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I am submitting herewith a thesis written by Young Mok Park entitled "Role of Plasmid DNAs in the Protection of Endosymbiotic X-Bacteria from Digestion by Amoebae." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Zoology.

Kwang W. Jeon, Major Professor

We have read this thesis
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Accepted for the Council:

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ROLE OF PLASMID DNAs IN THE PROTECTION OF ENDOSYMBIOTIC X-BACTERIA FROM DIGESTION BY AMOEBAE

A Thesis

Presented for the

Master of Science

Degree

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ABSTRACT

The symbiotic X-bacteria that have established an obligatory symbiosis with the xD strain of Amoeba proteus contain two plasmid DNAs, pHJ11 and pHJ12. In this study, the effect of high temperature on the two plasmid DNAs of X-bacteria was studied by electron microscopy and agarose gel electrophoresis.

By examining the growth rates of symbiotic and nonsymbiotic amoebae in single cell cultures at different temperatures and the average number of symbionts per amoeba, it is shown that symbiotic amoebae cultured at 27°C grow at the same rate as those grown at 22°C during the first 8 days, after which they cease to divide, duplicating only three divisions on the average, and then all cytolyze by the 14th day. At 27°C, the average number of symbionts per amoeba declines rapidly, and the symbionts disappear completely from amoebae after 10 days. The depletion of symbionts appears to be the cause of death of xD amoebae which closely follows the disappearance of symbionts.

The growth characteristics of amoebae in mass cultures at 27°C are somewhat different. They survive longer, i.e., over a month, and the average number of symbionts per amoeba in the mass culture at 27°C decreases slower than those in single-cell cultures. The reasons for these differences are not clear, but it may be that the high density of amoebae and of symbionts per unit area and unit volume of medium causes somehow favorable (compensation) effect on them so that they can alleviate the adverse effect of the higher temperature.

Agarose gel electrophoretic studies of DNAs show that the decrease of plasmids of X-bacteria can be detected from the 8th day of culture at 27°C, whereas X-bacteria still remain healthy. No plasmids were found in X-bacteria after 14 days of culture at 27°C. At that time X-bacteria started to show the symptom of digestion by amoebae as examined by electron microscopy; that is, lysosomes fused with symbiotic vesicles which contain X-bacteria. The symbiotic bacteria appear to be digested by amoebae after the symbionts have lost the plasmids which they normally harbor.

From this study, the role of plasmids in the symbiotic relationship between amoebae and X-bacteria can be speculated; the protection of X-bacteria from digestion by amoebae. It is possible that the plasmids found in X-bacteria encode some polypeptides which are somehow inserted into the bacterial outer membrane and/or the symbiotic vesicle membrane, so that X-bacteria and symbiotic vesicles are protected from digestion by amoebae. Thus, X-bacteria can succeed in the establishment of endosymbiosis by protecting themselves during initial stages of infection when the X-bacteria are directly exposed to the amoeba cytoplasm and X-bacteria can maintain successful symbiotic relationship with amoebae by prevention of lysosomal fusion with symbiotic vesicle membranes during sustained multiplication of both host and symbiont.

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CHAPTER I

INTRODUCTION

A plasmid is an extrachromosomal genetic element which is physically separated from the chromosome of the cell and is able to perpetuate itself within the cell. This plasmid is usually 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 (Broda, 1979; Bukhari et al., 1977; Clowes, 1972; Levy et al., 1981; Timmis and Puehler, 1979).

The size of most plasmids falls in the range of one to a few hundred megadaltons. Plasmids usually exist as a supercoil of covalently-closed circles of DNA (CCC or form I) within the cell. Because of the sealed ends and the three dimensional characteristic of CCC molecules, plasmid DNAs have distinct physical and chemical properties different from those of open circular (OC, relaxed circle, or form II) or linear duplex (form III) DNA. Although the content of plasmid DNAs within cells is usually low, the special physicochemical properties of the plasmid molecules allow their separation from the linear duplex fragments of bulk chromosomal DNA. The physicochemical separation methods of plasmid include alkaline sucrose-density-gradient centrifugation (Studier, 1965), cellulose-nitrate adsorption chromatography (Cohen, 1969; Wohlheiter, 1966), dye-buoyant-density CsCl ultracentrifugation (Radloff et al., 1967; Bauer and Vinograd, 1968) and agarose gel electrophoresis (McDonell et al., 1977; Mickel et al., 1977).

Historically, the existence of plasmids in nature was undetected until World War II when antibodies 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. This view failed, however, to explain the resistance acquired, at the same time, to multiple drugs and a sudden gain or loss of such phenotypic traits. It has been shown that transfer of multiple drug resistance is attributable to a type of plasmid that is now called R factor (Akiba et al., 1960).

In nature, bacterial plasmids are found in many forms and are usually recognized by the functions they determine. There are three types of well-known plasmids: (1) F or sex factors which promote bacterial conjugation (Hayes, 1968), (2) R factors which, in addition, confer resistance to the host against single or, more frequently, multiple antibiotics (Falkow, 1975; Mitsuhiashi, 1977), and (3) Colicinogenic factors which produce bacteriocin that inhibits the growth of strains related to themselves but not to the host cell (Reeves, 1972). Besides the above well-known plasmids, it has only recently been recognized that plasmids are commonly found in bacteria (see the appendices in Bukhari et al., 1977). For instance, there are a variety of plasmids in a large number of different organisms possessing quite different functions, such as, degradative plasmids in Pseudomonas (Wheelis, 1975), tumorogenic plasmids of Agrobacterium (Tooze, 1973), antibiotics producing plasmid of Streptomyces (Hopwood and Mervick, 1977), gas vacuole and toxin-producing plasmids of cyanobacteria (Lau et al., 1980), man-made environmental pollutant dissimilation plasmids of Acromobacter (Ahmed and Fiocht, 1973), plasmids in yeast (Guerineau, 1979). Some closed circular DNAs are found in higher eukaryotes, such as tabacco (Wong and Wildman, 1972), Drosophila (Stanfield and Helinski, 1976) etc, even though their functions are unknown.

The reason for the existence of so many plasmids 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 whereas 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 of those genes (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. Anytime there arose a need for the function of these genes by changing environmental circumstances they could be spread quickly to all the cells of a progeny population either if the plasmid carried a transfer factor as the plasmid intergrated, or if other rare cells contained transfer factors which could interact with other nontransmissible plasmids to form transmissible plasmids (Anderson, 1968). 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).

Interestingly, plasmid DNAs have also been found in the symbiotic bacteria of a number of hosts including plants and protozoa; i.e., Rhizobium bacteria of plants in the legume family (Bishop et al., 1977; Verma and Long, 1982), a number of Kappa symbionts of Paramecium tetraurelia (Dilts, 1977), X-bacteria of Amoebae proteus (Han and Jeon, 1980) and xenosome, a gram-

negative symbiotic bacteria, of Paramecium acutum (Soldo, 1983). From the viewpoint of "species-wide control of gene-pool synthesis", if these plasmids of symbionts carry any of the genes which produce proteins, and if these proteins are involved in a series of complex interactions between the hosts and symbionts for the establishment of stable endosymbiosis (such as surface recognition, entry, avoidance of digestion, and sustained multiplication of hosts and symbionts), the symbiont population would economically and enormously increase the total gene pool which is necessary for the growth and multiplication of the symbionts inside hosts.

The best-known symbiotic bacteria harboring plasmids are the Gram-negative bacteria, Rhizobium species, whch have a symbiotic relationship with legumes. Many important legume crop plants such as soybeans, groundnuts, beans, peas, clover and alfalfa possess the symbiotic nitrogen-fixing relationship between Rhizobium and their roots. The infection procedure begins by penetration of the bacteria at the tip of root hairs. An infection thread is formed within the root hair by invagination of the plant cell wall. The bacteria multiply inside this thread. As the nodule develops the infection threads continue to penetrate the host cells and the bacteria near the tips are pinched off, surrounded by plant membrane, and liberated into the cytoplasm. These forms of Rhizobium are known as bacteroids; owing to the loss of much of bacterial cell wall they are pleimorphic and much larger than free-living Rhizobium. The bacteroids synthesize nitrogenase which yields ammonia that is exported to the plant cytoplasm for assimilation and subsequent transportation to the rest of the plant.

Since Nuti et al. (1977) first demonstrated in R. tritolii and R. leguminosarum, the presence of plasmids of 100 megadalton, it has become clear that such plasmids are widespread in other species also. There does not appear to be a pea nodulation plasmid of uniform molecular weight. Several reports have shown that genes required for nodulation (nod) and for nitrogen fixation (fix) are plasmid-carried. In some species the components of the nitrogenase complex (nit gene) have been shown to be plasmid-carried. It is also apparent that the genes concerned with the infection process are clusted in one plasmid (Johnston et al., 1981).

Similar to the plasmids of Rhizobium, the plasmids of the other symbiotic bacteria have been suggested to play important roles in the symbiotic relationship between host and their symbionts; for example, the roles of the plasmid of xenosome of Paramecium in the infective process (Soldo, 1983), and those of the plasmid in the Kappa symbionts of paramecium in the killing trait (Preer et al., 1974; Dilts, 1977) have been suspected.

Two plasmids were also isolated from symbiotic X-bacteria by Han and Jeon (1980). The initial observation of a symbiotic relationship between an unidentified strain of Gram-negative rod-shaped bacteria and a strain of Amoeba proteus was made in 1966 (Jeon and Lorch, 1967). The D strain of A. proteus (D amoebae) was first noted to be infected with a large number of rod-shaped bacteria (X-bacteria). The relationship between X-bacteria and the infected amoebae (xD amoebae) was shown to be a harmful intracellular parasitic relationship. The infecting bacteria were initially virulent to host cells. However, within a few years, the relationship between the host and symbionts

changed into an obligatory endosymbiotic relationship as shown by the results of micrugical studies (Jeon, 1972; Jeon and Jeon, 1976).

The symbiotic association has bestowed some characteristics on the host: xD amoebae (1) have lost the capacity to grow at temperatures above 26°C, and (2) have a decreased capacity to resist starvation. In view of these findings, the amoeba-bacteria symbiosis has been considered as a model system for the study of partner integration at the subcellular level and the possible origin of eukaryotic cell organelles (Margulis, 1976; Smith, 1979; Taylor, 1979).

At present each symbiotic xD amoeba contains about 45,000 X-bacteria in membrane-bounded symbiotic vesicles which separate them physically from the amoeba cytoplasm (Jeon and Jeon, 1976). X-bacteria have the following characteristics: they cannot grow outside the amoebae, they have rigid cell walls so that they can resist digestion either by amoebae or by other enzymes such as amylase, cellulase, lipase, and trypsin (Han, 1979), they cannot grow very well at temperatures above 26°C (Jeon and Ahn, 1978), and they carry two plasmid DNAs, designated as pHJ11 and pHJ12, with molecular weights of 39×10^6 and 14×10^6, respectively (Han and Jeon, 1980).

The roles of 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 (Han and Jeon, 1980). One prerequisite for X-bacteria to establish endosymbiosis with the amoebae is to avoid digestion by the amoebae. Therefore, X-bacteria should require a protective device during the initial stages of infection when they are directly exposed to the amoeba cytoplasm (Ahn and Jeon, 1979).

X-bacteria seem to have a protective device within their cell wall, which has the ability to resist several digestive enzymes and a concentrated supernatant from amoeba homogenate. Hence, the cell wall of X-bacteria may possess a unique chemical composition and/or structure (Han and Jeon, 1980). It may be that the plasmid DNAs found in X-bacteria encode some of the unique protein components of the outer membrane like other bacteria (Costerton, 1977; Olsen et al., 1979; Shapiro et al., 1980; Bishop et al., 1977)). The X-bacteria treated with plasmid-curing agents support this view indirectly. When the X-bacteria within amoeba were treated with plasmid-curing agents such as ethidium bromide or acridine orange, no plasmid was detected in the bacteria and the bacteria were unable to infect D amoebae (Han, 1979).

A more direct approach to determine the involvement of plasmids in the protection of X-bacteria from digestion by amoebae would be to check if X-bacteria being digested in xD amoebae grown at 27°C still have their plasmids. If one or both of the plasmids disappear from X-bacteria before the latter are digested, it might suggest that the plasmids are needed for the protection of X-bacteria.

The main objective of this study was to test the hypothesis that either one or both of X-bacteria plasmids were responsible for the maintenance of the symbiotic relationship between amoebae and X-bacteria, that is, to determine the relationship between the disappearance of the two X-bacteria plasmids and the loss of viability of X-bacteria at temperatures above 27°C.

CHAPTER II

MATERIALS AND METHODS

A. Culture of Organisms

1. Amoebae

In this study, two strains of Amoeba proteus, D and xD, were used. The D strain originated from Sister Monica Taylor (Glasgow, Scotland) in 1948, and the xD strain, a bacteria-infected variant strain, was first noted in D amoeba cultures in 1966 (Jeon and Lorch, 1967).

Mass culture. To obtain a large number of amoebae, the xD strain of Amoeba proteus was cultured with axenically grown Tetrahymena as a food organism in Pyrex baking dishes (34x22x4 cm) containing about 1 liter (ca. 2cm in depth) of modified Chalkley's medium (Jeon and Jeon, 1975). For convenience of handling and maintenance of high amoeba growth rate, the population of amoebae in each dish was kept at about 3.5×10^5. Amoeba culture dishes were changed every other day to maintain cleanliness; the old culture medium was aspirated, and the scum, molds and floating amoebae were decanted to another dish. The cells that attached to the bottom of the dish were transferred to a clean dish with fresh Chalkley's medium. Then the amoebae were fed every day or every other day, depending on the need of amoebae, with freshly harvested Tetrahymena (4.6×10^6 Tetrahymena for every other day feeding or 2.6×10^6 Tetrahymena for every day feeding per dish containing 3.5×10^5 amoebae). Amoebae were cultured at 22°C and 27°C.

After the amoebae attained a maximum growth number, they were starved for 3 days to eliminate undigested food materials. The starved cells were then harvested by centrifugation for 7 seconds at 150 g and washed once with 0.9% NaCl to remove divalent cations from the medium. Each dish containing 3.5×10^5 amoebae yielded about 0.9 ml of packed cells. The packed amoebae were quickly frozen in dry-ice acetone and stored at -70°C until used to isolate of X-bacteria.

Single cell culture. Healthy amoebae of both strains from mass culture were transferred singly to Syracuse watch glasses containing 1 ml Chalkley's medium (Hawkins and Danielli, 1963). Amoebae were counted, washed, and fed three times a week, by adding medium containing a concentration of 1,000 Tetrahymena / ml Chalkley's. Both strains of amoebae were grown in single cell cultures for two weeks at 22°C and 27°C. The mean generation (MGT) of each experimental group was calculated by the following formula (Buchanan and Fulmer, 1928).

$$MGT = \frac{t \cdot \log 2}{\log b - \log a}$$

a = initial number of amoebae
b = final number of amoebae
t = number of days of experiment

2. Tetrahymena pyriformis (GL strain)

Tetrahymena was cultured axenically in the medium described by Goldstein and Ko (1976). The culture medium consisted of 4% of proteose peptone (W/V), 0.4% of liver concerate (W/V), and salt and vitamin solutions. After inoculating 5ml of fully grown Tetrahymena culture into 200 ml of medium in a 1000-ml flask, the cells were grown for 2 days at room temperature in a shaker bath. For harvesting, Chalkley's solution equal to one-half volume of culture (100 ml) was added and the culture was left shaking for 10 min to

minimize an osmotic shock. Tetrahymena were harvested by centrifugation for 1 min at 350 g and washed twice in Chalkley's medium. The yield was about 6 to 8 ml of packed Tetrahymena from a 200-ml culture. The packed Tetrahymena were then suspended in 150 times volume of the Chalkley's medium which yielded a density of about 1.3×10^5 Tetrahymena / ml (optical density 0.4 at 515 nm).

3. Other bacterial strains

Plasmids from four strains of bacteria were used as molecular weight markers for the study of the two X-bacteria plasmids. Two were strains of E. coli, strain GM8 (pBR322) (Bolivar et al., 1977) and strain GM8 (RSF2124) (So et al., 1966) harboring pBR322 and RSF2124 plasmid, respectively. The other two strains of bacteria, E. coli V517 bearing 8 plasmids (pVA517A, 517B, 517C, 517D, 517G and 517H) (Macrina et al., 1978) and Pseudomonas putida (arivilla) strain ATCC 23793 (Williams and Murray, 1974) maintaining TOL plasmid were kindly provided by Dr. G.S. Sayler, Department of Microbiology, The University of Tennessee.

Both of the two E. coli GM8 strains were cultured in antibiotic broth medium containing antibiotics at 37°C. E. coli V517 were grown in antibiotics medium without antibiotics at 37°C. Pseudomonas were cultured in basic salt solution (pH 7) containing 5 mM m-Toluate as a carbon source to maintain TOL plasmid at 30°C. The basic salt solution contained 4.0 gm of $NaNO_3$, 1.5 gm of K_2HPO_4, 0.5 gm of Na_2HPO_4, 0.0005 gm of $FeCl_2$, 0.2 gm of $MgSO_4 \cdot 7H_2O$, and 0.01 gm of $CaCl_2 \cdot 2H_2O$ per liter of glass-distilled water. All the bacterial strains were harvested during their logarithmic growth phase (O.D. 550 of 0.9-1.0).

B. Bacteria Counts

To examine any changes in the X-bacteria population per amoeba culture at 27°C, the number of bacteria per amoeba was counted every two days for two weeks. For single cell culture, to obtain enough amoebae to count symbionts population during the examination period, hundreds of amoebae were cultured in a Falcon plastic dish (60x15 mm). Twenty healthy amoebae were picked up from both the Falcon-dish culture and mass culture and washed 3 times in Chalkley's by transferring from dish to dish. The washed amoebae were put on a depression slide with a minimum amount of medium, lysed in 20 ul of 2N NaOH, and the bacteria were counted on a Petroff-Hausser bacteria counter under the phase-contrast microscope (Jeon and Hah, 1977).

After the isolation and purification of X-bacteria (see below), the number of X-bacteria was also counted under phase-contrast microscope using the Petroff-Hausser bacteria counter for the adjustment of number of X-bacteria in lysate solution.

C. Isolation of X-bacteria

Ten volumes of ice-cold isolution buffer (50 mM Tris / 50 mM EDTA / 100 mM NaCl, pH 8.0) containing 0.2% Triton X-100 were added to frozen packed amoebae, and the cells were allowed to thaw. All the procedures were carried out at 4°C. The thawed cells were vortexed briefly to resuspend those cells which had precipitated during thawing. Then they were immediately ultrasonicated twice for 30 seconds with 1-minute interval at 60% maximum intensity using a 10-mm-tip probe (intermediate tip) on a Fisher Dismembrator, Model 300.

By freezing-thawing and sonication, rupturing of the cells as well as cellular organelles such as nuclei, mitochondria, DNA bodies, and bacterial vesicles was satisfactorily achieved. Only X-bacteria, characteristic amoeba crystals and few cell membrane debris were found under phase-contrast microscope after the above treatments. To remove crystals and heavy membrane debris, the lysate was centrifuged for 5 min at 150 g. The supernatant was collected. Some X-bacteria were coprecipitated with the cell debris, and so this initial pellet was treated once more as above. With this procedure, more than 99% of the crystals and cell debris were removed. The combined supernatants were centrifuged for 10 min at 10,000 g. The resulting pellet containing the X-bacteria was washed 3 times with fresh ice-cold isolation buffer by centrifugation for 10 min at 10,000 g. Following the above procedure more than 90% of X-bacteria was isolated from amoebae, i.e., about 1.4×10^{10} X-bacteria could be isolated from 3.5×10^{5} amoeba culture. No further steps of X-bacteria purification were necessary for this study. The washed X-bacteria were frozen at -70°C until the isolation of plasmids was initiated.

D. Isolation of Plasmids

1. Lysis of X-bacteria

The method of lysis of X-bacteria was taken from the procedure of Han and Jeon (1980) with a slight modification to increase efficiency of lysis (Figure 1). About 5×10^{10} X-bacteria were usually suspended in 1.2 ml of "lysing buffer" (25% Sucrose, 50mM Tris, pH 8.0) by vigorous vortexing. The volume of each solution was adjusted proportional to the starting number of X-bacteria. All procedures were carried out at 4°C except where noted. First 0.6 ml of

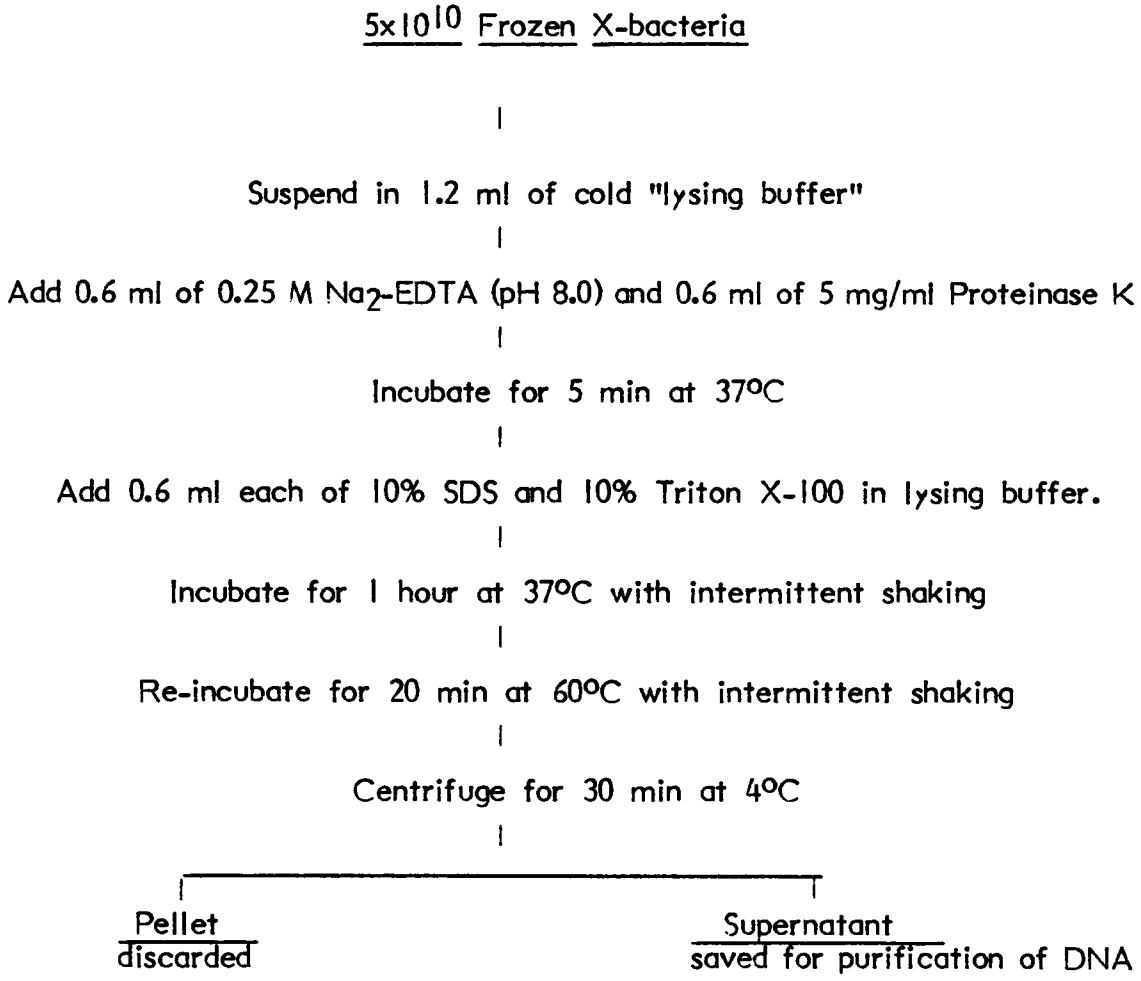

Figure 1. Preparation of X-bacterial lysate.

0.25 M Na_2-EDTA (pH 8.0) and 0.6 ml of Proteinase K (5 mg/ml in lysing buffer freshly prepared) (Hansen, 1974) were added to the bacterial suspension. The suspension was incubated for 5 min at 37°C, and then 0.6 ml of 10% SDS and 10% Triton X-100 in lysing buffer were added. The mixture was incubated for 1 hour at 37°C with intermittent shaking. The mixture was re-incubated for 20 min at 60-65°C with intermittent shaking and immediately cooled to 4°C. By this time the bacteria were lysed and the suspension became clear and showed an increased viscosity. If these results were not observed an additional incubation was done for 30 min at 37°C.

2. Lysis of other bacteria

The method of Marmur (1961) was followed except that 2% Triton X-100 was used instead of 2% SDS. Bacteria from their logarithmic growth phase culture (about $5x10^9$ cell) were harvested and washed once with 5 ml of ice-cold 10 mM Tris, 1 mM EDTA (pH 8.0) by centrifugation. The pelleted cells were first resuspended in 1 ml of cold lysing buffer (25% Sucrose in 50mM Tris, pH 8.0), and then 0.2 ml of lysozyme (10 mg/ml in 250 mM Tris, pH 8.0; freshly prepared) were added. The mixture was incubated for 5 min at 4°C after which 0.4 ml of 0.25 M EDTA, pH 8.0 were added. The suspension was kept at 4°C for another 5 min after which 2% Triton X-100 in 250 mM Tris, pH 8.0 was added and incubated for 5 min at 4°C. Finally lysis was brought about by additional incubation for 5 min at 37°C during which time the suspension showed an increasing viscosity.

3. Clearing of chromosomal DNA

To increase the plasmid-to-chromosomal DNA ratio in the total lysate of X-bacteria, E. coli and P. putida, chromosomal DNA had to be removed.

This was done by either of the two procedures. The first was the method of Gurrey et al. (1973). To the lysate 0.1 volume of 10% SDS plus 0.2 volume of 5 M NaCl were added and this mixture was kept overnight at 4°C, after which it was centrifuged for 30 min at 17,000 g at 20°C. This step removed about 90% of the chromosomal DNA and left the plasmid DNAs in the supernatant. The second procedure involved high-speed centrifugation (Godson and Vapnek, 1973). The lysate was centrifuged for 1 hr at 30,000 g at 4°C, and then the supernatant was carefully decanted so as not to disturb any chromosomal DNA which was precipitated with the cell pellet. With this high-speed centrifugation, most of the chromosomal DNA was successfully removed.

4. DNA purification

Proteins were removed from nucleic acid solution by the combined methods of Marmur (1961) and Kirby (1957). To the lysate, 1 volume of phenol was added and gently shaken for 15 min at room temperature. To the above mixture 1 volume of chloroform was added and gently shaken for another 15 min at room temperature. In this case "phenol" means redistilled phenol equilibrated with an equal volume of buffer (once with 0.2 M Tris, pH 8.0 followed with 10 ml Tris, 1 mM EDTA, pH 8.0) until the pH of aqueous phase is 7.6. "Chloroform" means a 24: 1 (v/v) mixture of chloroform and isoamyl alcohol. The combining phenol/chloroform mixture extraction procedure made the organic phase sufficiently dense so that it was at the bottom and also allowed a more rapid separation of the phases and permitted easy removal of aqueous phase at the end, thereby minimizing the transfer of phenol (especially in handling small volume of lysate), which in turn reduced the need to do repeated ethanol precipitations. The mixture was then centrifugated for 5

min at 1500 g at room temperature and the aqueous phase was collected. The interphase or protein gel was added to fresh Tris (100 mM, pH 8.0) buffer and re-extracted with the 1:1 phenol/chloroform mixture. The aqueous phase from the two extracts was pooled and further extracted with an equal volume of chloroform by gentle shaking. The emulsion was broken by centrifugation for 5 min at 1,500 g at room temperature. Using 3 M Sodium acetate (pH 5.5), the aqueous phase was brought to 0.3 M Sodium acetate, and exactly 2 volumes of cold (-20°C) ethanol were added and kept overnight at -20°C. When small amounts of DNA were present in the mixture, the period of storage was extended and the temperature was lowered to -70°C. To collect the DNA, the mixture was centrifuged for 30 min at 12,000 g. Ethanol was decanted and the tube was placed in an inverted position on a layer of absorbent paper to allow as much of the supernatant as possible to drain away. Using a gentle stream of N_2 gas, the remaining ethanol was evaporated. The DNA pellet was usually dissolved to a desired concentration in buffer containing 10 mM Tris, 1mM EDTA (pH 8.0). The final solution was stored at -70°C until use.

5. Quantitation of nucleic acid

For quantitating the amount of nuclei acid and the estimate of the purity of the nucleic acid, the solution containing DNA was read at wavelengths of 260 nm and 280 nm (Warburg and Christian, 1941; Maniatis et al., 1982). The reading at 260 nm allows for calculation of the concentraton of nucleic acid in the sample. An OD 260 of 1 corresponds to approximately 50 ug/ml for double stranded DNA, 40 ug/ml for single stranded DNA and RNA. The ratio between the reading at 260 nm and 280 nm (OD 260/OD 280) provided an estimate for the purity of the nucleic acid. A pure preparation of DNA and RNA has OD 260/OD 280 of 1.8 and 2.0 respectively.

E. Other Methods

1. Agarose-gel electrophoresis

Ethanol-precipitated DNA samples from phenol extraction were subjected to analytical agarose-gel electrophoresis (McDonnell et al., 1976; Mickel et al., 1977). 0.3% Agarose gels (Seakem ME agarose) were prepared in a horizontal slab-gel apparatus following the method of Fangman (1978). The dimension of the gels used for analytical purpose was 8.4x15x0.5 cm. 0.3% Agarose was poured into a "box" of 0.7% agarose made first by pouring a 2-mm slab, then four sides 0.5 mm thick and 0.6 cm wide, the "box" allowed dilute 0.3% gels to be picked up and moved for photography and other manipulations. To avoid over heating and characteristic deformation of the gel, a low ionic strength buffer, 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA adjusted to pH 7.7 with HCl and a low temperature (4°C) were used. Agarse was melted briefly (1 to 3 min) in electrophoresis buffer in an autoclave and mixed well and cooled to around 50°C before pouring. After the gel was set and while the DNA samples were adjusted to 5% glycerol and 0.025% bromophenol blue (BPB), the gel was equilibrated by pre-running it for 30 min at 40 V. After equilibration, approximately 25 ul of samples containing 0.1 to 0.5 ug DNA were loaded into each well. The power was then applied for 30 min at 30 V, or long enough for the samples to enter the gel. After introducing DNA samples to the gel, electrophoresis was carried out at 40 V for 20 hours at 4°C, or until the tracking dye reached the end of the gel using a VoKam power supply, Model 2541. A low-voltage gradient and a low-percent gel were used to facilitate the resolution of large plasmid DNAs. At the end of

a run, the gel was placed in a solution containing 0.5 ug EtBr/ml of electrophoresis buffer for 30 min. The DNA bands were visualized under UV light by virtue of the fluorescence emitted by DNA-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).

2. Electron microscopy

After washing and centrifugation, the packed amoebae were fixed in cacodylate-buffered (0.1 M, pH 7.4) paraformaldehyde-glutaraldehyde (Karnovsky, 1965) for one hour at 4°C and then washed 3 times at 5-min intervals in cold 0.1 M cacodylate buffer, pH 7.4. The cells were left overnight in buffer at 4°C. The amoebae were then post-fixed in 1% buffered osmium tetroxide for an hour at room temperature. Fixed cells were dehydrated in a graded series of ethanol. Toluene was used as a transitional agent between the alcohol series and the embedding mixture. Finally the amoebae were embedded in Epon 812 (Luft, 1961). Sections were cut on a Porter-Blum MT-2 ultramicrotome. Before thin sectioning, thick sections (0.3 µm thick) were cut and stained with 0.25% Azure II on a glass slide (Jeon, 1965) and examined under a light microscope. The thin sections with silver or golden-yellow color were mounted on copper grids and double-stained with saturated uranyl acetate and lead citrate for 15 and 5 min, respectively (Reynold, 1963). The sections were examined and photgraphed in a Hitachi H-600 electron microscope.

CHAPTER III

RESULTS

A. The Purity of Isolated X-bacteria

Amoeba lysates usually contain a large amount of cell debris such as membrane fragments, various vesicles, nuclei, mitochondria and dense crystals. Consequently, no single-step isolation procedure could be used to obtain pure X-bacteria. With conventional methods for isolating X-bacteria, i.e., freezing-thawing-shearing with a syringe, sonication and Ficoll-gradient centrifugation (Han and Jeon, 1980), or sonication, filtering through screens and sucrose-step-gradient centrifugation (Ahn and Jeon, 1982), the procedures were time-consuming, and the yield of X-bacteria after isolation was lower than that of the routine procedures used in this study. Cell organelles, such as nuclei, mitochondria and DNA bodies which also have their own DNA and interfere with this work were not detectable after the first ultrasonification. The lysate then was washed three times so as to remove these cell organelle contents. The purified X-bacteria, prepared by this method are shown in Figure 2. No detectable contamination of cell organelles and debris was found by phase-contrast microscopic examination. The steps applied for isolation of X-bacteria in this study were milder than those of other procedures so that the X-bacteria could retain their viability and infectivity.

B. Effect of High Temperature on the Growth of Amoebae

To study the effect of high temperature on the amoeba population, both D and xD strains of amoebae were grown singly at 22°C and 27°C. In the

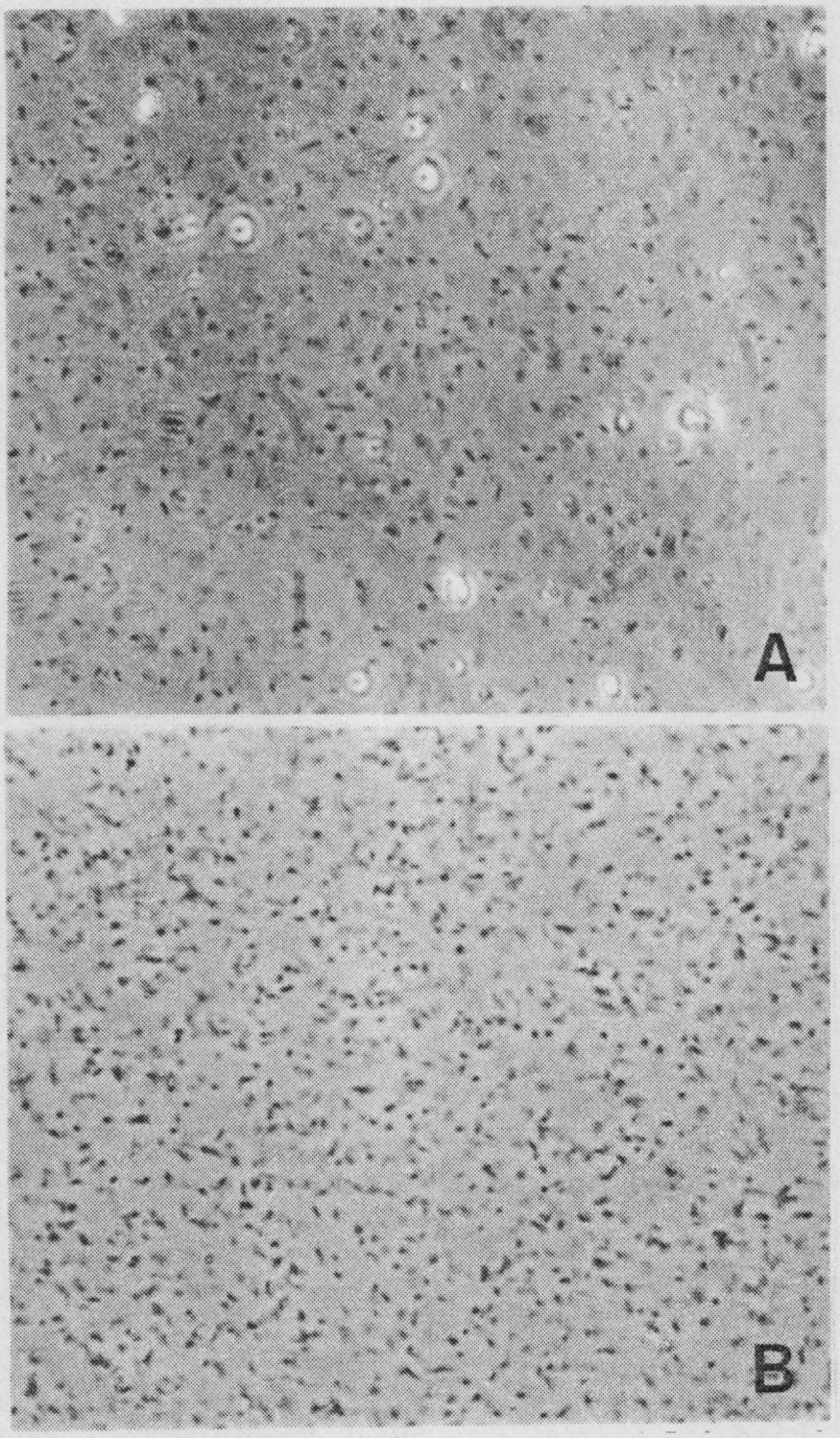

Figure 2. Purity of X-bacteria (Magnification, X2030)

Phase-contrast micrographs of X-bacteria before purification (A) and after purification (B) by centrifugation.

beginning all amoebae were picked up from the same mass culture and observations were made every other day except on weekends.

Growth rates of amoebae are shown in Figure 3. During the study period all amoebae in the two groups did not show any abnormality in feeding Tetrahymena. Both D and xD amoebae grown at 22°C and D amoebae at 27°C were healthy and well attached to the bottom of the dishes. The xD amoebae grown at 27°C were healthy until the 8th day of culture. Thereafter, however, they became abnormal (dark and round) in shape and degenerated by cytolysis.

Until the 7th day, both D and xD amoebae in two groups grew rapidly and divided more than 3 times on the average. The mean generation times of amoebae were almost the same until this day; 1.61 (D amoebae at 22°C), 1.62 (xD amoebae at 22°C), 1.7 (D amoebae at 27°C) and 1.76 (xD amoebae at 27°C). From the 10th day xD amoebae showed a sharp decrease in number, and most of them showed serious sick conditions on the 12th day, i.e., they attached poorly to the bottom of the dish, aggregated and dark and abnormal in shape. After the 14th day none of them were alive. At 27°C D amoebae showed a normal growth pattern as D and xD amoebae grown at 22°C with a slightly longer mean generation time (2.675) until the 12th day. However, after that some of them also showed a symptom of decrease in population. This symptom was somewhat different from that observed by Hawkins and Danielli (1963), who showed that several strains of amoebae grew well at temperatures up to 27°C.

At 20°C, both D and xD amoebae grew normally with mean generation times of 2.29 and 2.19 days respectively. For comparison, growth rates of

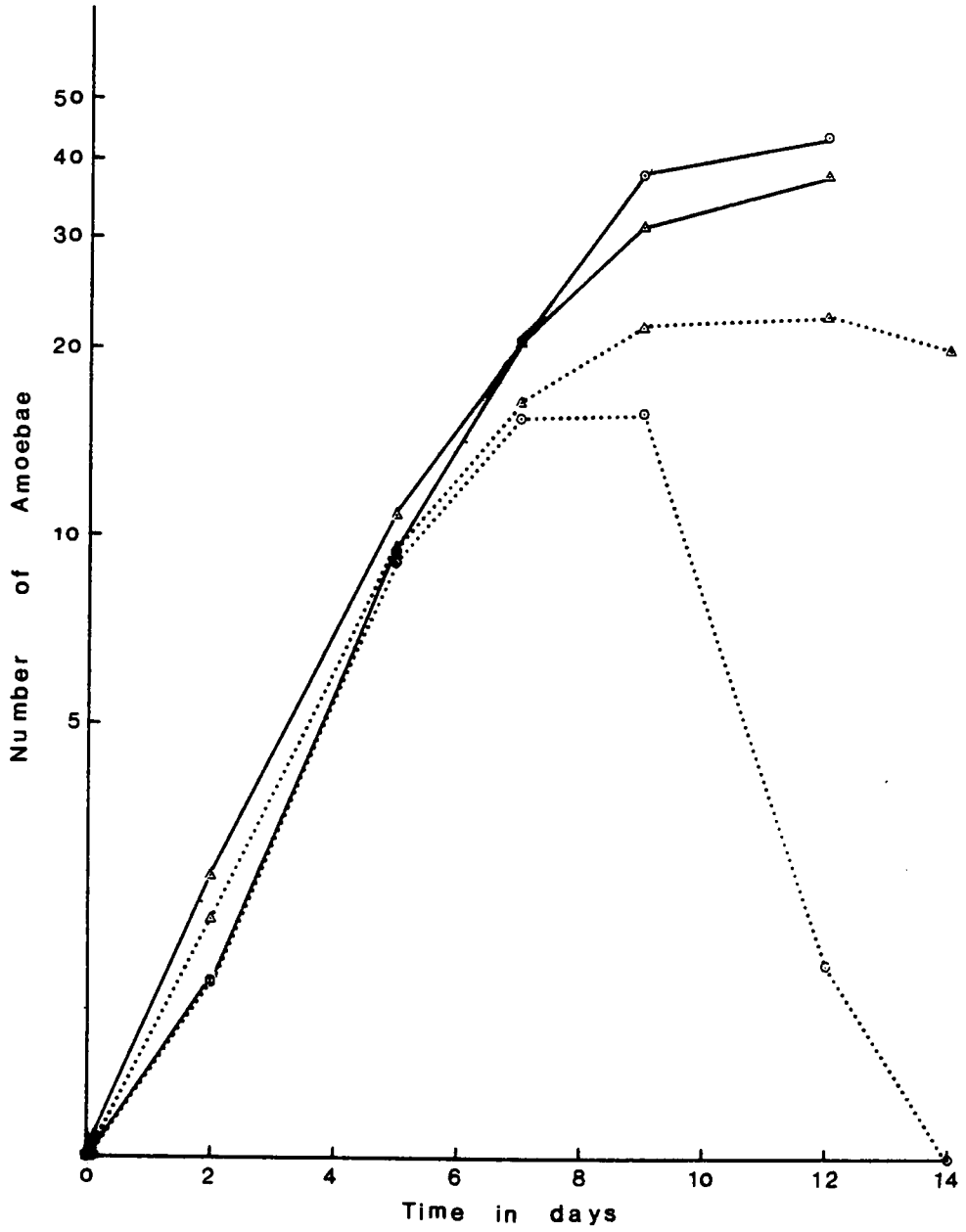

Figure 3. Growth rates of amoebae in single cell cultures at different temperatures.

△——△——, D amoebae at 22°C
⊙——⊙——, xD amoebae at 22°C
.....△....., D amoebae at 27°C
.....⊙....., xD amoebae at 27°C

single cell cultures at different temperatures are shown in Table I.

When xD amoebae were cultured at 27°C in mass culture for the isolation of plasmids from X-bacteria, the growth pattern was different from that observed in single cell cultures. In the mass culture at 27°C, some xD amoebae were quite healthy until the 14th day like those grown at 22°C. They ingested Tetrahymena normally and well attached to the bottom of the dishes, showing characteristic cytoplasmic streaming and there were no symtoms of abnormality in shape or degeneration. Moreover, some survived more than a month at 27°C. A similar phenomenon was also found in small Falcon-dish cultures which were grown for the bacteria counts (see Chapter II). Until the 8th day at 27°C in the small Falcon dishes, xD amoebae were normal and from the 12th day at 27°C they became abnormal in shape, but were healthier than those of single cell cultures. After the 18th day they did not survive any more. Since the only difference among single cell cultures, Falcon-dish cultures and mass cultures was the size of culture dishes and the number of amoebae per dish, the dimension of each culture dish and the density of each culture were calculated for comparison.

As shown in Table II, xD amoebae survived longer at 27°C with an increasing density per unit area (cm^2) and per unit volume (ml) of medium ranging from single cell cultures to mass cultures.

C. Effect of High Temperature on Symbionts

1. Bacterial population

To study the effects of high temperature on the xD amoeba population and the symbiont population, the number of X-bacteria per amoeba from both single cell and mass cultures at 27°C was counted. At the beginning of the

TABLE I

GROWTH OF AMOEBAE AT DIFFERENT TEMPERATURES

	22°C		27°C	
Days	D	xD	D	xD
0	1	1	1	1
2	2.83±2.09*	1.82±0.65	2.41±1.05	1.86±1.03
4	11.61±6.99	9.39±4.28	9.54±6.51	9.05±4.25
7	20.28±12.29	20.04±8.55	17.59±12.96	15.68±9.60
9	31.16±18.15	37.78±13.68	21.50±19.19	16.04±10.44
12	37.61±17.24	43.66±16.58	22.41±21.65	2.04±3.03
14	—	—	19.90±20.26	0.04±0.20

Note * Average cell number ± S.D.

TABLE II

COMPARISON OF DIFFERENT CULTURES

	Single cell culture	Falcon dish culture	Mass culture
Shape of culture dish	round	round	rectangle
Size of culture dish (cm)	1.3x1.0	6.0x1.5	34x22x4
Area/dish (cm^2)	1.3	28.3	748
Vol. of medium/dish (ml)	1	20	1000
Amoebae/dish	1-40	300-1000	$3x10^5$ - $4.5x10^5$
Density of amoebae/cm^2	0.76 - 30.3	10.6 - 35.4	401 - 602
Density of amoebae/ml of medium	1 - 40	15 - 50	300 - 450
Length of survival at 27°C	14 days	18 days	> 1 month

experiment, for the bacteria count of single cell culture, a few hundred xD amoebae were picked up from mass cultures and grown in a Falcon dish (60x15 mm).

In the single cell culture, the average number of symbionts per amoeba declined rapidly at 27°C (Figure 4). While xD amoebae themselves grew normally until the 7th day (see Figure 3), the average number of symbionts per amoeba decreased rapidly and reached fewer than half of that per amoeba grown at 22°C. By the 10th day at 27°C, most symbionts disappeared from amoeba cytoplasm (less than 1/10 of that per amoeba at 22°C), and no symbionts were detected after that.

In the mass culture at 27°C, the average number of symbiots per amoeba decreased more slowly as compared with that of single-cell cultures. Until the 6th day the average number of symbionts per amoeba decreased slowly, but after that they declined rapidly as in single-cell cultures (Figure 4). Until the 8th day at 27°C, xD amoebae in mass culture had more than 70% of symbionts as those of amoebae at 22°C. On the 14th day the amoebae in mass culture at 27°C had more symbionts than those in single-cell cultures found on the 8th day, and were quite healthy as mentioned above. Even though further examination was needed with the above observation of both single-cell cultures and mass cultures, it seemed that xD amoebae at 27°C could maintain a healthy condition if they had more than 1/3 of their original symbiont population.

2. Morphological change of bacteria

In addition to usual cellular organelles xD amoebae had many vesicles containing hundreds of bacteria as shown in Figure 5, 6 and 7. The vesicular

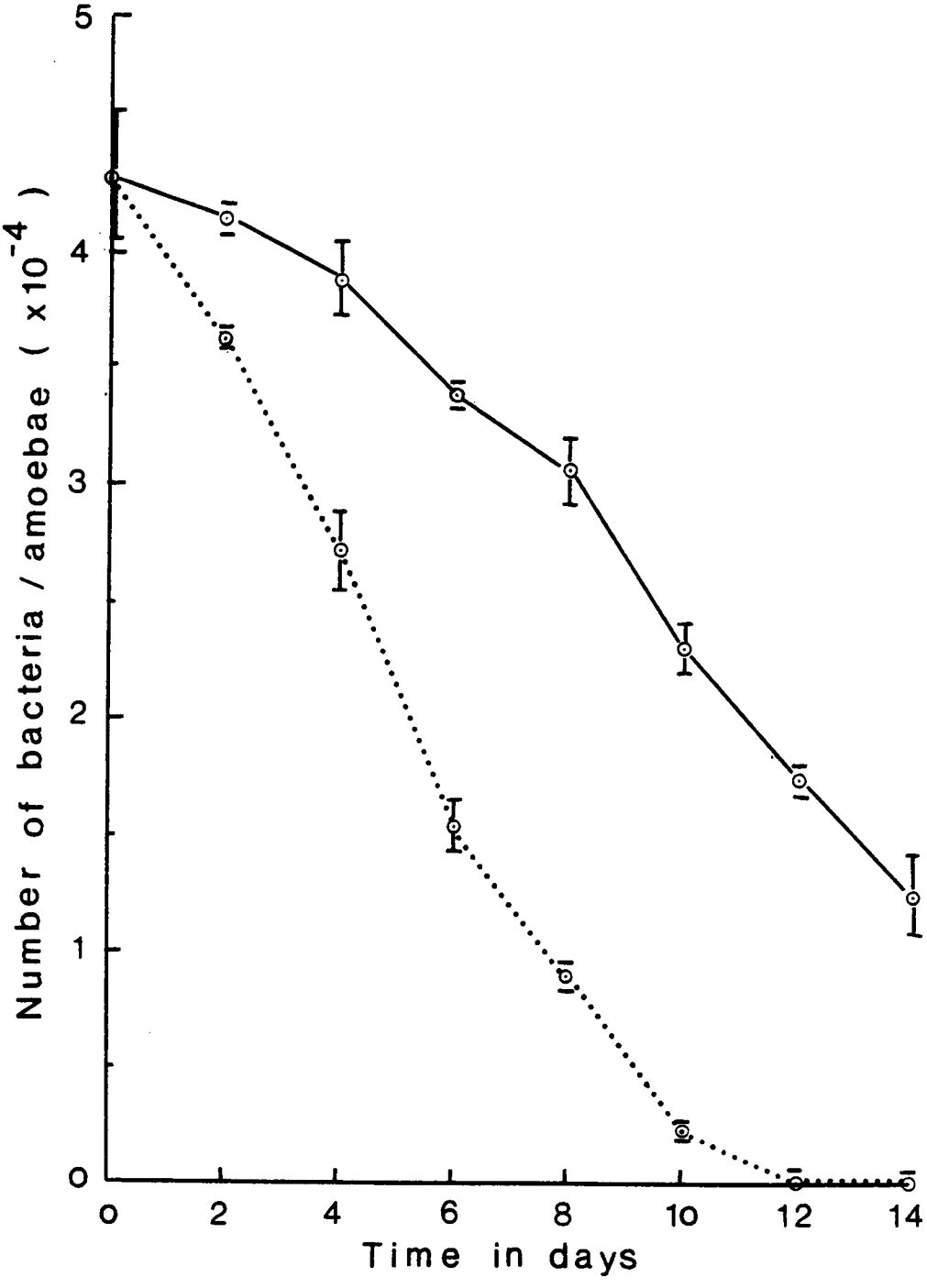

Figure 4. The average number of X-bacteria per amoeba at 27°C. The vertical bars represent standard deviations.

·····⊙·····, Single cell culture
——⊙——, Mass culture

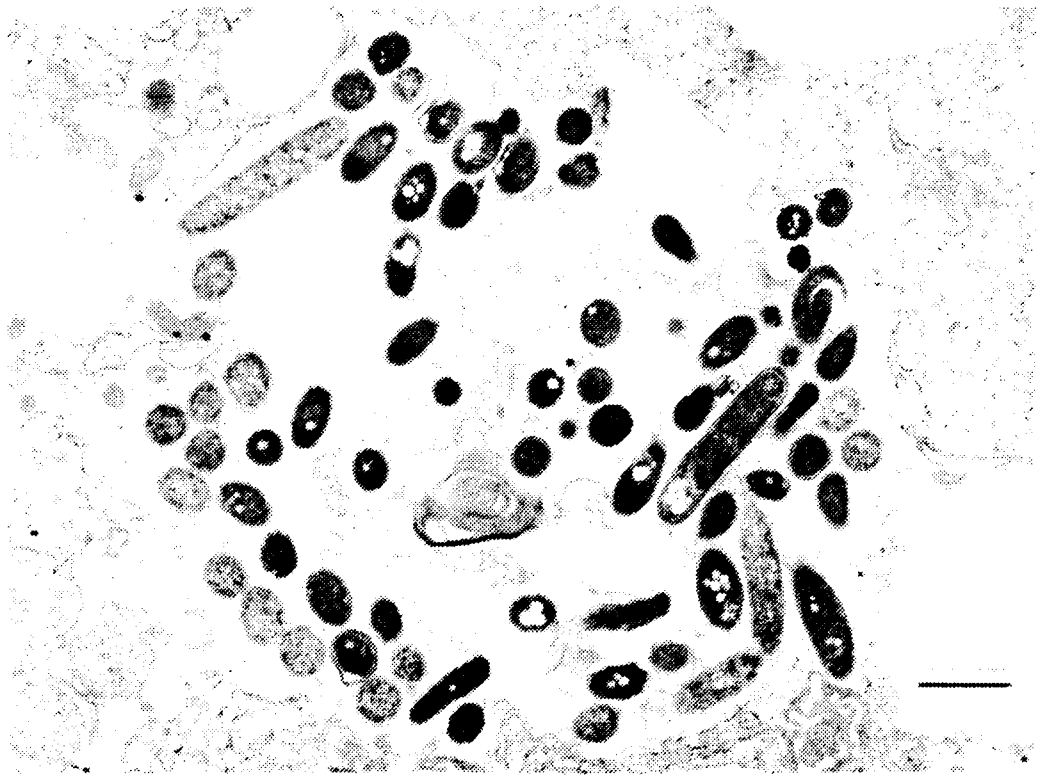

Figure 5.

Figure 5-7. Electron micrographs of symbiotic vesicles and X-bacteria in xD amoebae cultured at 22°C and 27°C. The bars represent 1 µm.

Figure 5. A typical symbiotic vesicle containing X-bacteria in amoeba grown at room temperature (X12,500).

Figure 6. A symbiotic vesicle in amoebae grown for 8 days at 27°C (X19,200).

Figure 7. A large symbiotic vesicle containing apparently degenerated X-bacteria after the amoeba was grown for 14 days at 27°C (X30,000).

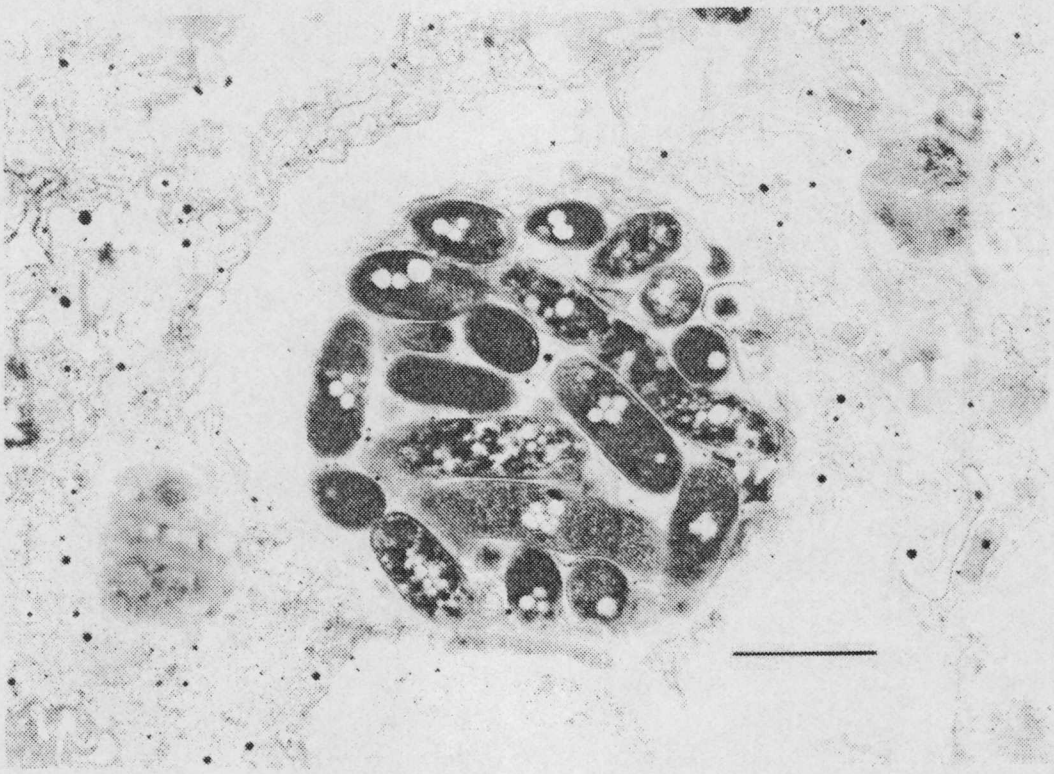

Figure 6.

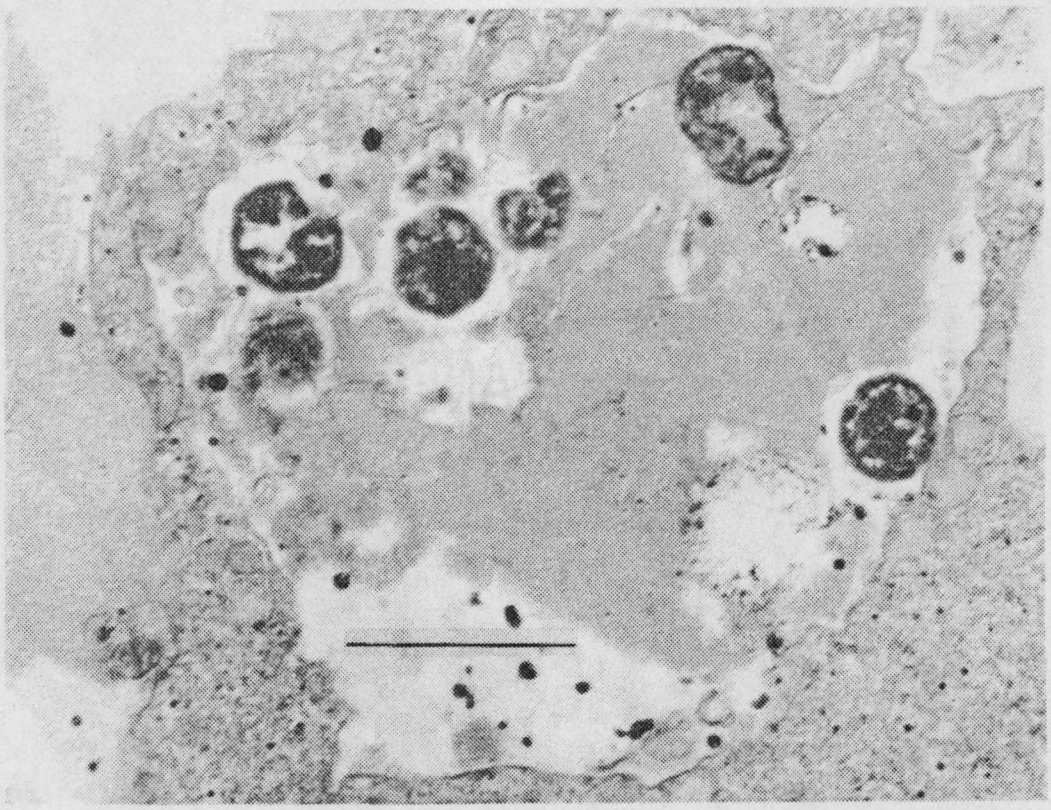

Figure 7.

membrane, which was conspicuous in comparison with the cell membrane, separated the symbiotic bacteria from the host cytoplasm. The characteristic plasmalemmal structure of amoebae, which has glycocalyx on the external surface (Ahn and Jeon, 1982), was not distinguishable in the membrane of the vesicles containing bacteria. A dense matrix filled the interspace of the vesicle. This structure was also noted by Ahn (1977) and Jeon and Hah (1977). The dense matrix appeared to develop from channeling structure which was also observed in the symbiotic vesicles following experimental infection. Bacteria were enclosed either singly or in various numbers in vesicles. The rod-shaped bacteria measured approximately 0.5 x 2 m. Each bacterium had a cell wall structure outside the plasma membrane. An electron-lucid intermediate zone was observed between the cell wall and the plasma membrane. Inside the plasma membrane the symbionts contained particles which could be considered as the ribosomal structure of the bacteria. The electron-light area, which represented the nuclear zone, had a network of filaments. The growing bacteria appeared to divide by constriction of the cell membrane.

Figure 6 shows an xD amoeba which was grown for 8 days at 27°C in mass culture. Until the 8th day at 27°C, xD amoebae had no discernable morphological changes as compared with those grown at 22°C except for the reduced size of symbiotic vesicles and the decreased average number of symbiotic bacteria per vesicle. The size of amoeba was similar, and there were no signs of abnormality in the structure of cell organelles. Although most symbiotic vesicles were intact like those found in xD amoebae grown at 22°C, a few symbiotic vesicles appeared to fuse with lysosomes. However, there was still no symptom of morphological change in bacteria detectable under the electron microscope.

xD amoebae grown for 14 days at 27°C also had similar structures to those of amoebae grown at 22°C. Some of the vesicles containing X-bacteria had similar structures to those of amoebae grown for 8 days at 27°C, but other symbiotic vesicles began to show abnormalities as shown in Figure 7. After an apparent fusion with lysosomes a few X-bacteria were found in the large symbiotic vesicles and channeling structure and dense matrix inside symbiotic vesicles disappeared. X-bacteria showed symptoms of apparent degeneration, i.e., they were densely stained giving a higher contrast and showed an extended intermediate zone between cell wall and plasma membrane.

From electron microscopic observations it could be seen that as the culture of amoebae was prolonged at 27°C, the size of symbiotic vesicle and the average number of bacteria per symbiotic vesicle decreased, accompanied by the decrease in the total average number of X-bacteria per amoeba. In addition, some of the X-bacteria degenerated inside the symbiotic vesicles at least from the 14th day at 27°C because of the fusion with lysosomes. It appeared that X-bacteria were digested by amoebae.

D. Effect of High Temperature on Plasmid DNAs of Symbionts

The two plasmid DNAs of X-bacteria were subjected to electrophoresis in 0.3% agarose gel with four plasmid DNA markers of known molecular weights from E. coli and Pseudomonas putida. Two plasmids, pBR322 (M.W. 2.6×10^6) and RSF2124 (M.W. 7.3×10^6), from E. coli strain GM8, plasmid pVA517A (M.W. 35.8×10^6) from E. coli strain V517, and TOL plasmid (M.W. 75×10^6) were used as molecular weight markers. The electrophoretic pattern of these plasmid DNAs on agarose gel is shown in Figure 8-A. All the plasmid DNAs were

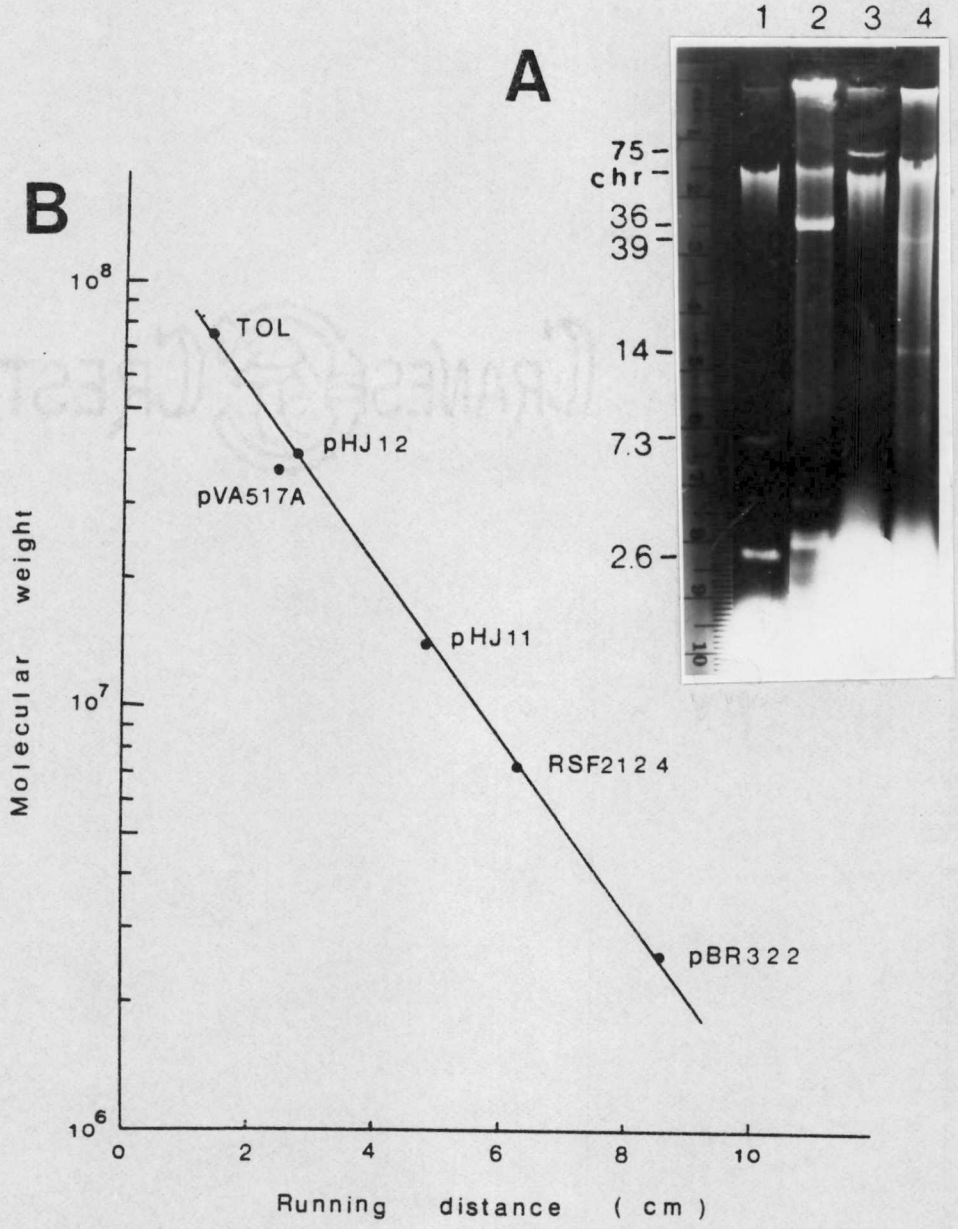

Figure 8. Electrophoretic pattern of plasmid DNAs in 0.3% Agarose gel.

A. Agarose gel electrophoresis of plasmid DNAs.

Lane 1: Top band, chromosomal DNA; middle band, RSH2124 (M.W. 7.3×10^6); lower band, pBR322 (M.W. 2.6×10^6).
Lane 2: Lower band, pVA517A (M.W. 36×10^6).
Lane 3: Top band, TOL (M.W. 75×10^6).
Lane 4: Middle band, pHJ11 (M.W. 39×10^6); lower band, pHJ12 (M.W. 14×10^6).

B. Molecular weight calibration curve. The molecular weights of plasmid DNAs were plotted against running distance.

isolated by high-speed centrifugation chromosomal-DNA-clearing method except for X-bacteria plasmids (Lane 4) which were isolated by the SDS-salt precipitation method (Guerry et al., 1973). Because of the lower efficiency of high-speed centrifugation method (Godson and Vapnek, 1973) for removing chromosomal DNAs as compared with SDS-salt precipitation method, the SDS-salt precipitation method was used for the isolation of the two plasmid DNAs from X-bacteria throughout this study. When the molecular weight of each plasmid was plotted against its running distance on a logarithmic scale, all the plasmids fell on a straight line except for pVA517A as shown in Figure 8-B.

Figure 9 shows the electrophoretic patterns of plasmid DNAs isolated from X-bacteria of xD amoebae grown in mass culture under different conditions. Each lane represents the plasmid DNAs obtained from 3×10^9 X-bacteria. It appears that the amount of the two plasmid DNAs of X-bacteria from amoebae grown for 11 days or longer at 27°C (Lane 2) is smaller than that of amoebae from at 22°C. No plasmids were detectable in X-bacteria from amoebae grown for 14 days at 27°C. The staining density of plasmid bands in Lane 3 suggested that the smaller plasmid (pHJ12) may disappear sooner than the larger plasmid (pHJ11) from X-bacteria of amoebae grown at 27°C.

In case of 22°C culture the two plasmid DNAs from 1×10^9 X-bacteria could easily be detected as bands like in Lane 1. However, even when plasmid DNAs from 2×10^{10} X-bacteria of amoebae grown for 11 days at 27°C were subjected to electrophoresis, it was not possible to detect the small plasmid band. Therefore, even considering the limitation of detection of DNA as a band after agarose-gel electrophoresis with ethidium bromide strains (ng as a single band, Maniatis et al., 1982) and the low-yield efficiency of isolation of

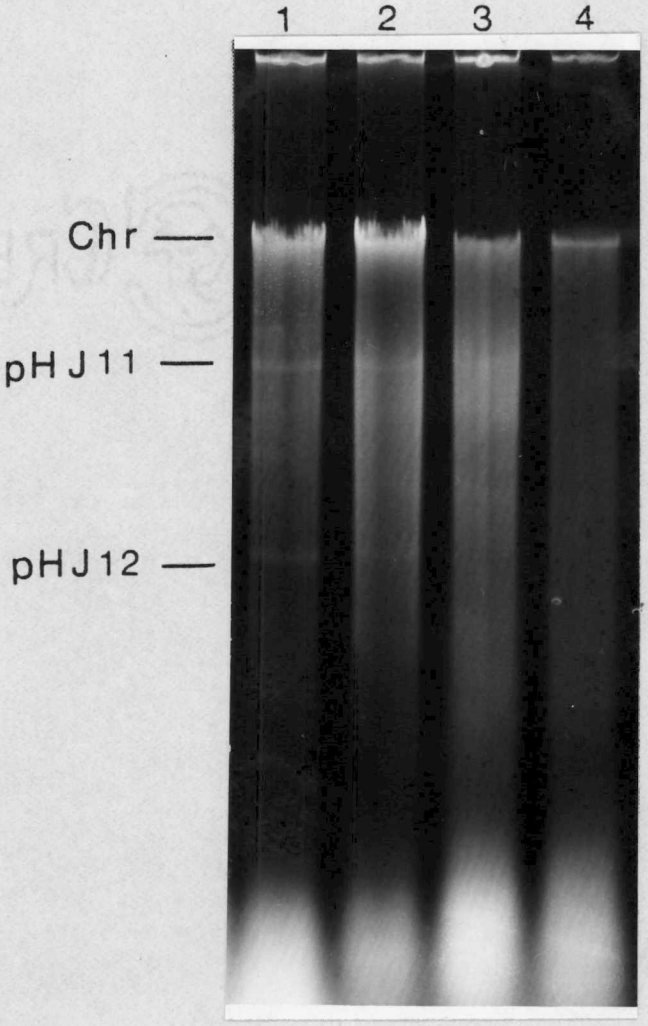

Figure 9: Electrophoretic pattern of plasmid DNAs isolated from X-bacteria of xD amoebae grown under different conditions.

Lane 1: plasmid DNAs of X-bacteria from room temperature culture.
Lane 2: plasmid DNAs of X-bacteria grown for 8 days at 27°C.
Lane 3: plasmid DNAs of X-bacteria grown for 11 days at 27°C.
Lane 4: plasmid DNAs of X-bacteria grown for 14 days at 27°C.

Each lane represents the DNAs isolated from 3×10^9 X-bacteria.

plasmids from X-bacteria, it was obvious that not more than one X-bacteria out of 20 could have had the small plasmid DNA if they were grown for 11 days at 27°C. From the above observation the value should be much lower in the case of X-bacteria of amoebae grown for 14 days at 27°C.

CHAPTER IV

DISCUSSION

The organismic relationship of X-bacteria with the xD strain of Amoeba proteus which started as a parasitism on D strain (Jeon and Lorch, 1967) changed to obligatory symbiosis after 5 years of association (Jeon, 1972, 1973). Through the nuclear transplantation study on this amoeba, the X-bacteria were considered as cytoplasmic components of this amoeba (Jeon and Jeon, 1976). As it has been pointed out by others (Margulis, 1976, 1981; Smith, 1979; Taylor, 1979, 1982; Whatley, 1976), the mutualistic relationship which is a necessary prerequisite in the evolution of intracellular organelles has been established in a very short time in the xD amoeba.

The dependence of host amoebae on the symbionts for survival has also been shown by the death of xD amoebae following selective removal of X-bacteria with the use of antibiotic drugs, such as chloramphenicol (Jeon and Hah, 1977) and trimethoprim (Jeon, 1977). Recently, another way has been found to demonstrate that the survival of xD amoebae requires X-bacteria (Jeon and Ahn, 1978). Due to the psychrophile-like characteristics of X-bacteria (they cannot grow at elevated temperatures; Morita, 1975), X-bacteria disappear from amoebae when the latter are cultured at or above 26°C. Following the disappearance of X-bacteria, xD amoebae stop growing and die in a few days. Thus, the dependence of the xD amoebae on their symbionts has led to the aquisition of a new cell character, temperature sensitivity.

Unlike other bacteria (Savant and Pavillard, 1964), X-bacteria are immune to digestion in the amoebae when they are introduced into the cytoplasm by

microinjection or into food vacuoles by induced phagocytosis (Ahn and Jeon, 1979). Killing of Salmonella within vacuoles of amoebae is almost exponential for the first 6 hours. X-Bacteria are an exception but the mechanism is not known as to how they avoid destruction by their host cell, which is known to be rich in lysosomal enzymes. Even though the in vitro culture of these bacteria is not possible (Freesh, 1975), they are known to maintain viability for a certain period outside the host cell (Ahn and Jeon, 1979). Meanwhile, it was found that X-bacteria contain two plasmid DNAs designated as pHJ11 and pHJ12 (Han and Jeon, 1980). It was also noted that these plasmids are possibly related to the bacteria's resistance to enzymatic digestion and the rigidity of their cell walls. The X-bacteria treated with plasmid-curing agents support this view indirectly. When the bacteria within amoebae were treated with agents such as ethidium bromide or acridine orange, no plasmids were detected in the bacteria and the bacteria were unable to infect D amoebae (Han, 1979).

Thus, the objective of this study was to test the hypothesis that one or both of X-plasmids are directly involved in the maintenance of the symbiotic relationship between amoebae and X-bacteria: That is, to determine the relationship between the loss of viability of X-bacteria and the disappearance of the two X-bacteria plasmids at 27°C.

By examining the growth characteristics of xD amoebae in comparison with D amoebae, the change in character of X-bacteria can be followed. One of the adverse effects of X-bacteria found in 1966 (Jeon and Lorch, 1967) was a low clonability of hosts in single cell cultures. After 5 years of symbiosis, the adverse effect of the bacteria diminished showing 95.6% clonability in amoeba infected by microinjection (Jeon, 1972). The adverse effect of X-bacteria on the mean generation time of xD amoebae in comparison with D

amoebae has also changed. In 1977 the mean generation time of D and xD amoebae was 3.17 and 4.19 days at 20°C, respectively. It was shown that D amoebae grew faster than xD amoebae at that time (Ahn, 1977). Now, however, the mean generation times of D and xD amoebae are almost the same at 22°C (2.28 and 2.19 days, respectively) and even at 27°C (1.70 and 1.76 days, respectively) until the 7th day (See Table I, p. 24).

The presence of symbiotic bacteria within xD amoebae still bestows adverse effects on the growth of xD amoebae at 27°C after 8 days, however. From then on their population decreases, most of them are poorly attached to the bottom of the dish, aggregated and dark and abnormal in shape showing sick conditions on the 12th day. All cytolyze by the 14th day at 27°C in case of single cell cultures. In single-cell cultures kept at 27°C, the average number of symbionts per amoeba declines rapidly and the symbionts disappear completely from all amoebae by the 10th day. The depletion of symbionts appears to be the cause of death of amoebae, which closely follows the disappearance of symbionts. The death of amoebae cannot be attributed to the elevated culture temperature alone, since nonsymbiotic amoebae (D strain) keep increasing in number until 2 weeks of culture at 27°C, having divided more than four times on the average (Figure 3, p. 22).

The observation that xD amoebae in mass cultures survive longer (> 1 month) than those in single cell cultures at 27°C could be explained by the fact that the average number of symbionts per amoeba in the mass culture decreases slower than that in the single cell culture at 27°C. The reasons why the xD amoebae in mass culture survive longer at 27°C and why the symbionts in mass culture decrease slower at 27°C are not clear, but it may be that the high density of amoebae and symbionts per unit area (cm^2) and unit

volume (ml) of medium (Table 2, p. 25) causes somehow favorable (maybe compensation) effect on them so that they can reduce the adverse effect of high temperature.

The current average number of X-bacteria per xD amoeba (about 42,000; Jeon and Jeon, 1976) is much smaller than 60,000-150,000 found in the early years of infection (Jeon and Lorch, 1967). A host's control over the symbiotic population is expected to be operative in any stable symbiotic association and at least four mechanisms have been proposed to account for the observed control; expulsion of symbionts (Taylor, 1969; Steele, 1976), digestion (Fankboner, 1971; Weis, 1976), regulation of symbiont growth (Reisser, 1980; McHaley, 1981) and synchronization (Preer et al., 1974; Muscatine and Pool, 1979).

However, when the culture condition was changed the loss of symbionts from host was found in some cases. The best-known endosymbionts, Kappa particles, which are considered to be under the control of host genome, multiply at a maximum rate of 2.0 duplications per day. When Paramecia are well fed, too rapid a multiplication of the host results in a dilution of the number of endosymbionts and their eventual loss of symbionts (Preer et al., 1974). Electron microscopic study (Figure 7, p. 29) showed that the reason for the disappearance of the endosymbionts from amoebae during prolonged culture at 27°C was because of the degeneration of X-bacteria inside symbiotic vesicles which was apparently caused by digestion after fusion with lysosomes. The inability of X-bacteria to remain viable at high temperatures suggests that the symbiotic bacteria are like psychrophiles (temperature sensitive bacteria; Morita, 1975). Thus, it is evident that the inability of xD amoebae to grow

at 27°C was due to the loss of their symbionts, which was in turn caused by the inability of growth of X-bacteria at 27°C.

Why then are X-bacteria unable to grow at 27°C while they grow normally at 22°C? This might be explained by of the loss of the two plasmids of X-bacteria at 27°C. The symbiotic bacteria appeared to be digested by amoebae in turn after the symbionts had lost the plasmids they normally harbor, i.e., the signs of loss of plasmids in X-bacteria were observed after 11 days of culture at 27°C and few or no plasmids were found in X-bacteria after 14 days of culture at 27°C. At that time some X-bacteria started to show the symptom of digestion by amoebae (Figure 7 and 9, p. 29 and p. 34). Thus, it appears that the temperature sensitivity of symbiotic amoebae is related to the psychrophilic properties of the plasmid DNAs present in symbiotic bacteria, although such a relationship has not been definitively proven.

The psychrophilic properties of plasmid DNAs have been demonstrated in E. coli (Jacob et al., 1963), in Staphylococcus aureus (May et al., 1964), in Proteus vulgaris, E. coli, Salmonella typhimurium (Terawaki et al., 1967), and Rhizobium species (Zurkowski and Lorkiewicz, 1978; Kiss et al., 1980). All the plasmids from the above species of bacteria including X-bacteria show their temperature sensitivity when the temperature of bacteria culture is raised 5-6°C higher than that of normal culture. The temperature sensitivity of plasmid DNAs is explained that the autonomously-replicating plasmid DNAs whose doubling rates are equal to the cell-doubling rate at the temperature of normal culture are more or less suppressed at higher temperatures, i.e., temperature sensitive replication. Under these conditions the plasmids would be diluted out so that they are inherited unidirectionally in a "one or none" manner (May et al., 1964; Zurkowski and Lurkiewicz, 1978).

Then the next questions naturally follow: What is the role of the two plasmids of X-bacteria? How do the two plasmids enable X-bacteria to maintain symbiotic relationship with amoebae? At least one function of the plasmids might be explained from this study; the protection of X-bacteria from digestion by amoebae. Because of this function X-bacteria may succeed in the establishment of endosymbiosis by protecting themselves during initial stages of infection when they are directly exposed to the amoeba cytoplasm (Ahn and Jeon, 1979) and may maintain successful symbiotic relationship with amoebae by prevention of lysosomal fusion with symbiotic vesicle (SV) membrane during sustained multiplication of both partners (Jeon, 1983).

Other experiments support the above views indirectly. When X-bacteria are treated with plasmid-curing agents such as ethidium bromide and acridine orange, the bacteria are unable to infect D amoebae (Han, 1979). The SDS gel electrophoretic pattern shows that the SV membranes contain at least one polypeptde component on the cytoplasmic surface which is not found in either plasmalemma or phagopolysomes (Ahn and Jeon, 1982). Thus, it is possible that these plasmids found in X-bacteria encode some polypeptides which are somehow inserted into the bacterial outer membrane and/or the symbiotic vesicle membrane, so that bacteria and/or symbiotic vesicle can be prevented from digestion by amoebae.

Plasmid-encoded surface components of bacteria have been found in several cases. The plasmid RP-1 of Pseudomonas encoded surface components as a somatic receptor for phage (Olsen et al., 1977). The plasmids of some Gram-negative bacteria encode cell envelope components as a barrier to antibiotics (Costerton, 1977). The degradative plasmids of Pseudomonas produce

membrane components of cell envelope as a substrate utilization system (Shapiro et al., 1980). The plasmids of Rhizobium, one of the symbiotic bacteria like X-bacteria, code for a surface antigen which is involved in the initial step of infection of a legume (Bishop et al., 1977).

To conduct further studies on the two X-bacteria plasmids, it is necessary to detect, isolate and map the genes of the plasmids, which might encode the protein(s) that plays an important role in endosymbiosis between X-bacteria and amoebae. However, the difficulty in studying X-bacteria plasmids is to prepare an ample quantity of material for analysis. The reasons for the difficulty are that: (1) It is difficult to obtain a sufficient number of X-bacteria since they do not grow in vitro. (2) The two plasmid DNAs are present fewer than one copy per X-bacterium and are not amplifiable. (3) It is difficult to lyse X-bacteria both with conventional methods (Marmur, 1961) and with clear-lysate methods (Godson et al., 1967). The SDS-salt-precipitation method (Guerry et al., 1973) has been adopted to isolate plasmid DNA selectively from X-bacteria chromosome, but unfortunately such treatment removes a large portion of the plasmid DNA (Han and Jeon, 1980).

To solve this problem several different approaches should be made. During this study several methods were found to be applicable to increase the yield of isolated X-bacteria plasmids. A 0.5% or lower concentration of sodium lauryl sarcosinate which lyses cytoplasmic membranes while leaving cell walls intact (Filip et al., 1973) was very effective for the lysis of amoebae. However, the use of this method to get pure X-bacteria remains to be developed. Alkaline SDS method (pH 12.6) at elevated temperature (Kado and Lin, 1981) was more effective to lyse and eliminate chromosal DNA than the method used in this study, but there is a possibility of loss of large plasmid pHJ11

during isolation and so it needs improvement. 8 M Guanidine HCl (Cox, 1968) and 4 M guanidine thiocyanate (Chirgwin, 1979) were very effective to lyse X-bacteria and so these can be used for the study of X-bacteria RNA and are applicable for the study of the plasmid DNAs. Electrophoresis in 0.3% agarose gel is recommended for the preparative purpose of X-bacteria plasmids partially because of a longer running distance and a better separation between the two X-bacteria plasmid DNAs and chromosomal DNA, and partially because the large pore size of 0.3% gel makes it easier to remove plasmid DNAs from the gel.

The last approach would be cloning of each of the two X-bacteria plasmids using either suitable cloning plasmid vectors or charon phages (Leder et al., 1977). If cloning of the two X-bacteria plasmids can be achieved, the excessive amount of amoeba culture which requires a lot of work and time will not be needed to get ample amounts of plasmids for further studies of plasmid DNAs such as physical mapping and gene mapping.

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