount of solid CsCl or buffer required to change the density of a given volume, $\bar{V}$, by a specific increment, $\Delta\rho^{25^{\circ}\text{C}}$:

$$\text{CsCl (g)} = \Delta\rho^{25^{\circ}\text{C}} \times \bar{V} \times 1.32$$

$$\text{Buffer (ml)} = \Delta\rho^{25^{\circ}\text{C}} \times \bar{V} \times 1.52$$

Agarose Gel Electrophoresis

Analytical Gel

To detect plasmid DNA as a satellite band on the agarose gel, DNA purified by phenol extraction or dye-buoyant density centrifugation

was subjected to electrophoresis in 0.7% agarose gel according to the procedure of McDonnell et al. (1976). The gel chamber, the buffer chamber, and the well-forming comb were made according to the description of McDonnell et al. The dimension of gel used for analytical purpose was 8.4 x 15 x 0.5 cm. Before loading, the DNA samples were adjusted to 10% glycerol and 0.025% bromophenol blue (BPB). Approximately 25 μ l of sample containing 0.1-0.5 μ g DNA was loaded into each well. Using a buffer system of 40 mM-Tris, 5 mM-sodium acetate, and 1 mM-EDTA (pH 7.7), electrophoresis was carried out horizontally at 4°C and 120 V for 4 hours or until the tracking dye neared the bottom of gels. To prevent evaporation during the run, each well was sealed with hot agar solution after the DNA entered the gel and the chamber was covered with a tightly fitting cover. At the end of a run, the gel was placed in a solution containing 0.5 μ g EtBr/ml of running buffer for 30 min. The DNA bands were visualized under UV light by virtue of the fluorescence emitted by bound EtBr. The gels were laid directly on the UV-transilluminator (Model 51), Ultraviolet Product Inc., San Gabriel, California) and photographed with a camera equipped with a Kodak orange gelatin filter (Cat. No. 149 5548). Polaroid Type 57 Land film or Type 667 Land film was used.

Preparative Gel

To collect plasmid DNA by electrophoretic separation, preparative agarose gels were prepared according to McDonnell et al. (1976). The gels were 12 cm long, 9 cm wide and 1.5 cm thick. The dimension of sample wells was 0.3 cm wide, 7 cm long, and 1.4 cm deep. A DNA sample of about 3 ml prepared by ethanol precipitation

or CsCl/EtBr density gradient centrifugation containing glycerol and BPB was electrophoresed overnight at 80V and 4°C. At the end of a run, the gel was soaked in EtBr solution (0.5 µg/ml) for 15 min. in order to stain only surface of gel, thus protecting the remaining plasmid DNAs in the gel from dye-mediated nicking in the presence of light. The plasmid DNA band visualized by UV illumination was cut into slices and the DNA was electrophoretically eluted from the slice as described below.

Elution of DNA from the Gel Slice

Using a modified procedure adopted from Galibert et al. (1975) and Strongin et al. (1977), DNA was eluted electrophoretically in a large scale with a device constructed from a 15 ml plastic conical centrifuge tube with a diameter of 1.2 cm. The tip of the centrifuge tube was cut to provide a 10-cm long tube, tapering to a narrow end with 0.3-cm diameter. The narrow end was then slid into boiled dialysis tubing of 1-cm diameter, creating a watertight seal. The tubing was tied about 3 cm below the tube tip, and was filled with about 5 ml of buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.7). After removing air bubbles, a boiled cotton plug was placed in the conical tube end separating the tube from the bag. Gel slices to be eluted were extruded from a syringe (without needle) into the tube, with periodic addition of buffer to avoid air bubbles. The tube was suspended vertically from a rack with the bag below, immersed in an anode buffer tank. The top of the tube was connected by a "U"-tube to a second buffer tank containing the cathode. Electrophoresis of DNA from the gel into the bag was carried out at 4°C and 100V and was

completed in 10 to 15 hours. At the end of a run, the polarity of current was reversed (i.e. upper tank anode, lower tank cathode) and run for 15 min. to release any DNA tightly bound to the dialysis tubing.

The bag was pierced with a hypodermic needle, and the remaining buffer solution above the bag membrane was collected into a syringe. The bag was carefully removed from the tube and cut into several pieces, and soaked in about 2 ml of buffer for 1-2 hours with shaking at 4°C. The DNA contained in the pooled buffer was ethanol precipitated and the DNA pellet was dissolved in Tris buffer (10 mM, pH 8.0) containing 1 mM EDTA. The DNA was kept frozen at -20°C until use.

Estimation of the Molecular Weights of Plasmid DNAs

Agarose Gel Electrophoresis

For the estimation of molecular weights, six marker plasmid DNAs with known molecular weights from E. coli and P. aeruginosa listed in Table 1 (page 16) were co-electrophoresed with the sample plasmid DNAs from x-bacteria in 0.7% agarose gel. The calibration curve was constructed according to Meyer et al. (1976) by plotting markers molecular weights against their relative mobilities. From this graph the molecular weights of sample plasmid DNAs were directly read. Relative mobilities were expressed as the distance each plasmid DNA migrated from the origin divided by a distance the dye (BPB) front migrated from the origin.

Electron Microscopy

Plasmid DNAs purified from equilibrium CsCl-dye density gradient centrifugation and agarose gel electrophoresis were mounted

for electron microscopy by Kleinschmid's aqueous spreading technique (1968) as modified by Davis et al. (1971), the drop diffusion method of Lang and Mitani (1970) as modified by Trendelenburg et al., 1976), or the formamide spreading method of Davis et al. (1971) as modified by Kolodner and Tewari (1970).

The plasmid DNAs used in this experiment were covalently closed circles (CCC, Form I) and were first converted into relaxed open circles (Form II) either by irradiation with 1000 rads of X-ray (Sharp et al. 1972) or by nicking with DNase I (Hudson et al., 1969). In the latter case, 50 µg/ml plasmid DNAs were incubated with DNase I (bovine pancreas) at a concentration of 2×10^{-5} g/ml in 0.015 M NaCl, 0.012 M $MgCl_2$, 0.007% BSA, 0.001 M EDTA, and 0.01 M Tris pH 8.0 for 30 min. at 30°C. After the treatment, the film made up of DNA and a basic amphiphilic protein (cytochrome C) was picked up on grids according to the procedure outlined in Table 2. To minimize experimental variations from the film pick up procedure, a grid holder attached to a micromanipulator was made using 100 µl microcapillary pipette whose one end was sealed with a small piece of double stick tape. This grid holder enabled the grid to be handled horizontally in an accurate manner.

The grids were dehydrated and stained with 5×10^{-5} M uracyl acetate in 90% ethanol for 30 seconds followed by 10 seconds in 90% ethanol after which excess liquid was removed by touching a piece of filter paper on the edge. During these steps only the sample face of each grid was touched with the liquids. After the staining, the grids were shadow cast with an 80% platinum/20% palladium mixture

TABLE 2

Conditions for DNA Pick-up for Electron Microscopy

Methods	Description
<u>1. Aqueous Spreading</u>	
Spreading vessel	A Falcon dish 1" x 2" and a dry, acid-cleaned glass slide as a ramp
Spreading phase	Ammonium acetate 0.5 M pH 7.5, Na ₂ EDTA 0.001 M, cytochrome C 100 µg/ml, total 50 µl
Hypophase	Ammonium acetate, 0.15 M pH 7.5
Supporting grids	200 mesh copper grids, covered with a film of 3.5% (W/V) Parlodion in amyl acetate formed on a water surface
Condition	From a micropipette (50 µl) attached to a screw dispenser, spreading solution was slowly run on a 30° angled slide starting 1 cm above the surface of the hypophase. One or two minutes after the spreading, the grids were touched horizontally to DNA-protein film for 1 sec. at a distance of 0.5 cm from the slide-solution boundary.
<u>2. Formamide Spreading</u>	
Spreading phase	Tris-HCl 0.1 M pH 8.5, Na ₂ EDTA 0.01, M cytochrome C 100 µg/ml, formamide 50%, DNA 1 µg/ml, total 50 µl.
Hypophase	Tris-HCl 0.01 M pH 8.5, Na ₂ EDTA 0.001 M formamide 20%
Condition	Same with aqueous spreading
<u>3. Drop Diffusion</u>	
Vessel	A Falcon dish 1" x 3" and a clean parafilm circle at the center where sample was placed. Water drops at the edge to prevent a sample from drying.
Sample drop	Ammonium acetate 0.2 M pH 8.0, Na ₂ EDTA 0.001 M, cytochrome c 10 µg/ml, DNA 1 ng/ml, total 50 µl
Supporting grids	200 mesh copper grids deposited with a thin carbon film in a vacuum evaporator and activated by glow discharge at 200 µm Hg for 20 min.
Condition	The grids were touched face down to the surface of sample drop for 1 sec. at the interval 1, 3, 10, 40 hours.

at an angle of 6° C, and distance of 8 cm from the metal source (Davis et al., 1971).

The DNA molecules were photographed using an RCA 4 EMU-4 microscope with a 50- μ m objective aperture and 50 kV accelerating voltage. The resulting negatives were enlarged and the contour lengths were measured on the tracings with a map measurer. Fifteen to fifty molecules were measured for each plasmid DNA. As internal standards pBR322, RSF2124, linear λ DNA and T_4 ligase-closed λ DNA were mounted on the same grid together with the plasmid DNAs from x-bacteria. The magnification of the electron micrograph was calibrated with a replica plating grid (Ernest Fullam, Inc., 54, 864 lines/inch) for each examination.

The linear λ DNA used in this experiment was converted into a circular form by T_4 -ligase. The reaction mixture consisted of 1 μ g λ DNA, 10 units of enzyme, 0.5 mM ATP, 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 10 mM dithiothreitol (Heyneker et al., 1976). The incubation was done for 1 hour at 20° C.

Cleavage of DNA by Restriction Endonucleases

The DNAs collected by CsCl/EtBr centrifugation or preparative gel electrophoresis were digested with restriction endonucleases. The buffers used were 100 mM Tris-HCl (pH 7.5) 50 mM NaCl, 5 mM $MgCl_2$, and 100 μ g/ml BSA (Hedgpeth et al., 1972) for Eco RI, 150 mM NaCl, 6 mM Tris-HCl (pH 7.3) 6 mM $MgCl_2$, and 100 μ g/ml BSA for Bam HI (Roberts et al., 1977) and Sal I (Arrend et al., 1978), and 6 mM NaCl, 7 mM $MgCl_2$, 7 mM Tris-HCl (pH 7.4) and 100 μ g/ml BSA for Hind III (Old et al., 1975). The reaction mixture contained about 1 μ g DNA

in the above buffer, and 2 μ l of endonuclease. The total reaction volume was 20 μ l. Reaction mixtures were usually incubated for 2 hours at 37°C. The reaction was initiated by the addition of enzyme and terminated by the addition of 5 μ l of a solution containing 2.5% sodium dodecyl sarcosinate, 50% glycerol, and 0.025% bromphenol blue 0°C.

Curing of Plasmid

Treatment with Intercalative Dye

Using acridine orange and ethidium bromide as plasmid-curing agents (Hahn and Ciak, 1972), an attempt was made to eliminate plasmid from x-bacteria. For this purpose, xD amoebae were treated with acridine orange (8×10^{-6} M) or ethidium bromide (8×10^{-5} M) for 1 week followed by 3 days in the normal culture medium, and then x-bacteria were isolated. The x-bacteria were lysed, and plasmid fraction was obtained by SDS/salt-precipitation method. The assessment of plasmid curing was based upon the loss of plasmid bands in agarose gels after electrophoresis and staining with ethidium bromide.

Temperature Treatment

As a physical agent for plasmid curing (DiJoseph et al., 1973; Eichenlaub 1979; Latta et al., 1978), x D amoebae were treated with high temperature (27°C) which had been known to be nonpermissive for the survival of both x-bacteria and symbiotic amoeba (Jeon and Ahn, 1978). For this purpose, xD amoebae were cultured at 27°C for 3 weeks and allowed to recover for 1 week. During the recovery period, x-bacteria were labeled with ^3H -thymidine as described above. The x-bacteria were then isolated and lysed to extract DNA. Partially

purified plasmid DNA fraction was prepared by SDS/salt-precipitation method. After gel electrophoresis, the curing of x-bacterial plasmids was tested by the presence or absence of plasmid band in stained gels or by plasmid DNA peaks in radioactivity counting.

CHAPTER III

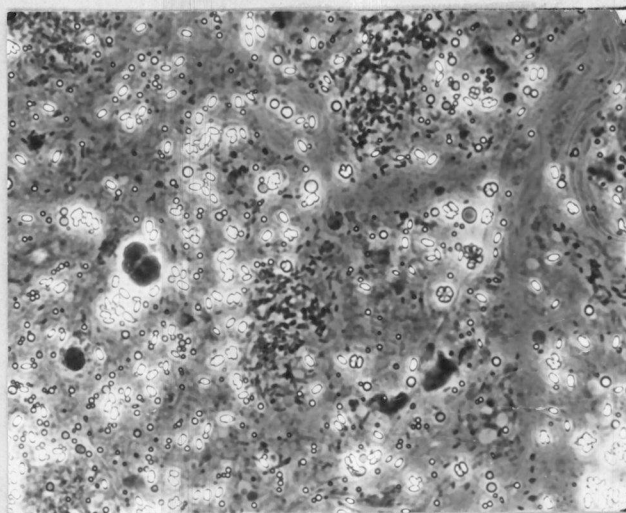
RESULTS

The Purity of x-Bacteria After Isolation

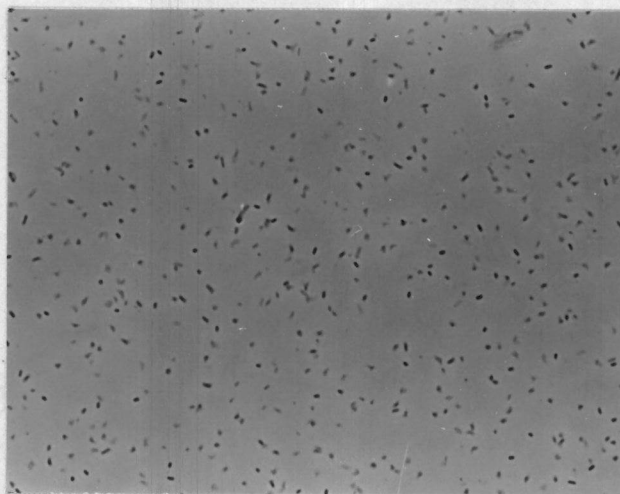
Amoeba lysates usually contained a large amount of cell debris such as membrane fragments, various vesicles, nuclei and dense crystals. Consequently, no single-step isolation procedure could be used to obtain pure x-bacteria. The routine procedures used in this work were arrived at after many preliminary experimentations. By this method, clean preparations of x-bacteria were obtained, as shown in Figure 3 by phase-contrast microscopic examination. The x-bacterial preparation isolated by the routine procedures employed retained their viability and infectivity as evidenced by their ability to infect other amoebae and to establish symbiosis.

Lysis of x-Bacteria

During the preliminary studies, x-bacteria were found to be insensitive to lytic enzymes such as lysozyme, α - and β -amylase, cellulase, glucuronidase and lipase. Also the x-bacteria showed considerable cell rigidity to physical methods of cell disruption such as repeated freezing and thawing, osmotic shock treatment, sonication and French pressing. Treatment of x-bacteria with nonionic detergents such as Triton x-100 and Brij were of little effect in bringing about cell lysis. Therefore, conventional methods for lysing E. coli and other bacteria could not be directly applied to x-bacteria. However, a combination of SDS or Sarkosyl at high concentration and



A. x-Bacteria before isolation, x 1320



B. x-Bacteria after isolation, x 528

Figure 3. Purity of x-bacteria

A. Observation under the phase contrast microscope. x-bacteria before isolation; B. x-bacteria after isolation.

proteolytic enzymes with a prolonged incubation produced partial lysis of x-bacteria, whereby their cell walls became leaky to macromolecules such as DNAs. Among various proteolytic enzymes tested such as pronase, trypsin and elastase, Proteinase K (Hansen, 1974) was found to be most effective in bringing about cell lysis. After such treatment, x-bacteria became sensitive to osmotic shock and mild sonication. Therefore, the leaky cells were further treated with osmotic shock and brief sonication to increase the recovery of DNA.

DNA Content of x-Bacteria

In Figure 4-A the standard curve for DABA (diaminobenzoic acid) fluorometry is presented to show the linearity between the amount of DNA and fluorescence emission at 520 nm. The maximum amount of DNA used in Figure 4-A was 1 μ g which emitted 30 units of fluorescence, but the linearity was also observed even when the DNA was increased tenfold. From this standard curve, the DNA content of x-bacteria was calculated to be 0.023 pg DNA per cell equivalent to 5.19×10^{-17} moles of DNA phosphorus, assuming that average phosphorus content in DNA is 7%. From this value, the molecular weight of the x-bacteria's chromosome was calculated to be 13.8×10^9 assuming that isolated x-bacteria were in the log growth phase. If they were in the stationary phase of their cell cycle, the molecular weight would be 6.9×10^9 . For comparison, the DNA content of other bacteria is listed in Table 3.

TABLE 3

DNA Content of x-Bacteria and Others

Organisms	DNA/cell pg	Size of Chromosome Giga Daltons	Reference
x-Bacteria	0.023	13.8 ^a	
<u>Bacillus cereus</u>	0.021		Fitz-James and Young (1959)
<u>Bacillus subtilis</u>	0.028		Cairg <u>et al.</u> (1969)
<u>E. coli</u> , B (log phase)	0.016	9.6	Harold and Ziporin (1958)
<u>E. coli</u> , B (stationary phase)	0.026		Harold and Ziporin (1958)
<u>E. coli</u> , K (log phase)	0.0047	2.5	Cairns (1962)

^a The value was not corrected for cell cycle stages.

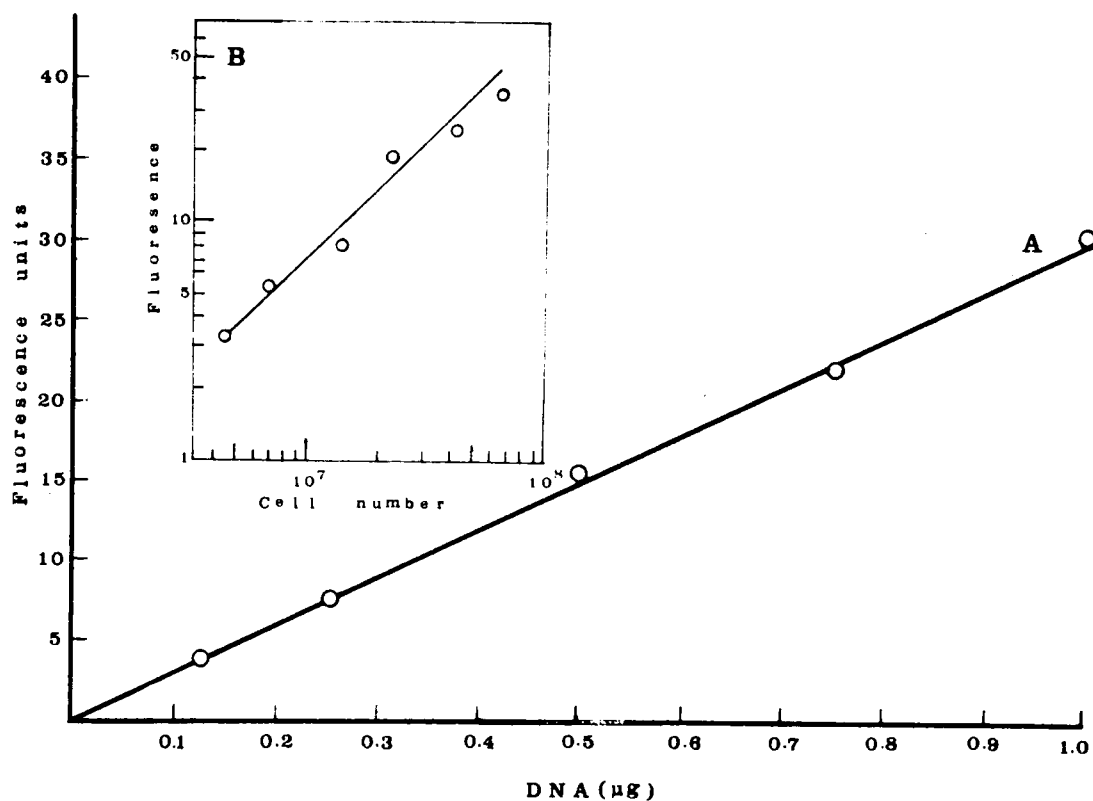


Figure 4. Determination of DNA by DABA Fluorometry

A. Constructed standard curve. B. Determination of DNA in x-bacteria. Samples of varying concentration of isolated x-bacteria were extracted as described in the text, and the residue was allowed to react with 100 μ l of 300 mg/ml DABA. The intensity of sample fluorescence which was proportional to the concentration of x-bacteria was converted into quantity of DNA from a standard curve.

CsCl-EtBr Density-Equilibrium Centrifugation

Centrifugation Profile of *E. coli* DNA

A typical pattern of CsCl-EtBr gradient centrifugation of plasmid DNA is shown in Figure 5-A. In *E. coli* strain GM8(pBR322) and strain GM8(RSF2124), plasmid DNAs were amplified by treatment with chloramphenicol. Total cell lysates from the two strains were pooled, processed and subjected to dye-density gradient centrifugation as described in the Materials and Methods section. In Figure 5-B, the density distribution throughout the gradient after centrifugation is shown, where the density of each fraction was determined by refractometry as described earlier. Two plasmid DNAs were located as simple, diffuse band below the clear chromosomal band. The density of the plasmid band was about 0.04 g/m greater than that of the chromosomal band. This result was in good agreement with other observations (e.g., Meynell, 1977). The two plasmid DNAs (pBR322 and RSF2124) could be resolved according to their molecular weights into individual plasmid DNA bands by agarose gel electrophoresis (see below).

Centrifugation Profile of x-Bacterial DNA

In order to detect and isolate plasmid DNA from x-bacteria, the lysate of x-bacteria was subjected to CsCl-EtBr centrifugation. The distribution of x-bacterial DNAs after initial centrifugation is shown in Figure 6-A. The chromosomal DNA band in region 1 was a clear band, while no clear satellite band was visible in region 2 at this stage, apparently due to an insufficient amount of plasmid DNA being present. To visualize plasmid DNA as a clear band, region 2 in Figure 6-A was pooled from more than 10 tubes and subjected to a second centrifugation

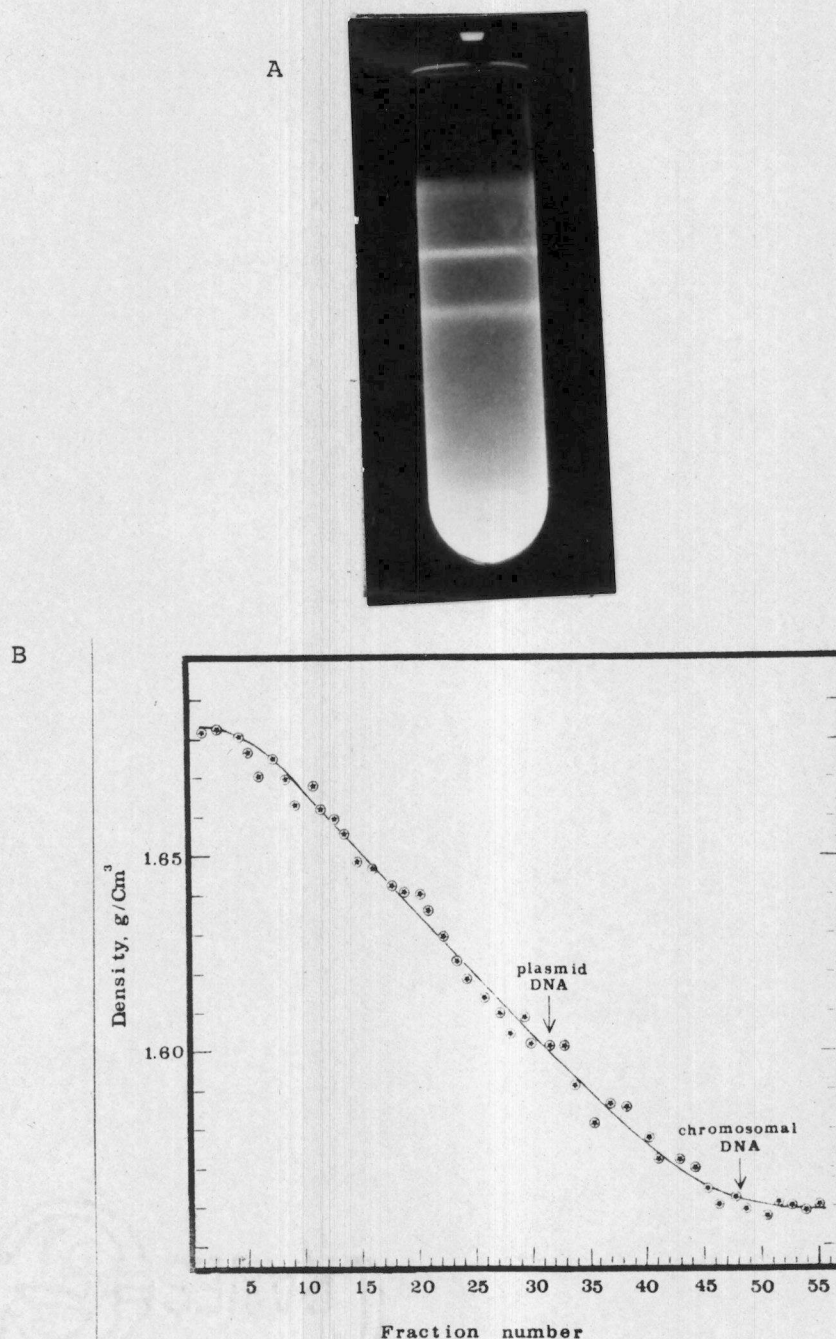


Figure 5. CsCl-EtBr buoyant density-equilibrium centrifugation of *E. coli* lysate.

A. Two DNA components seen after centrifugation. Upper band; chromosomal DNA. Lower band; plasmid DNA. B. Density distribution of tube content and position of two DNA components after centrifugation.

After lysis, *E. coli* DNAs were brought into 1.609 g/ml in density, 300 µg/ml in ethidium bromide and centrifuged for 60 hours at 40,000 rpm and 15°C using Ti-50 fixed angle rotor. After the run, fraction was collected by bottom puncture and the density was determined for each fraction.

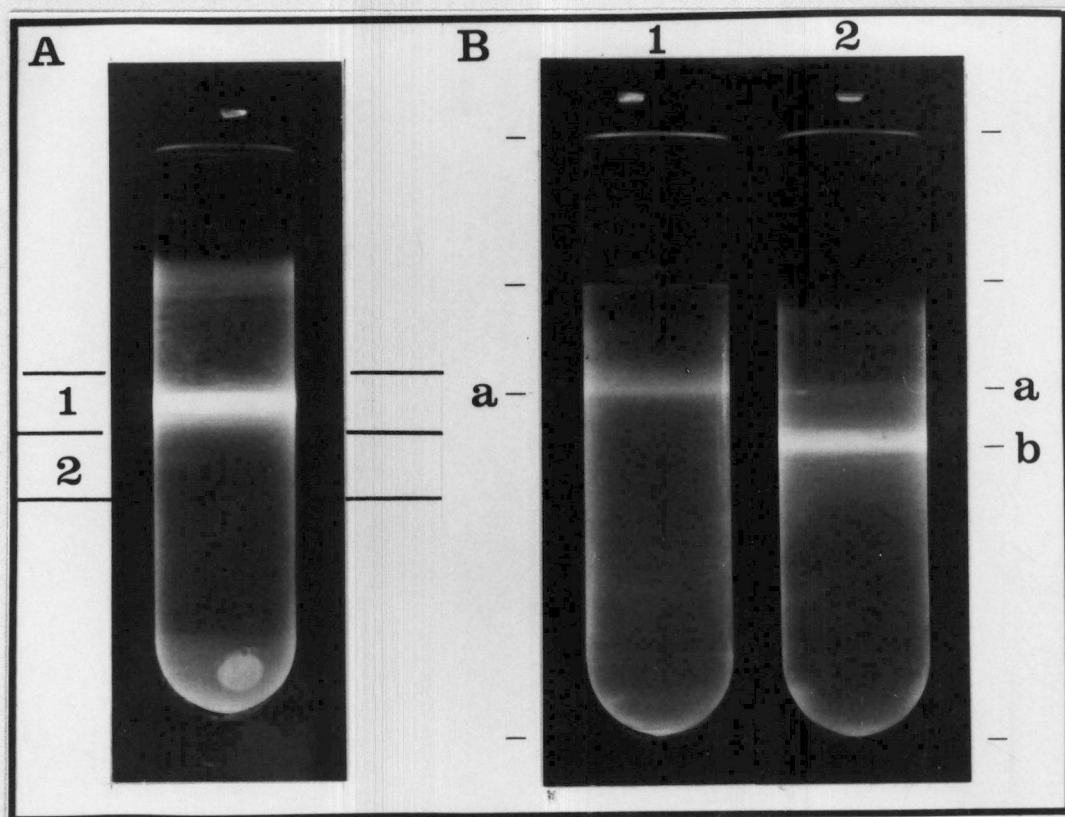


Figure 6. CsCl-EtBr buoyant density-equilibrium centrifugation of x-bacterial lysate.

A. After initial centrifugation; A1: Chromosomal DNA. A2: Presumptive region containing plasmid DNA which is not visible at this stage. RNA pellet is seen at the bottom of tube. B. Second centrifugation of fraction 1 and 2 from the initial centrifugation. Tubes B1 and B2 were centrifuged under identical conditions. B1: Chromosomal DNA (a) from tube A. B2: Fraction A2 pooled from 10 tubes. Note the visible plasmid DNA band (b). A faint band of contaminating chromosomal DNA (a) is also visible.

as described in Materials and Methods. This resulted in a clearly visible satellite band under U.V. illumination as shown in Figure 6-B, 2.

On the other hand, RNA was seen as a pellet at the bottom of the centrifuge tube (Figure 6-A) after initial centrifugation, but was absent after the second centrifugation (Figure 6-B).

Agarose Gel Electrophoresis

Electrophoresis Pattern of x-Bacterial DNA

The electrophoretic pattern of x-bacterial DNA is shown in Figure 7 together with that of pBR322 and RSF2124 of *E. coli* for comparison. When the total DNA extracted from x-bacteria by phenol extraction was electrophoresed, bulk chromosomal DNA obscured the other minor components (Lane 1). Two groups of ribosomal RNA are also shown in Lane 1 as diffuse bands together with other RNA components as a long tail. After some of the chromosomal DNA was removed by SDS-salt precipitation method (Gruerry, 1967), two plasmid DNA bands were clearly detectable above and below the chromosomal DNA band (Lane 2). When the DNA sample from CsCl-EtBr centrifugation was used, the two x-bacterial plasmid bands could be distinguished more clearly (Lane 3). The chromosomal DNA band in Lane 3 was due to contamination during fractionation of the plasmid DNA. RNA was completely removed from this sample and therefore its band is absent from Lane 3. The reference plasmids, pBR322 and RSF2124, could be directly detected after electrophoresis of the total DNA without removing chromosomal DNA (Lane 6), although these bands were not as

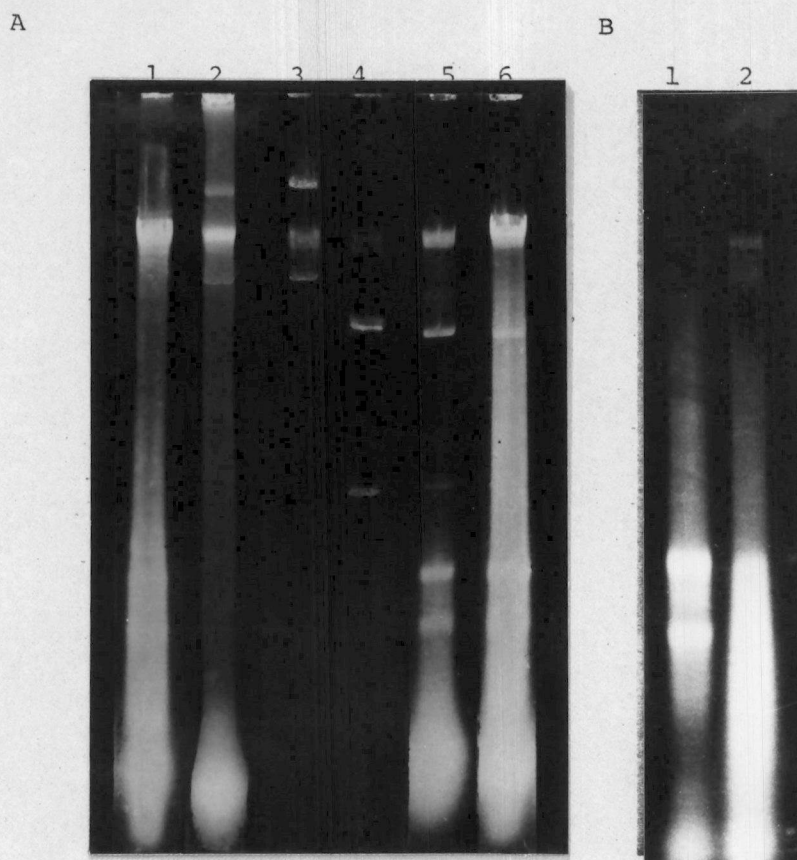


Figure 7. Agarose gel electrophoresis of DNA extracted from x-bacteria and *E. coli*.

A. Electrophoresis pattern of bacterial DNA. Lane 1: Total DNA from x-bacterial lysate. Only chromosomal DNA is seen in the upper portion. Lane 2: DNA of x-bacteria, after partial removal of chromosomal DNA by the SDS-salt clearing method. Two plasmid DNA bands are visible, one each above and below the chromosomal DNA band. Lane 3: DNA of x-bacteria, isolated by two cycles of CsCl-EtBr centrifugation. The two plasmid DNA bands are more clearly visible. RNA bands seen near the bottom in Lane 1 and 2 are absent. Lane 4: Combined DNA from two *E. coli* strains harboring pBR322 and RSF2124. DNA was isolated by CsCl-EtBr centrifugation of cleared lysate of *E. coli* cells. Top band; RSF2124 lower band; pBR322. Lane 5: Same as Lane 4 except that chromosomal DNA was partially removed by SDS-salt clearing method. Lane 6: Same as Lane 4, except that total DNA was isolated by phenol extraction and ethanol precipitation like DNA in Lane 1. Two plasmid DNAs are visible in this case. B. Electrophoresis pattern of amoebae DNA. Extensive degradation of DNA during the isolation of x-bacteria is shown. Lane 1: DNA trim xD residue after x-bacteria isolation. Lane 2: DNA from D residue after the same treatment with xD amoebae.

clearly distinguishable as those seen after chromosomal DNA had been removed by SDS-salt precipitation method or by CsCl-EtBr centrifugation (Lanes 4 and 5).

In a separate experiment, DNA from the residue of the xD amoebae was extracted after removal of x-bacteria and electrophoresed in agarose gels, in order to assure that the satellite bands present in x-bacterial DNA preparations were not the result of contaminating amoeba DNA. Bands corresponding to those of x-bacteria were not observed. In addition, DNA from nonsymbiotic D amoebae showed no corresponding satellite band when extracted and electrophoresed in the same manner.

Isolation of Plasmid DNA by Preparative Gel Electrophoresis

Plasmid DNAs could be obtained in a large scale by preparative agarose gel electrophoresis. The advantage of this method over CsCl-EtBr centrifugation was its simplicity and quickness. The lengthy centrifugation procedure could be avoided by this technique. However, in the case of x-bacterial DNA, chromosomal DNA had to be removed first by the SDS-salt precipitation method before electrophoresis. In Figure 8, the electrophoretic pattern of E. coli DNA after total DNA extraction is presented (A), together with that of x-bacterial DNA after SDS-salt precipitation (B). Each plasmid band was sliced with a razor blade and DNA was electrophoretically eluted from each gel slice by the procedure described in Materials and Methods section.

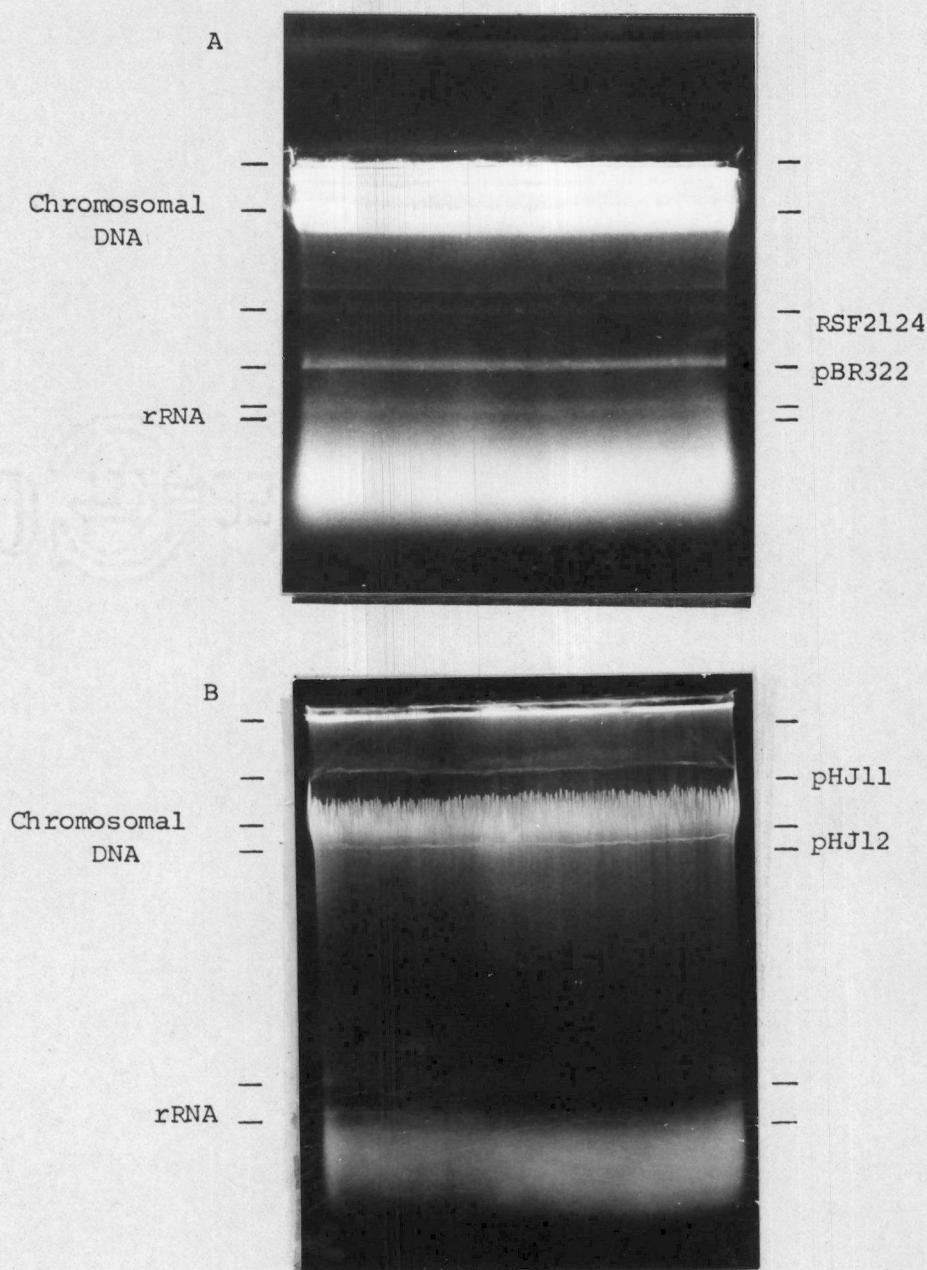


Figure 8. Preparative agarose gel electrophoresis of DNA extracted from x-bacteria and *E. coli*.

A. Total DNA from *E. coli*, strain GM8(pBR322) and *E. coli* strain GM8(RSF2124). B. DNA from x-bacteria after SDS-salt precipitation procedure. About 3 ml of a DNA sample was electrophoresed overnight at 80V, 4°C. After the run, only the surface of the gel was stained with EtBr (0.5 µg/ml) and visualized by UV illumination.

Molecular Weights of x-Bacterial Plasmid DNAs as
Determined by Gel Electrophoresis

The two plasmid DNAs of x-bacteria were electrophoresed in agarose gels with the six plasmid DNA markers of known molecular weight from E. coli and P. aeruginosa. The mobilities of these plasmid DNAs on agarose gel are shown in Figure 9-A. The six plasmids used as standards were: R1162 and pMG7, which were isolated by the Brij-DOC cleared lysate method; P3, R2, RSF2124 and pBR322 which were isolated by the SDS-salt precipitation method.

When the molecular weight of each plasmid was plotted against its mobility on a logarithmic scale, most of the plasmids fell on a straight line as shown in Figure 9-B. The mobility of each plasmid was expressed as its R_f value relative to the dye front and each point was obtained from at least three separate measurements. From the calibration curve in Figure 9-B, the molecular weights of the two x-bacterial plasmids designated as pHJ11 and pHJ12, were determined to be about 39×10^6 and 14×10^6 , respectively.

Electron Microscopy of Plasmid DNAs

λ DNA and Plasmid DNAs of E. coli

As internal standard, the electron micrograph of bacteriophage λ DNA (Figure 10-A) was converted into a circular form (Figure 10-B) by T_4 -ligase as described in the Materials and Methods section. Both types of λ DNA were mounted on grids by the formamide spreading method, in which further base pairing between sticky ends of linear

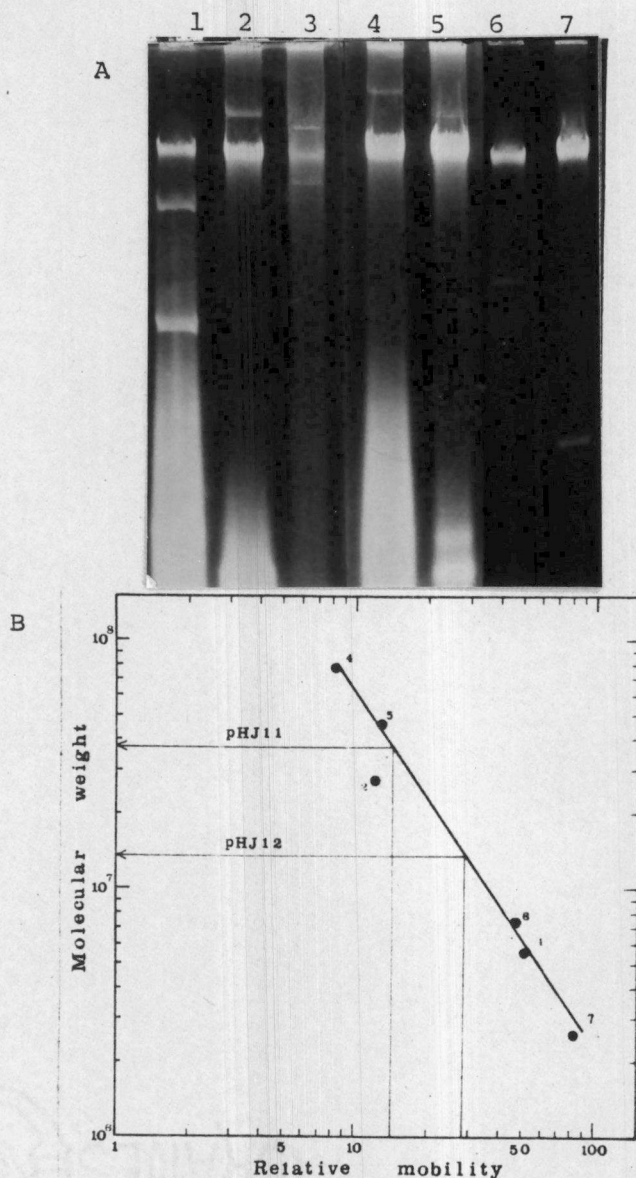


Figure 9. Determination of molecular weight by agarose gel electrophoresis.

A. Agarose gel electrophoresis of marker plasmid DNA. Conditions for plasmid isolation are described in the text. After phenol extraction, the DNAs were subjected to electrophoresis in 0.7% agarose gel and stained with ethidium bromide. A. Lane 1: Top band, chromosomal DNA; middle band, an unidentified component; lower band, R1162 (M.W. 5×10^6). Lane 2: Top band, pMG7 (M.W. 28×10^6). Lane 3: Top band, pHJ11; lower band pHJ12. Lane 4: Top band pIP64 (M.W. 78×10^6). Lane 5: Top band, R2 (M.W. 45×10^6). Lane 6: Lower band, RSF2124 (7.3×10^6). Lane 7: Lower band pBR322 (M.W. 2.6×10^6). B. Molecular weight calibration curve. The molecular weights of plasmid DNAs were plotted against mobilities. Each number represents plasmid DNA corresponding to that in Figure A.

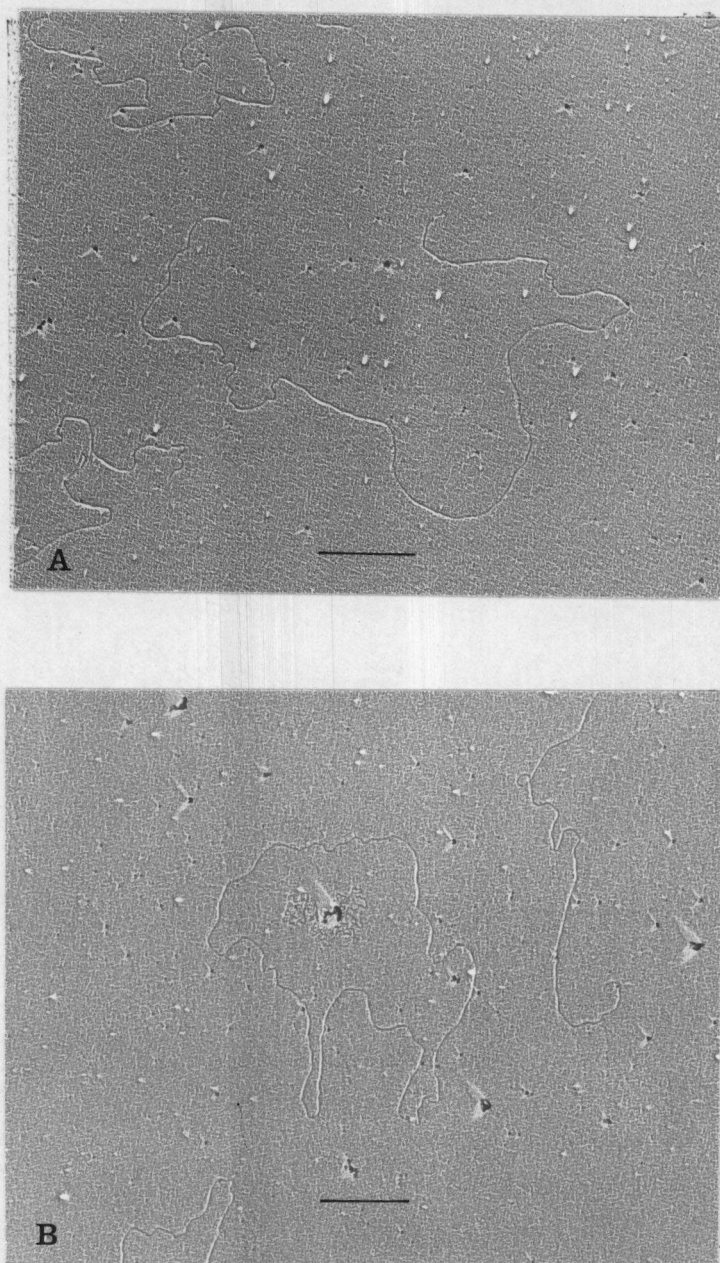


Figure 10. Electron micrograph of λ DNA.

A. Linear λ DNA. B. Circularized λ DNA by T_4 ligase. DNA was spreaded in the presence of formamide, picked on a parlodion-coated grid, and shadow cast with palladium-gold mixture at 6° angle. The bar represents 1 μ m.

λ DNA is interfered with. The measured contour length of both linear and circular λ DNA was 15.45 μm which was equivalent to 31.99 megadaltons, according to the known conversion factor of 1 μm = 2.07×10^6 daltons (Meyers et al., 1976). This molecular weight agreed with the known value of λ DNA (Fiandt et al., 1971).

As additional internal standards, pBR322 (Figure 11-A) and RSF2124 (Figure 11-B) from E. coli were examined together with x-bacterial plasmids on the same grid. These E. coli plasmids existed as open circular forms and thus no prior treatment for nick introduction was necessary. The contour lengths of these two plasmids were $1.328 \pm 0.08 \mu\text{m}$ for pBR322 and $3.655 \pm 0.01 \mu\text{m}$ for RSF2124. These lengths agreed with previously reported figures (Bolivar et al., 1977, So et al., 1976). Both electronmicrographs were taken with samples prepared by aqueous spreading technique using parlodion grids.

Plasmid DNAs of x-Bacteria

When satellite DNA of x-bacteria collected after CsCl-EtBr centrifugation was examined under the electron microscope after aqueous spreading, supercoiled molecules were observed as shown in Figure 12-A. After digestion with DNase-I or irradiation with X-ray, the plasmid DNAs were converted into open circular forms and were clearly identifiable (Figure 12-A and B). The longer plasmid DNA, pHJ11, had a contour length of $19.08 \pm 1.69 \mu\text{m}$ whis is corresponding to 39.5 ± 3.5 megadalton and the smaller plasmid, pHJ12, had a contour length of $6.84 \pm 1.11 \mu\text{m}$, equivalent to 14.15 ± 2.3 megadaltons. This is in good agreement with the electrophoresis data. The numbers were

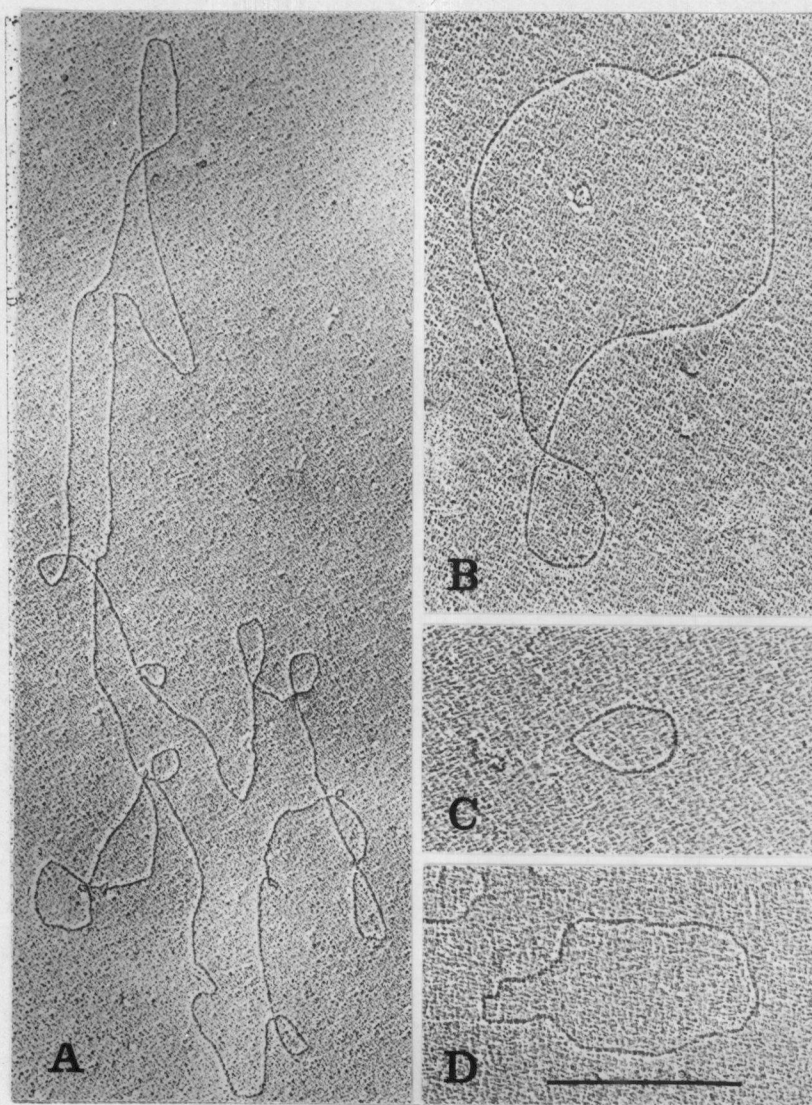


Figure 11. Electron micrograph of plasmid DNAs from x-bacteria and E. coli.

A. Large plasmid DNA (pHJ11) of x-bacteria with a molecular weight of 39×10^6 . B. Small plasmid DNA (pHJ12) of x-bacteria with a molecular weight of 14×10^6 . C. pBR322 of E. coli (M.W. 2.6×10^6). D. RSF2124 of E. coli (M.W. 7.3×10^6). pBR322 and RSF2124 were added as internal standards. The bar represents 1 μm .

The plasmid DNAs were mounted on parlodion-coated grids by aqueous spreading method and shadow cast with platinum-palladium mixture as described in Materials and Methods section.

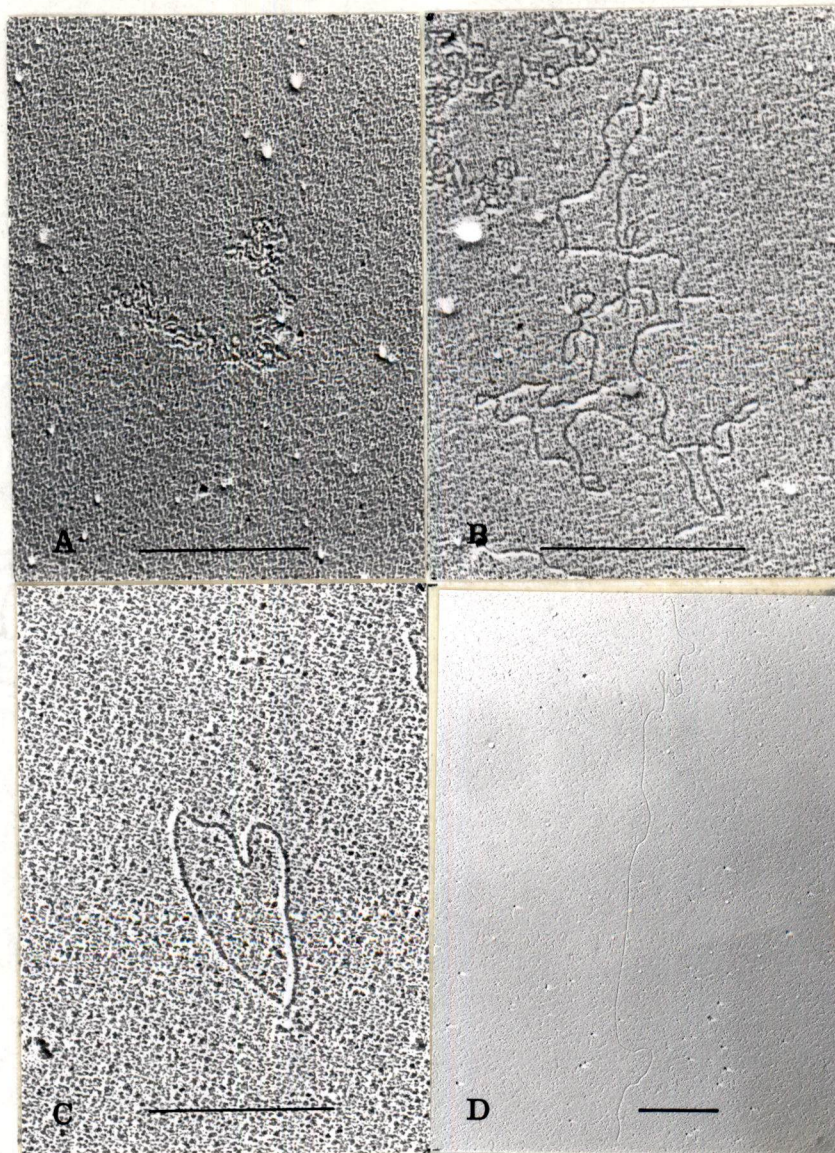


Figure 12. Electron micrograph of x-bacterial DNA

A. One of x-bacterial plasmid DNAs in tightly twisted form before nicking. B and C. Two minor groups of x-bacterial plasmid DNA components which were not resolved in agarose gel after electrophoresis. The molecular weight was 24.38×10^6 and 5.28×10^6 respectively. D. One of random fragments of chromosomal DNA from x-bacteria. The bar represents $1 \mu\text{m}$.

A, B and C were prepared by the aqueous spreading method using parlodion-coated grids and D was prepared by the drop-diffusion method using a carbon grid.

obtained from 15 to 50 measurements for each plasmid. In addition to pHJ11 and pHJ12, two other circular DNAs were observed as minor components (Figure 12-B and C). These minor groups of circular DNAs were not resolved in the gel after electrophoresis. The randomly fragmented chromosomal DNA from x-bacteria and E. coli had a size range of 10 to 30 megadaltons. One of these linear duplexes is shown in Figure 12-C which was prepared by the drop diffusion method using a copper grid covered with carbon film.

Pattern of Cleavage by Restriction Endonucleases

Chromosomal DNA

The patterns of cleavage in x-bacterial chromosomal DNA by four restriction endonucleases are shown in Figure 12-A. In this gel the chromosomal DNA was cleaved into at least 60 countable fragments by Eco RI, 70 fragments by Bam HI, 50 fragments by Hind III, and 30 fragments by Sal I.

Plasmid DNA

The cleavage pattern of x-bacterial plasmid DNAs is shown in Figure 13-B. The cleavage patterns of λ DNA, pBR322, and RSF2124 are also shown for comparison. The λ DNA was cleaved into 6 fragments by Eco RI, 5 fragments by Bam HI, 6 fragments by Hind III, and 2 fragments by Sal I (Figure 13-B, Lane 2 through 5) while pBR322 and RSF2124 were cleaved at one site by Eco RI, Bam HI, Hind III, and Sal I (Figure 13-B, Lane 11 through 14), which were in agreement with other published results. (Hedgpeth et al., 1972, Roberts et al., 1977, Old et al., 1975; Arrend et al., 1978). On the other hand, x-bacterial

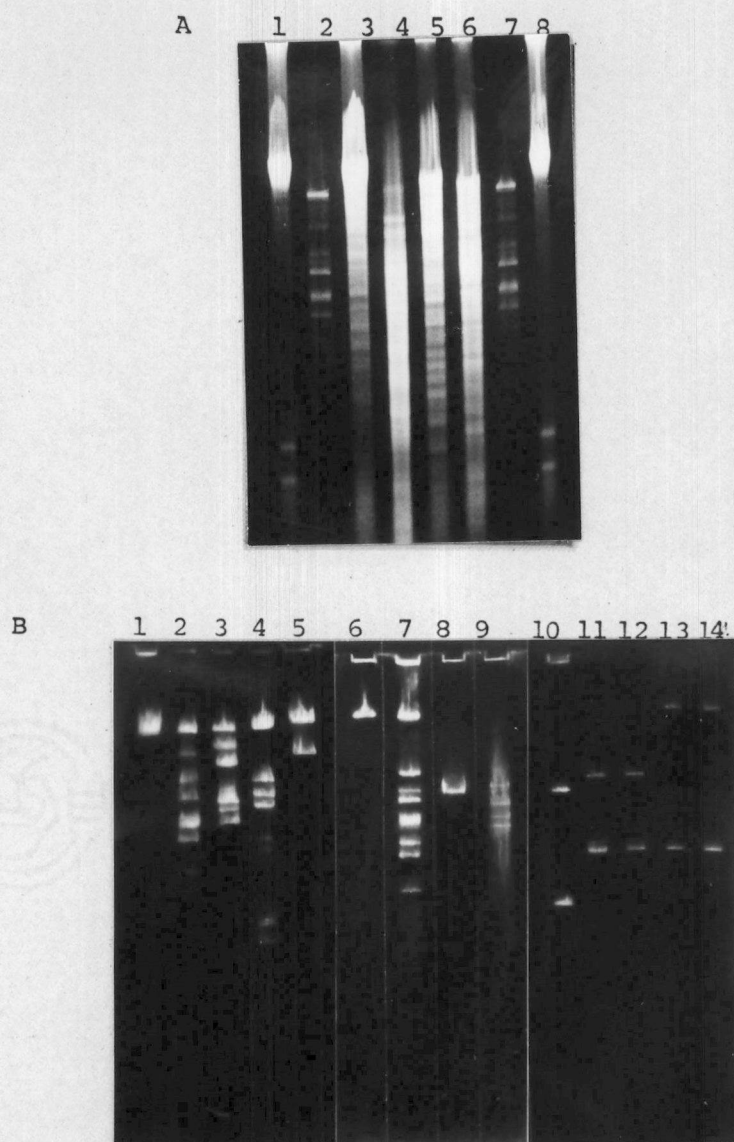


Figure 13. Pattern of cleavage by restriction endonucleases

A. Cleavage pattern in x-bacterial chromosomal DNA. Lane 1. Uncleaved x-bacterial chromosome DNA, Lane 2, λ DNA on Eco RI, Lane 3. x-bacterial chromosome on Sal I, Lane 4. x-bacterial chromosome on Hind III, Lane 5. x-bacterial chromosome on Bam HI, Lane 6. x-bacterial chromosome on Eco RI, Lane 7. λ DNA on Eco RI, Lane 8. Uncleaved x-bacterial chromosomal DNA. The two cleaved λ DNAs were put for comparison. B. Cleavage pattern in λ , RSF2124, pBR322, and x-bacterial plasmid DNAs. Lane 1 Uncleaved λ DNA, Lane 2. λ on Eco R-I, Lane 3. λ on Bam HI, Lane 4. λ on Hind III, Lane 5. λ on Sal I, Lane 6. Uncleaved pHJ11, Lane 7. pHJ11 on Bam HI, Lane 8. Uncleaved pHJ11, Lane 9. pHJ11 on Eco RI, Lane 10. Uncleaved RSF2124 (Top) and pBR322 (Bottom), Lane 11. RSF2124 and pBR322 on Eco RI, Lane 12. RSF2124 and BR322 on Bam HI, Lane 13. RSF2124 and pBR322 on Hind III, Lane 14. RSF2124 and pBR322 on Sal I.

plasmids of pHJ11 and pHJ12 was cleaved into several fragments by Bam HI and Eco RI respectively as shown in Figure 13-B, Lane 7 and Lane 9. For correct information regarding number of cleavage site, more experiments should be done.

Curing of Plasmid DNA

Curing with Intercalative Dyes

The concentration of a dye to kill 50% of xD amoebae during 1 week dye treatment and additional 3 days for recovery was 8×10^{-6} M for acridine orange (AO) and 7×10^{-6} M for ethidium bromide (EB). At these concentrations, surviving xD amoebae from AO treatment were less healthy than those from EB treatment. Also an AO-treated amoeba contained more x-bacteria than an EB-treated one and this seemed to be due to a higher cytotoxicity of AO to amoebae. On the other hand, the number of x-bacteria within EB-treated amoeba seemed unchanged as shown in Table 4. In both cases, no plasmid DNA bands were detected from the treated x-bacteria, even when the chromosomal DNA was removed by SDS/salt method as shown in Figure 14-C and D.

Curing by Temperature

After the incubation of xD amoeba at 27°C for 2 to 4 weeks, several changes in bacterial patterns were observed within amoeba: (1) reduced bacterial number per amoeba, (2) a change in morphology from rod shape to elongated, thread-like bacteria with multiple forms, and (3) a changed susceptibility to cell-lysing agents. The bacteria were dissolved both in 2N NaOH or 1% SDS. On the other hand, the number of bacteria decreased to one fourth of the original level

TABLE 4

The Elimination of pHJ11 and pHJ12 from x-Bacteria

Curing Agent	LD ₅₀ ^a (M)	Number of x-bacteria/xD amoeba Expressed as Ratio to untreated xD	Plasmids
Acridine orange	8 x 10 ⁻⁶	1.24	-
Ethidium bromide	7 x 10 ⁻⁵	0.94	-
High temperature (27°C) ^b	-----	0.24	-

^aLD₅₀ = the concentration of dye to kill 50% xD amoebae during 1 week treatment and 3 additional days for recovery.

^bxD amoebae were grown at 27°C for 3 weeks and allowed to recover for 1 week at 20°C.

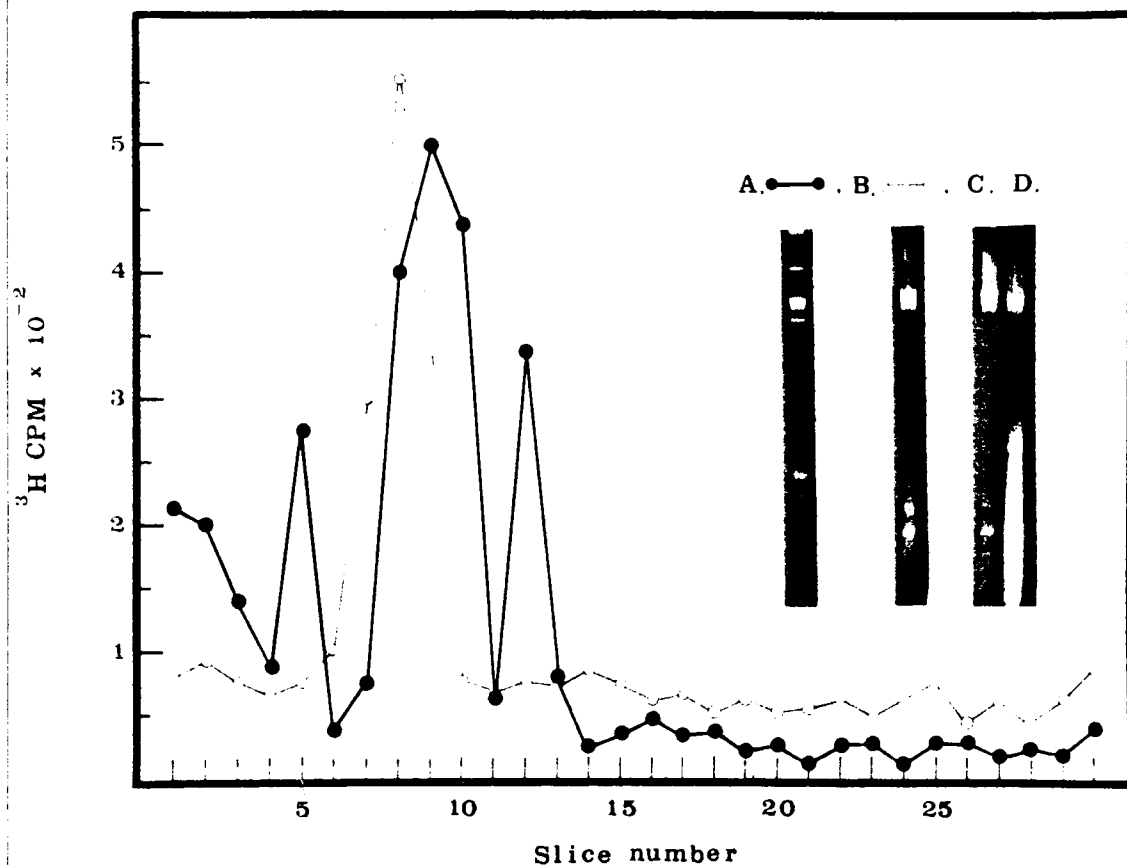


Figure 14. Curing of x-bacterial plasmid DNA by temperature treatment and intercalative dyes.

A. Electrophoretic pattern of partially purified plasmid DNA from untreated x-bacteria. B. DNA from high temperature (27°C) treated x-bacteria lacking both two plasmid DNA peaks. C and D. DNA from ethidium bromide and acridine-orange treated x-bacteria, respectively, showing the absence of both x-bacterial plasmid DNA bands.

after 1 week recovery in 20°C as presented in Table 4. However, no plasmid DNA peak was detectable from the DNA preparation after SDS/salt precipitation method. This is shown in Figure 14-B.

CHAPTER V

DISCUSSION

Detection and Isolation of Plasmid DNAs from x-Bacteria

The results presented in this dissertation clearly demonstrate that x-bacteria harbor two distinct species of plasmid DNAs. Throughout this work, three difficulties were encountered in detecting and isolating plasmid DNAs from x-bacteria. The first and foremost obstacle was the difficulty in obtaining sufficient amounts of x-bacteria. The second difficulty was the small amount of plasmid DNA present in x-bacteria. The final problem was x-bacteria's resistance to conventional bacteria lysis agents. Because of these difficulties, special methods were required to minimize plasmid loss and to detect the plasmids with small copy numbers.

To detect cryptic plasmid DNA molecules such as pHJ11 and pHJ12, simple agarose gel electrophoresis after extraction of total DNA from cell lysate is quite inadequate, because bulk chromosomal DNA obscures the minor components of two plasmid DNAs as shown in Figure 7, lane 1 (page 41). This method and simple sucrose gradient centrifugation seem to be applicable only to bacteria which harbor multiple copies of plasmid DNAs like pBR322 or RSF2124 of E. coli as shown in Figure 7, lane 6. To detect and isolate plasmid DNAs, especially those with small copy numbers, the chromosomal to plasmid DNA ratio should be reduced by an appropriate means. The most simple and commonly used method for preferential extraction of plasmid DNAs from the bacteria is

the cleared lysate method in which lysozyme and the nonionic detergent such as Brij-58 are used to produce a leaky spheroplast from which plasmid DNA can escape (Godson and Sinsheimer, 1967). Unfortunately, this method was not applicable to x-bacteria because they were resistant both to lysozyme and nonionic detergents. To eliminate this problem, both the cellulose nitrate method (Wohlhieter, 1966) and the alkaline sucrose gradient centrifugation method (Currier, 1976; Freifelder et al., 1971) were tested, but unsatisfactory results were obtained with both methods. In the case of cellulose nitrate method, a very small amount of pHJ11 and pHJ12 DNA seemed to be coprecipitated and lost together with the centrifuged cellulose nitrate particles on which denatured single stranded chromosomal DNAs have attached. When alkaline sucrose gradient centrifugation technique was used after shearing of detatured chromosomal DNA, pHJ11 and pHJ12 were not detectable as satellite bands, both by the ethidium bromide and the absorption at 260 nm by spectrophotometer. They were not detected presumably due to the presence of insufficient concentration of plasmid DNAs.

In spite of a significant loss of plasmid DNAs and contamination of chromosomal DNA, the SDS-salt precipitation method (Guerry et al., 1973) was quite simple and useful in detecting pHJ11 and pHJ12 from x-bacteria (Figure 7, lane 2, page 41 and Figure 8, page 43). Precipitation of over-saturated SDS crystals at 0°C and facilitation of crystal formation by high Na⁺ ion concentration (1M) seemed to preferentially coprecipitate chromosomal DNA due to large size and linear conformation leaving compact, small plasmid DNAs in the supernatant after centrifugation. By using a weaker shearing force,

it was possible to reduce the DNA contamination from chromosomal DNA in the sample. The loss of plasmid DNAs could also be minimized by re-extracting the first SDS-salt pellet with 1 M NaCl solution followed by the same procedures described in Materials and Methods section.

Detection and isolation of x-bacterial plasmid DNAs in a large quantity and with comparatively small loss were also effectively made by an additional run of CsCl-EtBr centrifugation after pooling and concentrating presumptive plasmid fractions from the first CsCl-EtBr centrifugation (Figure 6, page 39). To visualize plasmid DNA as a band, this method seemed to be particularly useful in bacteria where isotopic labeling of DNA is difficult as in x-bacteria. The disadvantage of this method, however, lied in the long time periods required for two cycles of density equilibrium centrifugation. When the SDS-salt precipitation method after re-extraction of first SDS-salt pellet was combined with one cycle of CsCl-EtBr centrifugation, better results were obtained in terms of speed and DNA recovery.

Preparative gel electrophoresis alone after the SDS-salt procedure was very effective in quick preparation of x-bacterial plasmid DNAs, although there was severe loss of plasmid DNAs. This method, therefore, seems to be very useful when multi-copy plasmids like pBR322 of E. coli are used or when the isolation of small copy plasmid is started with sufficient amounts of sample DNA.

Other Characteristics of x-Bacterial Plasmids

The two x-bacterial plasmids, pHJ11 and pHJ12, exist as covalently closed circles as evidenced by CsCl-dye density gradient centrifugation (Figure 6). If the two plasmids were present as open

circles or linear duplex, they would not have resolved as a satellite band under the main chromosomal DNA band. The two plasmids also have a supercoiled structure, because when they were examined under electron microscope after CsCl-EtBr centrifugation, twisted coil structures were observed as shown in Figure 12-A (page 49). After the introduction of a nick either by X-ray irradiation or digestion with DNase I, open circles were clearly visible (Figure 11-A and b, page 48). Both pHJ11 and pHJ12 were spontaneously converted into open circles and the larger plasmid, pHJ11, was more unstable which was eventually converted into linear duplex.

After three months storage at -100°C either in the xD amoebae, in purified bacteria, or with isolated DNA, the pHJ11 was converted into open circles and resolved as a satellite band between the CCC and chromosomal DNA in agarose gel electrophoresis. After six months, the pHJ11 DNA migrated together with chromosomal DNA which indicated that pHJ11 was converted into linear duplex. The small plasmid, pHJ12, was more stable than pHJ11, but its mobility was also changed after six month storage indicating a conformational change within the plasmid DNA. The two plasmid DNAs also disappeared from x-bacteria when they were stored for more than two days at room temperature after purification from amoeba. This seems to be due to the degradation of plasmid DNA since they were not detected in OC or even in linear form. Because isolation of plasmid DNA molecule is mostly based on CCC structure, experiments during this investigation were done with x-bacteria harvested less than 1 month from xD amoeba culture.

According to Clewell and Helinsky (1969), plasmid DNAs have two forms in terms of complex formation with protein. In case of complexed

plasmid DNAs like Col E, CCC undergo conformation changes into OC when the plasmids are treated with any condition which denatures protein. In that case, their position after CsCl/EtBr centrifugation coincides with that of chromosomal DNA, and as a result, they are lost during this procedure. In this sense, both pHJ11 and pHJ12 are of the non complexed type of plasmid DNAs because they are resolved as satellite bands in CsCl centrifugation after the treatment with detergent (SDS), proteolytic enzymes, heat, and high concentration of salt (CsCl).

The GC content of the two plasmid DNAs seems to be the same as that of chromosomal DNA. Although the melting point or sedimentation rate in analytical centrifugation was not measured, the plasmid DNAs were not resolved as a satellite band in preparative CsCl centrifugation without intercalative dye.

Judging from the size of the two plasmid DNAs, 39×10^6 and 14×10^6 daltons, it is unlikely that the smaller plasmid DNA is a dissociation product of the larger one. On the other hand, two minor components of circular DNAs with molecular weights of 24×10^6 and 5×10^6 respectively taken from CsCl/EtBr centrifugation might originate by error during replication of pHJ11 or pHJ12 because their copy numbers are extremely small so that they are not resolved on gel after electrophoresis. According to the size spectrum of plasmid DNAs made by Clowes (1972), plasmids larger than 26×10^6 daltons are regarded to have sex-factor characteristic. In this sense, pHJ11 may be transmissible while pHJ12 might be nontransmissible although a transmissibility test could not be made due to the lack of any marker on the two plasmid DNAs.

An attempt was made to estimate plasmid copy number per x-bacterial chromosome using labeled DNA and agarose gel electrophoresis. In this experiment, the two plasmid (pHJ11 and pHJ12) were not properly resolved as satellite bands on the gel and correct estimation of copy number could not be made. However, the copy number of pHJ11 and pHJ12 appeared to be present less than one per chromosome. The copy numbers for both plasmid DNAs could not be increased by treatment with chloramphenicol. These results strongly suggest that replication and maintenance of both plasmids are under stringent control. This idea is further supported by Clowes' suggestion that plasmids larger than 35×10^6 daltons are generally under stringent control and have less than 1 copy per cell (Clowes, 1972; Clewell, 1972). At present, the relationship between the two plasmid DNAs is not clear, and it is also not known whether they are contained in the same bacterium or in different ones. Considering the small copy number of plasmid, it is believed that certain bacteria do not carry any plasmid DNA at all. This suggests that plasmid DNA in x-bacteria could be nonessential both for the survival of the bacteria and for the maintenance of endosymbiosis.

The large x-bacterial plasmid pHJ11 was cleaved into at least seven fragments by the restriction endonuclease Bam HI while the small plasmid pHJ12 was cut into three fragments by Eco RI. Both enzymes cut pBR322 and RSF2124 of E. coli at 1 site yielding each molecule of linear duplex plasmid DNA. The initial hope was to combine pBR322 or RSF2124 to pHJ11 or pHJ12 if one of the two x-bacterial plasmids had one cleavage site for these enzymes. To obtain such an enzyme by which pHJ11 or pHJ12 is singly restricted at a nonessential

part of the gene, more tests on the proper variety of enzymes are necessary.

Use of x-Bacteria in Gene-Cloning Experiment

For recombinant DNA research, four elements are essential:

(1) an enzyme system for breaking and rejoining DNA molecules, (2) a suitable cloning vehicles replicating itself with or without a foreign DNA segment, (3) a suitable means of transfection, and (4) a method for selecting a clone of recipient cells which have acquired the chimeric DNA (cohen et al., 1977).

If one is to carry out recombinant DNA research, a stringent safety system should be adequately prepared; since (1) the functioning of combined genes from different source is frequently unpredictable, (2) the outcome of this research could damage the biosphere, and (3) the technology could be a primary hazard to the human species because most research is being done in the strains of E. coli which can infect man (NIH Guide Lines, Curtis III, 1976).

To develop a biolotical containment system, Curtis III, in 1976 constructed a mutant strain of E. coli, χ 1776 which is now widely used as a safe host for recombinant DNA research (Curtis III et al., 1977). The properties of χ 1776 include (1) the inability to colonize and survive in intestinal tract of warm blooded animals or to grow nonlaboratory conditions because of the loss of the ability to synthesize a rigid cell wall, (2) the inability to synthesize DNA in the absence of thymine and resulting self degradation of DNA and (3) a high level nalidixic acid resistance by which the monitoring of host strain may be accomplished.

The x-bacteria in endosymbiosis with a strain of amoeba seems to possess a number of natural safety features. First of all they have lost their ability to grow outside of amoeba. This inability to grow outside the host which seems to be the result of a deletion mutation makes the x-bacteria safe for recombination DNA work. Moreover, the x-bacteria have a thermosensitivity whose permissive temperature for growth is below 27°C (Jeon and Ahn, 1978) so that they cannot infect man or other warm blooded animals. This thermosensitivity to animal was proven by the fact that x-bacteria injected into a rabbit was not able to survive (J.H. Tullock, unpublished result). This thermosensitivity provides an additional containment of x-bacteria.

The x-bacteria degrade their plasmid DNAs when they are stored outside the amoeba cytoplasm at room temperature for two days. The reason for this disappearance of plasmid DNAs is unknown; however, this fact obviously minimizes the chance of conjugal transmission of plasmid DNAs with or without a segment of foreign DNA to another bacteria.

The x-bacteria also have characteristics that permit monitoring of the strain. They are resistant to lysozyme in vitro and avoid digestion when they are fed to amoeba. These properties will allow selection of the x-bacteria when they are mixed with other strains of bacteria.

Although x-bacteria possess several natural features comparable to those of χ 1776, it also has some inadequate properties for recombinant DNA research. The main obstacle for such experiments is the difficulty in obtaining large amounts of plasmid DNAs from the

x-bacteria. This is mainly due to the fact that they can be obtained only from amoeba culture, they harbor plasmid DNAs in a small copy number, and they are hard to lyse. Due to these properties, x-bacteria system with present plasmid DNAs seems to be inadequate for use as a genetic engineering system. Such a system requires large amounts of gene products produced by a cloned gene. This problem could, however, be solved by introducing and using other known conventional plasmid DNAs into the x-bacteria. On the other hand, other gene cloning experiments which do not require large quantity of gene products could be done with this system. For example, studies of toxin do not require large quantities of gene products, and may therefore be a useable maker for use with the x-bacteria system.

Role of Plasmid DNAs in x-Bacteria

Characterization of the correct plasmid-determined function of the two x-bacterial plasmid DNAs was extremely difficult because the bacteria did not grow in culture conditions in vitro, therefore, tests have to be indirectly made within hosts cytoplasm. However, tests in vivo, such as antibiotic resistance test, were severely interfered with by other cytoplasmic components within the amoeba so that clear interpretation of the results was very difficult.

On the other hand, after a certain period of time from infection, x-bacteria are found in bacterial vesicles (Jeon and Jeon, 1976) which separate them physically from the amoeba cytoplasm. Due to this structure, x-bacteria may not need further protective devices from the digestive enzymes present in the amoeba cytoplasm. However,

during the early stage of infection, they would be directly exposed along with other ingested food to such digestive enzymes. In this case, they must have some mechanism to avoid digestion by amoeba, if they are to establish endosymbiosis.

In view of the resistance of x-bacteria to lysozyme in vitro, they must also be resistant to lysozyme present in amoeba cytoplasm during infection. However, this lysozyme resistance cannot alone explain the resistance of x-bacteria to digestion during early infection because other lysozyme-resistant bacteria such as Staphylococcus aureus were also digested by amoeba when fed to amoeba, as were other food organisms. Therefore, to destroy invading microorganisms, amoeba must have other enzyme(s), for example, lysostaphin in Staphylococcus aureus (Shindler, 1964) which can lyse cell walls resistant to lysozyme.

To explain the uniqueness of the bacterial cell wall, one must look to the protein composition of outer membrane or at the additional layers present outside the outer bacterial membrane (Glauert et al., 1969; Nikaido, 1973; Schleifer, 1972). In this regard, the device of x-bacteria to escape digestion by amoebae may be explained in terms of cell wall composition within bacteria. The cell wall of x-bacteria seems to be coated with an extra layer of protein. This view is supported by the observation that x-bacteria can be partially lysed by high concentrations of detergent combined with a proteolytic enzyme. Additional evidence includes a decreased cell wall rigidity after the removal of proteins, which causes the bacteria to be more susceptible to osmotic shock and mild sonication.

The protein component of the x-bacterial cell wall which might be responsible for this unique property during infection could be coded

by genes on the plasmid DNA (olson, 1977; Costerton, 1977). The x-bacteria treated with plasmid-curing agents supports this view indirectly. When the x-bacteria within amoeba were treated with agents such as ethidium bromide or acridine orange, no plasmid was detected in the bacteria and the bacteria were unable to infect the D amoeba. However, it is also possible that the agents could affect infectibility by damaging the viability of x-bacteria.

The other indirect evidence to support above view is the connection between the copy number of plasmid per cell, which is less than one, and the infectivity of isolated x-bacteria. When isolated, all x-bacteria were not capable of infecting amoeba; instead, only a small fraction was able to infect the amoeba while a considerable fraction seems to be destroyed by the amoeba (Ahn and Jeon, 1979). The fractions which were digested by amoeba could be x-bacteria which might have arisen from spontaneous curing of plasmid DNA or segregant lacking the DNA. A long time after the establishment of endosymbiosis, compartmentation of x-bacteria within bacterial vesicles could provide x-bacteria with a chance for spontaneous curing of plasmid DNA. Among such mixed populations, a fraction of x-bacteria which still had plasmid DNA would infect other amoeba and establish new symbiosis.

The most direct way to prove the involvement of plasmid DNAs in the establishment of endosymbiosis may be to incorporate the plasmid DNAs into other bacteria which are normally destroyed by amoeba and see if resistance arises after infection into D strain of amoeba. However, due to the lack of a phenotypic marker on the x-bacterial plasmid DNAs, there is almost no way to select the

bacteria which pick up the plasmid DNAs. To solve this problem, a chimeric plasmid molecule between x-bacterial plasmid DNA and other known plasmid DNAs with known phenotype, such as pBR322 might be made first. After the transfection of such recombinant plasmid DNA, the bacteria which harbor the new plasmid DNAs can be selected for by the known phenotype of the marker plasmid after which a resistance test could be easily made.

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