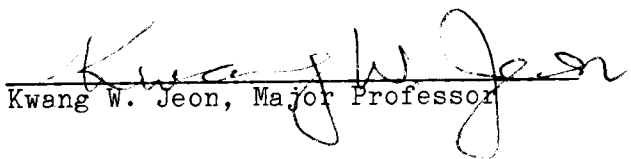
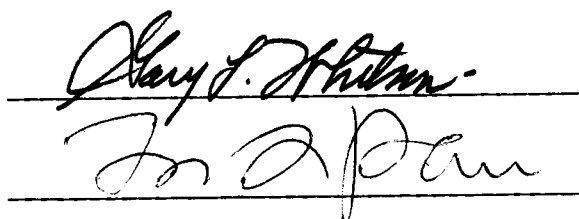
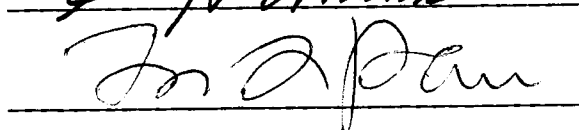


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ROLE OF PLASMID DNAS IN THE ESTABLISHMENT
OF SYMBIOSIS BETWEEN AMOEBAE
AND BACTERIA

A thesis
Presented for the
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ABSTRACT

The possible roles of plasmid DNAs in the establishment of symbiosis between symbiotic X-bacteria and xD strain of Amoeba proteus were studied by testing the infectivity of X-bacteria after curing with plasmid-curing agents. For the purpose of this test, plasmids were eliminated from X-bacteria in vivo and in vitro. First, plasmid DNAs were cured by culturing xD amoebae in a medium containing 10^{-6} M acridine orange or 10^{-5} M ethidium bromide for up to 2 weeks. For curing in vitro, isolated X-bacteria were incubated with plasmid-curing agents for up to 12 hours at 4 °C. After treatment with plasmid-curing agents, the relationship between the loss of infectivity and the absence of plasmid DNAs was determined. Infection kinetics were studied by poly-L-lysine-induced phagocytosis, and the presence or absence of plasmid DNAs was verified by agarose-gel electrophoresis.

By studying the infection kinetics of the plasmid-cured X-bacteria, it was shown that X-bacteria which had been treated with plasmid-curing agents disappeared completely from the amoeba cytoplasm within a few days after experimental infection and failed to establish new symbiotic bacterial populations. X-Bacteria isolated from xD amoebae cultured with plasmid-curing agents for 7 days and 14 days disappeared within 72 and 48 hours respectively after experimental infection. When X-bacteria had been cured for longer than 9 hours in vitro, no X-bacteria were detected after 96 hours of infection.

Agarose-gel electrophoretic studies of DNAs showed that the loss of

infectivity of X-bacteria in the symbiont-free amoebae was caused by the elimination of plasmid DNAs from the symbiotic bacteria. It appeared that the amount of plasmid DNA of X-bacteria, isolated from xD amoebae grown in the medium with plasmid-curing agents, decreased as the length of the culture period increased. No plasmid DNA was detected in X-bacteria isolated from xD amoebae that had been grown in plasmid-curing agents for longer than 7 days. At that time, X-bacteria lost their infectivity due to the subsequent loss of plasmid DNA which they normally harbor.

The results presented in this study support the idea that plasmid DNAs in the symbiotic bacteria play important roles in the establishment of symbiosis between amoebae and bacteria.

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CHAPTER I

INTRODUCTION

From the evolutionary standpoint, a symbiotic event represents the union of two or more previously divergent genomes into a new coevolutionary unit. The subsequent fate of this unit will depend on both the survival effectiveness of the new unit interacting with external selective forces and the continued integrative and competitive interactions between the host and symbiont.

The most significant evolutionary development which has been attributed to intracellular symbiosis is the origin of eukaryotic cellular organization. The "serial endosymbiosis theory" (Margulis, 1970; Taylor, 1979, 1983) proposes that the eukaryotic cell evolved from several intracellular symbiotic intermediates in the form of an oligogenetic intracellular ecosystem. After further coevolutionary intracellular adaptations, this evolution culminated in the homeostatically regulated system of today's eukaryotic cells.

The strongest evidence supporting the origin of chloroplasts and mitochondria from prokaryotic intracellular symbionts has come from sequence data of proteins and nucleic acids (Schwartz and Dayhoff, 1978; Dayhoff and Schwartz, 1980).

Concerning the origins of the chloroplasts and the mitochondria, the almost complete lack of a fossil record bearing on these events requires us to find our clues by unraveling the nature of the contemporary systems available to us. Fortunately, living forms

resembling the ancestral type required by the symbiotic theory can be found today. Thus, we know of aerobic bacteria as possible ancestors of mitochondria, prokaryotic algae as possible ancestors of chloroplasts, and Pelomyxa palustris as an ancestral host. Furthermore, characteristic forms transitional between the free-living and organellar stages are indicated by the endosymbiotic bacteria of Pelomyxa and by the cyanelles of anomalous algae-like Cyanophora paradoxa. Especially the giant amoebae, Pelomyxa palustris, provide us with a present-day model of a transitional stage which illustrates how mitochondria might have evolved from bacteria. The highly vacuolated cytoplasm lacks Golgi bodies and mitochondria, but contains three types of endosymbiotic bacteria. It is the larger of these bacteria which we are interested in with respect to the theory of serial endosymbiosis because of their apparently close structural integration with the host cell (Whatley, 1976).

Besides the possible ancestors of chloroplasts and mitochondria mentioned above, various forms of intracellular symbionts (Jeon, 1972; Preer et al., 1974; Beringer, 1979; Wille et al., 1981) are found in a variety of host cells, both protozoans and metazoans (Jeon, 1972; Richmond and Smith, 1979; Schell et al., 1979; Cook et al., 1980; Schwemmler, 1983; Horowitz, 1983). In most cases, no defined functions of intracellular symbionts are known. There are only a few known examples in which the hosts derive benefits from their intracellular symbionts (Soldo and Godoy, 1973; Preer et al., 1974; Verma and Long, 1983).

As one of the typical forms of symbiotic association, the Rhizobium bacteria reduce molecular nitrogen (N_2) into ammonia (NH_3), which the host plant can use as a nutrient (Bishop et al. 1977). Heckman (1975) found that "omicron particles" in Euplotes aediculatus were essential for the multiplication of the host, although the mechanism of dependence is not clearly understood.

Among protozoans, many different species and strains of free-living amoebae are known to have endosymbionts or infective organisms (Roth and Daniels, 1961; Chapman-Andresen, 1971). They are all rod-shaped and gram-negative like other bacterial symbionts. Most of them have been observed within vesicles formed by the host cell. As in other free-living amoebae, the xD strain of Amoeba proteus contains a large number of bacterial endosymbionts which originated by a spontaneous infection in the strain D in 1966 (Jeon and Lorch, 1967). These infectious bacteria were rod-shaped, measuring approximately $0.5 \times 2 \mu m$, and gram-negative. Under the electron microscope, two kinds of bacteria with different electron density were found. The bacteria were enclosed within vesicles of varying sizes of up to $40 \mu m$ in diameter. The infecting bacteria were initially virulent to the host cells. Infected amoebae became smaller in size, more fragile, extremely sensitive to starvation, longer in generation time, and lower in clone formation ability. However, some amoebae survived and within a few years the infecting bacteria became less virulent as the initially observed adverse effects on their host diminished. Symbiotic bacteria became integrated cell components by 1971 (Jeon, 1972), that is to say, the

host amoebae were unable to survive without their bacterial endosymbionts.

In studies by micurgy (Jeon and Jeon, 1976) and by treatment with antibiotics (Jeon and Hah, 1977) it was confirmed that the host nuclei are dependent upon live bacterial endosymbionts. Another way has been found to demonstrate that the survival of host amoebae requires endosymbionts (Jeon and Ahn, 1978). Due to the psychrophile-like characteristics of X-bacteria (Morita, 1975), X-bacteria disappear from amoebae when the symbiotic amoebae are cultured at or above 26°C. Following the disappearance of X-bacteria, xD amoebae stop growing and die in a few days. The reasons why xD amoebae are dependent on their symbionts are not known. The endosymbionts were suspected to produce and supply substances that might be required by the host cells (Jeon and Jeon, 1976).

As it has been pointed out by others (Margulis, 1976; Smith, 1979; Whatley, 1976; Taylor, 1983), the obligatory symbiotic relationship between xD amoebae and symbiotic bacteria is an excellent model system among many different endosymbiosis systems for the study of the establishment of intracellular organismic association which is a necessary prerequisite in the evolution of intracellular organelles. In comparison with other intracellular symbiotic relationships, xD amoebae have many advantageous characteristics which can lead to the understanding of development of intracellular symbiosis. First, they have a known history of association with the original host strain. Second, they have shown characterizable transformation from parasitic

infective organisms into required cytoplasmic components within a relatively short period of time (Jeon, 1972, 1973; Jeon and Jeon, 1976). Third, the development of the obligatory endosymbiotic relation can be easily manipulated by experimental infection of x-bacteria into symbiont-free amoebae. Fourth, X-bacteria have "fitness traits" which are necessary requirements in order to become intracellular symbionts. Therefore, a study of this symbiosis system has significance in understanding the organismic associations of bacterial symbionts with protozoans as well as in furthering our understanding as to how such components could give rise to cell organelles in the eukaryotic cell.

In order to become persistent and organismically dependent intracellular symbionts, the microorganisms have to solve various problems related to compatibility mechanisms (Taylor, 1974). One of the important mechanisms is the endosymbiont's ability to avoid destruction or ejection by the host cells (Taylor, 1976). In contrast, digestion or intracellular killing of microorganisms is one of the central mechanisms of the host defense (Sbarra et al., 1971; Klebanoff, 1975; Johnston and Lehmyer, 1977). After entering the host cells, the intracellular symbionts should have the "fitness traits" for intracellular survival (Moulder, 1974) by resisting digestion and killing (Armstrong and Hart, 1971; Friz, 1972; Jones and Hirsch, 1972), by preventing the lysosomal fusion (Karakashian and Karakashian, 1973; Karakashian and Rudzinska, 1981) or by escaping from the vacuole system (Bannister, 1979). Such a preventive mechanism appears to be specific to each symbiotic or parasitic relationship. Among the possible mechanisms, intracellular

symbionts in xD amoebae are thought to use the following two mechanisms; resistance to lysosomal enzymes and inhibition of lysosomal fusion (Jeon, 1983).

Plasmids are extrachromosomal genetic elements which are physically separated from the chromosome of the cell. They are distinct replicons; genetic units capable of replicating independently from the host chromosome. These genetic elements are usually nonessential for the growth and survival of host cells, so that under most conditions they may be gained or lost without inducing a lethal effect (Clowes, 1972; Broda, 1979; Glass, 1982).

Rigorous researches in Escherichia coli and other bacteria over the last thirty years have lead to the identification of a vast collection of these non-essential genetic elements, responsible for a number of different bacterial properties. The first extrachromosomal genetic element identified in E. coli was the F-plasmid (Glass, 1982). The F-plasmid is episome which replicates either independently or as part of the host genome. One third of genetic element of the plasmid is involved in conjugation (Clark and Warren, 1979). A second group encodes the genetic information for resistance to antibiotics, allowing both gram-negative and gram-positive bacteria harboring these R-plasmids to survive in the presence of antibacterial agents (Davies and Smith, 1978; Hopwood, 1978). A third group, the Col-plasmids, are responsible for the production of diffusable proteins, such as colicin, which is bactericidal to strains of E. coli lacking these genetic elements (Davies and Reeves, 1975 a, b; Hardy, 1975).

Besides the above well-known plasmids, there are various plasmids in a large number of different organisms possessing unique functions. For instance, there are dissimilatory plasmids in Psuedomonas (Wheelis, 1975), gas-vacuole and toxin-producing plasmids of yanobacter (Lau et al., 1980), and biodegradative plasmid of Acromobacter (Ahmed and Focht, 1973). Plasmids are found not only in prokaryotic cells but also in eukaryotic cells (Stanfield and Helsinki, 1971; Wang and Wildman, 1972; Guerinau, 1979). Thus we face the discovery of new pathways for the reassortment of genetic information and the possible evolution of chromosome structure in both higher and lower organisms.

Interestingly, plasmids have been found in the symbiotic bacteria in a variety of hosts from protozoa to plants, i.e., Rhizobium bacteria of plants in the legume family (Bishop et al., 1977; Verma and Long, 1983), a number of kappa symbionts of Paramecium tetraurelia (Dilts, 1977), and gram-negative symbiotic bacteria "xenosomes" in Paramecium acutum (Soldo, 1983).

Bacterial plasmids are more successful symbionts at the genetic level than bacterial viruses in that they never kill their hosts but rather confer on them a greater defense against adverse environments (Hayes, 1968; Reanney, 1976). Therefore, symbiotic bacteria which harbor plasmids coding for special proteins, which are involved in a series of important complex interactions to establish and maintain intracellular symbiosis, could grow and multiply in their hosts.

Two plasmids were also isolated from symbiotic bacteria of the xD strain of Amoeba proteus (Han and Jeon, 1980). The roles of these

plasmid DNAs are not completely understood, but indirect evidence suggests that they are involved in the establishment and/or maintenance of intracellular symbiosis (Han and Jeon, 1980; Ahn and Jeon, 1982). One prerequisite for X-bacteria to establish intracellular symbiosis within the amoebae is to avoid digestion by the host.

Since amoebae are phagocytosing cells rich in lysosomal enzymes (Chapman-Andresen, 1973) and destroy most exogenous material including microorganisms (Savanat and Pavillard, 1964; Casley-Smith and Savanat, 1966), X-bacteria should have some protective device to avoid digestion by amoebae during the initial stages of infection when they are directly exposed to the amoeba lysosomal enzyme system. In a test in vitro, the X-bacteria were found to be resistant to several digestive enzymes and some detergents (Han and Jeon, 1980), suggesting that X-bacteria have a protective device within their cell wall. In this regard, the device of X-bacteria to escape from the digestion by amoebae could be explained in terms of bacterial cell-wall composition. The cell walls of X-bacteria seem to have a unique protein component. This protein component of the X-bacteria cell wall was suggested to be encoded by genes in the plasmid DNA of X-bacteria (Han and Jeon, 1980) as has been found to be the case in other organisms (Costerton, 1977; Sansonetti et al., 1982). When symbiotic bacteria of xD amoebae are segregated into separate vesicles, they are not digested by lysosomal enzymes. Because lysosomes do not fuse with symbiotic vacuoles (Jeon and Jeon, 1976; Ahn and Jeon, 1982). Thus, these bacteria somehow prevent the fusion between the symbiotic vacuole membranes and lysosomes.

Ahn and Jeon (1982) made an attempt to elucidate the mechanism for the prevention of lysosomal fusion by comparing the protein composition of the membranes of symbiotic vesicles with that of the plasmalemma and of induced phagolysosomal membranes by means of SDS-polyacrylamide-gel electrophoresis and autoradiography. They found that the symbiotic vacuole membranes contained a polypeptide component on the cytoplasmic surface which was not found in either plasmalemma or phagolysosomal membranes. The origin and specific role of this unique polypeptide have not been determined, but this peptide could be encoded by the plasmids of X-bacteria.

The objective of present study was to test the hypothesis that either one or both of X-bacteria plasmids are responsible for the establishment and/or maintenance of the symbiotic relationship between amoebae and X-bacteria, that is, to determine the relationship between the disappearance of the plasmids and the loss of infectivity of X-bacteria to nonsymbiotic D amoebae. For the purpose of this test, plasmids were eliminated from X-bacteria in vivo and in vitro by plasmid-curing agents, ethidium bromide (EtBr) and acridine orange (A.O). In vivo, the plasmid was cured by culturing xD amoebae in a medium containing a sublethal concentration of plasmid-curing agents. In vitro, isolated X-bacteria were incubated in plasmid-curing agents at 4 C for a given period of time. A verification of the relationship between the loss of infectivity and the absence of plasmid DNA was made by using poly-L-lysine-induced phagocytosis by means of infection. Infection kinetics were studied by counting the number of X-bacteria at

intervals after the induced infection. Agarose-gel electrophoresis was used to detect the presence or absence of plasmids after treatment with plasmid-curing agents.

CHAPTER II

MATERIALS AND METHODS

A. Culture of Experimental Organisms

1. Amoebae

Strains of amoebae. In this study, two strains of Amoeba proteus, D and xD, were used. The D strain was originally obtained from Sister Monica Taylor (Glasgow, Scotland) in 1948. Some of the D amoebae were spontaneously infected with rod-shaped gram-negative bacteria of unknown origin in 1966 (Jeon and Lorch, 1967). The infected strain of Amoeba proteus was designated as the xD strain since that time.

Culture medium. The modified Chalkley's solution (Jeon and Jeon, 1975) was used in this study. The stock solutions were prepared as follows and kept at 4°C. Stock solution I (x1000); 0.5 M NaCl, 0.05 M KCl, 0.05 M NaHCO₃ and 7.2 mM Na₂HPO₃. Stock solution II (x2000); 0.1 M CaCl₂ 2H₂O. A working solution was prepared by adding 1 ml of Stock solution I and 0.5 ml of Stock solution II to 1 liter of glass-distilled water.

Stock cultures. Stock cultures were maintained in glass pie dishes (15.2 cm in diameter) at 20°C with Colpidium sp. as food organisms (Lorch and Danielli, 1953). Each culture was renewed once a month by placing 300-400 healthy amoebae in a clean dish with Colpidium sp. and 200 ml of Chalkley's solution. All renewed dishes were supplied with 5

wheat grains to support growth of Colpidium populations.

Mass culture. To obtain a large number of amoebae, both strains of Amoeba proteus were cultured with axenically grown Tetrahymena pyriformis as food organisms in Pyrex baking dishes (34 x 22 x 4.3 cm) containing 1 liter of Chalkley's solution at 22°C. Amoeba culture dishes were changed to keep them clean; the old culture medium was aspirated, and floating amoebae were decanted to another fresh dish. The cells attached to the bottom of dish were transferred to a clean dish with fresh medium. Amoebae were fed everyday or every other day with freshly harvested Tetrahymena to maintain steady growth conditions. After amoebae attained maximum growth, they were starved for 2 days by simply-changing the dishes with fresh medium to eliminate undigested food organisms, and harvested by centrifugation for 1 minute at 150 g and washed once with Chalkley's solution. Each dish yielded about 1.5 ml of packed cells containing about 5×10^5 amoebae. The packed amoebae were quickly frozen in dry-ice acetone and stored at 70°C until used to isolate X-bacteria.

2. Food Organisms

Tetrahymena pyriformis (GL strain). Tetrahymena were axenically cultured as described by Goldstein and Ko (1976). The culture medium consisted of 2 % proteose peptone (w/v), 0.2 % liver concentrate (w/v), supplemented with salt and vitamin solutions. Prepared medium was divided into 100 ml each in 500-ml flasks or 200 ml each in 1000-ml

flasks. Then medium was autoclaved for 20 minutes at 15 psi above atmospheric pressure. After inoculating 10 ml of fully grown Tetrahymena culture into 200 ml of medium in a 1000-ml flask, the cells were grown for 2-3 days with shaking at room temperature. Before harvesting, Chalkley's solution equal to one half volume of the medium was added and the culture was shaken for at least 20 minutes to minimize an osmotic shock. Tetrahymena were harvested by centrifugation for 1 minute at 350 g in an IEC centrifuge (Model PRJ) and washed three times in chalkley's medium. After washing, Tetrahymena were suspended in 150 times volume of the Chalkley's solution which yielded a density of about 1.3×10^5 cells/ml (optical density 0.4 at 515 nm). Stock cultures of Tetrahymena were kept in a medium consisting of 2 % proteose peptone and 0.1 % bacto yeast extracts in glass distilled water.

Colpium sp. Colpidium were cultured in glass pie dishes (15.2 cm in diameter) at 20°C, and renewed once a month. After filtering the fully grown Colpidium through a thin layer of absorbent cotton to remove wheat grains and mold debris, 30 ml of filtrate were transferred to a new pie dish with 15 boiled wheat grains and 200 ml of Chalkley's solution. The boiled wheat grains supported the growth of bacteria which served as food organisms for Colpidium.

3. Bacterial strains

Two plasmid-harboring strains of E. Coli, strain GM 8 (pBR 322) (Bolivar et al., 1977) and GM 8 (RSF 2124) (So. et al., 1966), were

used as sources of molecular markers for the study of the two X-bacterial plasmids. The bacteria were cultured in Luria-Bertani medium (LB) with appropriate antibiotics as follows; After inoculating 1 platinum-loop of bacteria inoculum from the agar slant culture into 2 ml of LB medium with ampicillin, 50 ug/ml of final concentration, bacteria were cultured overnight at 37°C with shaking. Next morning, 0.1 ml of first overnight culture was inoculated to 10 ml of LB medium with ampicillin and cultured for another 10-12 hours. One ml of culture in logarithmic growth phase were taken and inoculated to 100 ml of medium in a 500-ml Erlenmeyer flask. About 5-6 hours later, chloramphenicol was added to a final concentration of 170 ug/ml to amplify plasmids. The culture was kept at 37°C for another 12-14 hours before harvest. Extra care was given to this final period to prevent bacteria from lysing caused by an overgrowth. Bacteria were harvested by centrifugation for 5 minutes at 12,000 g in a JA-20 rotor. Centrifugation was performed in a Beckman centrifuge (Model J-21C). After washing twice with STE buffer (100 mM NaCl, 10 mM Tris-Cl, 1 mM EDTA, pH 8.0), bacteria were frozen and kept at -70°C for further experimental use.

B. Isolation of X-bacteria

Freshly harvested amoebae were suspended in twice the volume of isolation buffer (50 mM Tris-Cl, 50 mM EDTA, 100 mM NaCl, pH 8.0) containing 2 % Triton X-100 and vortexed vigorously at the highest

setting for 1 minute. After vortexing, 8 times the volume of isolation buffer without Triton X-100 was added to the amoeba suspension. Then, amoebae were kept at 4°C for 10 minutes, followed by 2 cycles of freeze-thawing. By vigorous vortexing in 2 % Triton X-100 and freeze-thawing, rupturing of the cells as well as cellular organelles such as nuclei and symbiotic vacuoles was satisfactorily achieved. The lysate formed was filtered through a 20-um-mesh nylon screen. Only X-bacteria, characteristic amoeba crystals and small debris were found under the phase-contrast microscope after the above steps. To remove crystals and small debris, the lysate was further centrifuged for 5 minutes at 750 g and the supernatant was collected. Some X-bacteria coprecipitated with the crystals, and the initial pellet was suspended in 10 ml of 1 N NaOH and vortexed vigorously. By vigorous vortexing, almost all crystals and membrane fragment were lysed. Bacteria recovered from the initial pellet were immediately centrifuged and washed 2 times with the washing buffer (20 mM Tris-Cl, pH 7.4). The resuspended bacteria were combined with the initial supernatant and were washed 3 times with fresh ice-cold washing buffer by centrifugation for 5 minutes at 12,000 g. When higher purity of X-bacteria was needed, the final bacteria pellet which contained only very traces of cell debris, they were further purified by sucrose density-gradient centrifugation. The final pellet was suspended in 2 ml of 10 % sucrose in the washing buffer, layered on top of a step gradient of 20, 30, 40, and 50 % sucrose in a Corex tube. Pure X-bacteria were collected from the 30/40 % layer after centrifugation for 20 minutes at 13,000 g and washed 3

times with the washing buffer to remove sucrose. During these isolation procedures, the purity of X-bacteria preparations were monitored by checking the bacteria suspension under the phase-contrast microscope. Isolated X-bacteria were then frozen at -20°C and used for DNA isolation or infectivity tests.

C. Bacteria Counts

To measure the yield of isolated X-bacteria or to adjust the initial concentration of X-bacteria for each induced infection, the number of isolated X-bacteria was determined. A drop of 1000-times-diluted bacteria suspension was placed on a Petroff-Hausser bacteria counter, and the number of X-bacteria was counted under a phase-contrast microscope. To determine the average number of bacteria per amoeba, 30 healthy amoebae were picked up from each experimental group and washed at least 3 times with Chalkley's solution by transferring from dish to dish to remove any contaminating bacteria in the medium. The washed amoebae were placed on a depression slide, and the excess medium was removed with a mouthpipette. Amoebae were lysed in 30 μl of 1 N NaOH, and bacteria were counted as described above.

D. Induced Infection

1. Bacteria preparation

After collecting isolated X-bacteria by centrifugation, 3 ml of

0.05 % poly-L-lysine was added to the bacteria pellet, vortexed, and left for 20 minutes at 4 °C. After incubation, bacteria were washed twice with Chalkley's solution by centrifugation for 5 minutes at 2,000 g and resuspended in Chalkley's solution to a final concentration of 1×10^7 bacteria per ml.

2. Infection

Healthy D amoebae were starved for 2 days and 1×10^3 amoebae were placed in a small Falcon tissue-culture dish (3.5 x 10 mm). After aspirating as much medium as possible, poly-L-lysine-treated bacterial suspension was added to each dish. After 2 hours, when D amoebae ingested the maximum number of X-bacteria, the remaining bacterial suspension was aspirated and amoebae were washed several times with Chalkley's solution. Furthermore, the dishes were changed to remove uningested X-bacteria left at the bottom of the dish.

E. Isolation of Plasmids

1. Lysis of X-bacteria

X-Bacteria were lysed by the method of Han and Jeon (1980) with some modifications. X-Bacteria isolated from 1 ml of freshly harvested amoebae were suspended in 200 ul lysis buffer (25 % sucrose, 50 mM Tris-Cl, pH 8.0) by vortexing. All procedures were carried out at 4 °C unless stated otherwise. First, 100 ul of 0.25 M Na₂EDTA (pH 8.0) and 100 ul of freshly prepared proteinase K (5 mg/ml in lysis buffer) were

added to the initial bacteria suspension. The suspension was incubated for 5 minutes at 37 °C, and then 100 ul of 10 % sodium dodecyl sulfate (SDS) in lysis buffer were added. The mixture was incubated for 20 minutes at 37 °C with intermittent shaking. The mixture was reincubated for another 40 minutes at 60 °C and immediately cooled to 4 °C. The mixture after showing increased viscosity was centrifuged for five minutes at 11,000 g in a micro-centrifuge (Fisher model 235 A), and the supernatant containing DNA was transferred to a fresh micro-centrifuge tube. The pellet contained a considerable number of unlysed X-bacteria. To recover more DNA, pellets were suspended again in 200 ul of lysis buffer and treated with proteinase K and SDS as before. The suspensions of X-bacteria were incubated for 10 and 20 minutes at 37 °C and 60 °C, respectively. After incubation, centrifugation was performed for 5 minutes in the micro-centrifuge. The second supernatant was combined with the first supernatant. When handling large amounts of X-bacteria, i.e. over 5×10^{10} bacteria, 15-ml size Corex tubes were used instead of micro-centrifuge tubes. Centrifugation was performed in a JA-20 rotor. The volume of each solution was also adjusted to the amount of X-bacteria.

2. Clearing of Chromosomal DNA

To increase the plasmid to chromosomal DNA ratio in the total lysate of X-bacteria, chromosomal DNA had to be removed. This was done by the method of Guerry et al. (1973). To each lysate, 0.2 volume of 5 M NaCl was added and the mixtures were kept overnight at 4 °C. Then,

the mixtures were centrifuged for 10 minutes in a micro-centrifuge.

3. DNA Purification

The cell lysate from SDS-salt precipitation step was subjected to deproteinization by the method of Marmur (1961). The supernatant obtained from lysis step was extracted twice with phenol-chloroform and once with chloroform-isoamyl alcohol (24:1, V/V). The supernatant after SDS-salt precipitation was extracted with 1 volume of saturated phenol by gentle rocking motion for 5 minutes. Then the DNA solution was centrifuged for 5 min in the micro-centrifuge to separate the aqueous (plasmid-containing) phase from the phenol (protein containing) phase. The aqueous layer was removed with a micropipetter and placed in a second 1.5-ml micro-centrifuge tube. After repeating the above steps once more, the DNA solution was reextracted with chloroform to remove the phenol. The DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.5) and 2 volumes of ice-cold ethyl alcohol. Ethanol precipitation of DNA was facilitated by standing at -20°C overnight. The DNA was next recovered by centrifugation for 10 minutes at 11,000 g in the micro-centrifuge. After removing the supernatant, the DNA was washed with 70 % ethanol at room temperature. Ethanol was removed by placing tubes in an inverted position on a layer of absorbent paper. The recovered DNA was dried by a gentle stream of nitrogen gas. Final DNA pellets were dissolved to desired concentrations in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) with 0.1 volume of RNase A (100 ug/ml in TE). These DNA solutions were stored at -20°C for further

determinations by electrophoresis.

F. Isolation of Plasmid DNA from E. coli

A rapid alkaline-extraction mini lysate method (Birboim and Doly, 1979) was adopted for the isolation of plasmid DNA from E. coli. Three ml of fully grown bacteria culture were spun down in a 1.5 ml micro-centrifuge tube, and the medium was carefully aspirated using a fine-tipped Pasteur pipette, and the cell pellet was loosened from the wall of tubes by vortexing tubes. The pellet was suspended thoroughly in 100 ul of lysozyme solution (50 mM glucose, 25 mM Tris, pH 8.0, 10 mM EDTA, pH 7.5, 5 mg/ml lysozyme, added freshly.) by vortexing immediately after each addition. The suspension was kept at 0 °C for 5 minutes. After adding 200 ul of alkaline SDS (0.2 N NaOH, 1 % SDS) at room temperature, the sample was mixed gently by inverting tubes several times and kept at 0 °C for 5 minutes. Then, 150 ul of high-salt solution (3M potassium acetate) was added, mixed gently, and kept at 0 °C for 15 minutes. A curd-like precipitate was pelleted down by centrifugation for 5 minutes. About 400 ul of supernatant was transferred to another fresh tube, care being taken to avoid disturbing the bulky pellet of chromosomal DNA and protein. Transferred supernatant was extracted with 400 ul of phenol:chloroform (1 : 1 v/v) by a gentle rocking motion for 10 minutes, and then centrifuged for 5 minutes. The upper aqueous phase was transferred to another clean micro-centrifuge tube, mixed with 400 ul of chloroform-isoamyl alcohol (24 : 1, v/v), and centrifuged for

5 minutes. At the end of deproteinization, 1 ml of cold 100 % ethanol (-20°C) was added to the sample, kept at -20°C for 20 minutes, and then centrifuged for 5 minutes at 11,000 g in a micro-centrifuge. The supernatant was removed carefully so as not to disturb the pellet. The tube was washed with 500 μl of 70 % ethanol, and ethanol was removed by pouring. Small droplets of ethanol inside of tube were aspirated with a fine-tipped Pasteur pipette. Furthermore, the pellet was dried completely by a gentle stream of nitrogen gas to prevent pellet from not dissolving into the buffer. The pellet was dissolved in 50 μl of TE buffer with 0.1 volume of RNase A (100 $\mu\text{g}/\text{ml}$). Five μl of DNA per well was used for electrophoresis.

G. Agarose-Gel Electrophoresis

To verify the presence or absence of plasmid DNAs, DNA samples were subjected to analytical agarose-gel electrophoresis (McDonnell et al, 1976; Meyers et al, 1976). Agarose-gel (Seakem ME agarose) was prepared in a horizontal slab-gel apparatus according to the procedure of Fangman (1978). The dimension of gel used for analytical purpose was 8.4 x 15 x 0.5 cm. Gel was prepared by melting 0.15 gm of agarose in 30 ml of an electrophoresis buffer (89 mM Tris-Cl, 89 mM boric acid, and 1.5 mM EDTA, pH 8.0) with a brief autoclaving or boiling in a waterbath and cooled to 50°C before pouring. After the gel was completely set (40 minutes at room temperature), the well-former was carefully removed. Before taking out the well-former, small amount of electrophoresis

buffer was added around the well-former in order to prevent the sample wells from deforming. Samples mixed with loading buffer, which contained 10 % glycerol and 0.025 % bromophenol blue as a tracking dye, were loaded into the sample wells. Before adding samples, the gel was equilibrated by prerunning it for 30 minutes at 50 volts. After adding samples, power was applied for about 30 minutes at 50 volts, or long enough for the samples to enter the gel. When all tracking dye entered the gel, the voltage was increased to 100 volts. Electrophoresis was carried out at 100 volts until the tracking dye reached the end of the gel, using a Vokam power supply, Model 2541. To prevent gel from drying, the chamber was covered with a fitting cover which had wet filter paper underneath. At the end of the run, the DNA bands were stained by immersing gel in a solution containing 0.5 ug ethidium bromide (EtBr) per 1 ml of distilled water for 30 minutes at room temperature. The DNA bands were visualized under UV light by virtue of the fluorescence emitted by bound EtBr (Sharp et al., 1973). The rinsed gel was directly laid 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).

H. Curing of Plasmids

Plasmid was eliminated from bacteria by either one of the two following procedures. (1) In vivo: Plasmids were cured by culturing symbiotic amoebae in a medium containing ethidium bromide (10^{-5} M)

(Bouanchaud et al. 1968) or acridine orange (10^{-6} M) (Hirota, 1960; Kawakami and Landman, 1965). xD amoebae were treated with plasmid-curing agents for 3 days, 7 days, and 14 days followed by 3 days in the normal Chalkley's medium. Then X-bacteria were isolated and used for infectivity test and DNA isolation. (2) In vitro: Isolated bacteria were incubated in a medium containing plasmid-curing agents for 3, 6, and 12 hours at 4°C. After incubation, bacteria were washed twice with the bacteria-washing buffer to remove plasmid-curing agents. Then bacteria were used for testing subsequent infectivity.

CHAPTER III

RESULTS

A. Effect of Curing Agents on the Hosts

To find the optimum concentrations of plasmid-curing agents which effectively remove the plasmid DNAs from the symbiotic bacteria without damaging the host cells, xD amoebae were cultured in Chalkey's solution containing various concentrations of plasmid-curing agents for up to 2 weeks. During the experiments, the number of amoebae was counted every other day. Growth rates of amoebae are shown in Figs. 1 and 2. When amoebae were treated with ethidium bromide at concentrations higher than 10^{-4} M, they rounded up and lysed within 4 days due to cytotoxic effects of the curing agents. However, when amoebae were treated with ethidium bromide at concentration lower than 10^{-5} M, only part of the amoebae were lysed. Unlysed amoebae in 5×10^{-6} M ethidium bromide multiplied and increased in number after 3-5 days of stationary phase during which they adjusted to the curing agent. xD amoebae cultured in acridine orange showed similar symptoms as amoebae treated with ethidium bromide. Even though 10^{-5} M ethidium bromide and 10^{-6} M acridine orange were not actual LD₅₀, they were chosen for plasmid-DNA curing since they were the highest dosages that allowed healthy survivors and did not show cytotoxicity for Tetrahymena, the food organism. The actual concentration of LD₅₀ during 2 weeks of curing agent treatment and additional 3 days for recovery was 5×10^{-5} M for ethidium bromide and 5×10^{-6} M for acridine orange, respectively.

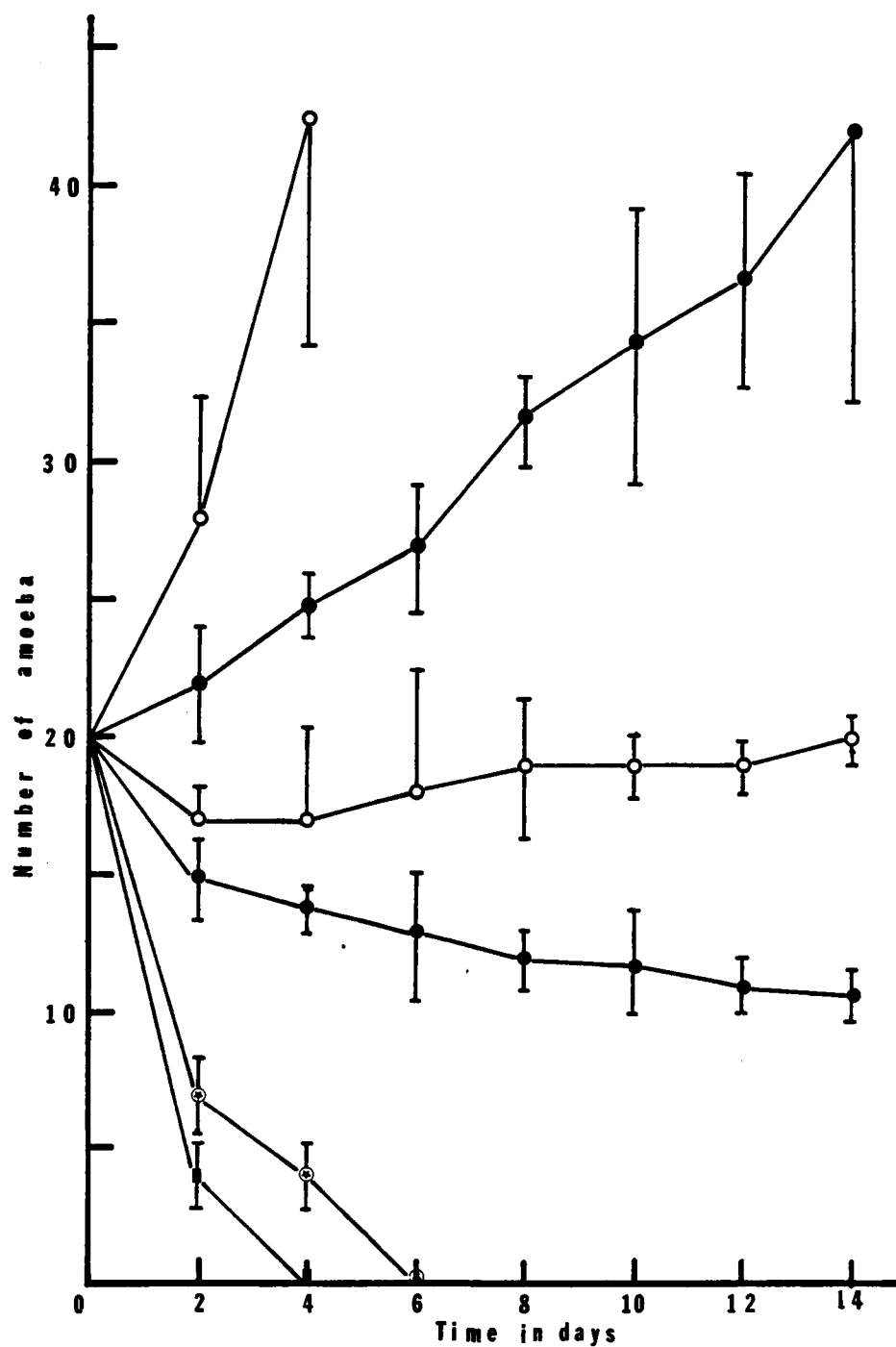


Figure 1. Number of xD amoebae during treatment with various concentrations of acridine orange.

- | | |
|---------------|------------------------|
| ○ Control | ● 5×10^{-7} M |
| ○ 10^{-6} M | ● 5×10^{-6} M |
| ⊕ 10^{-5} M | ■ 5×10^{-5} M |

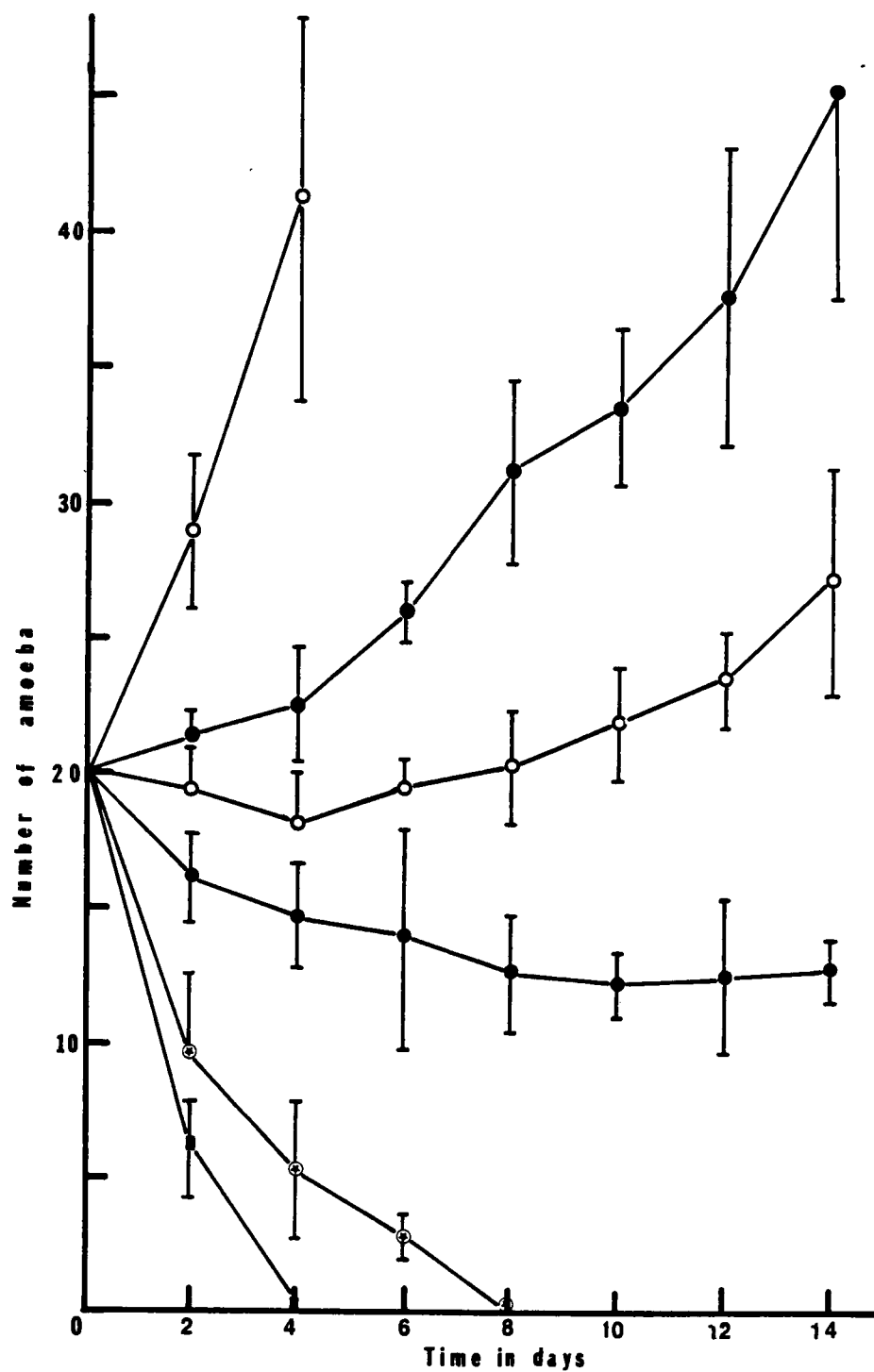


Figure 2. Number of xD amoebae during treatment with various concentrations of ethidium bromide.

- | | |
|---------------|------------------------|
| ○ Control | ● 5×10^{-6} M |
| ○ 10^{-5} M | ● 5×10^{-5} M |
| ⊗ 10^{-4} M | ■ 5×10^{-4} M |

B. Effect of Curing Agents on Symbionts

In order to test the effects of plasmid-curing agents on symbiotic bacteria during the study period, the number of symbiotic bacteria per amoeba was counted every other day. Thirty amoebae were picked up from the mass culture just before changing the medium, which contained plasmid-curing agents. During the early part of the study period (2 - 4 days), the number of bacteria per amoeba declined rapidly (Fig. 3). At the same time, the hosts also showed adverse symptoms, including lysis, floating, and increased sensitivity to starvation. During later culture periods (5 - 14 days), the number of X-bacteria per amoeba was maintained at about 40 % of their original number. During the same period, hosts showed a certain degree of adaptation to the cytotoxicity caused by the plasmid-curing agents. A similar phenomenon was also found in small Falcon dish cultures. However, the declining pattern of the average number of bacteria per amoeba was somewhat different from that of the mass culture. Until the 10th day, bacteria showed a sharp decrease in average number. From the 10th day on, the average number per amoeba was about 40 % of the original number per amoeba (Figs. 4 and 5).

C. Induced Infection

In order to characterize plasmid-harboring X-bacteria as infective agents and to examine the structural and physiological aspects of the

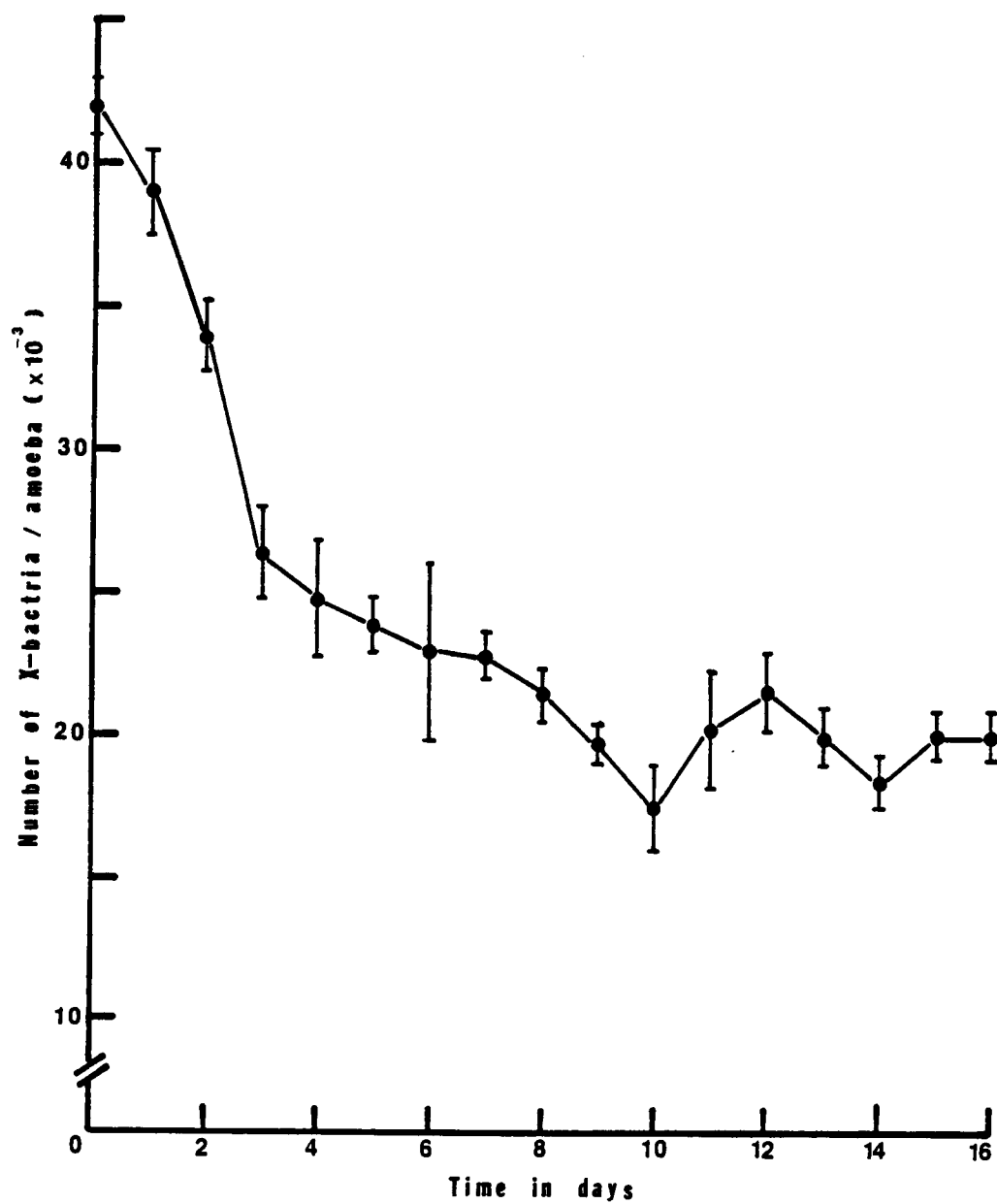


Figure 3. The average number of X-bacteria per amoeba in a mass culture during treatment with acridine orange (10^{-6} M).

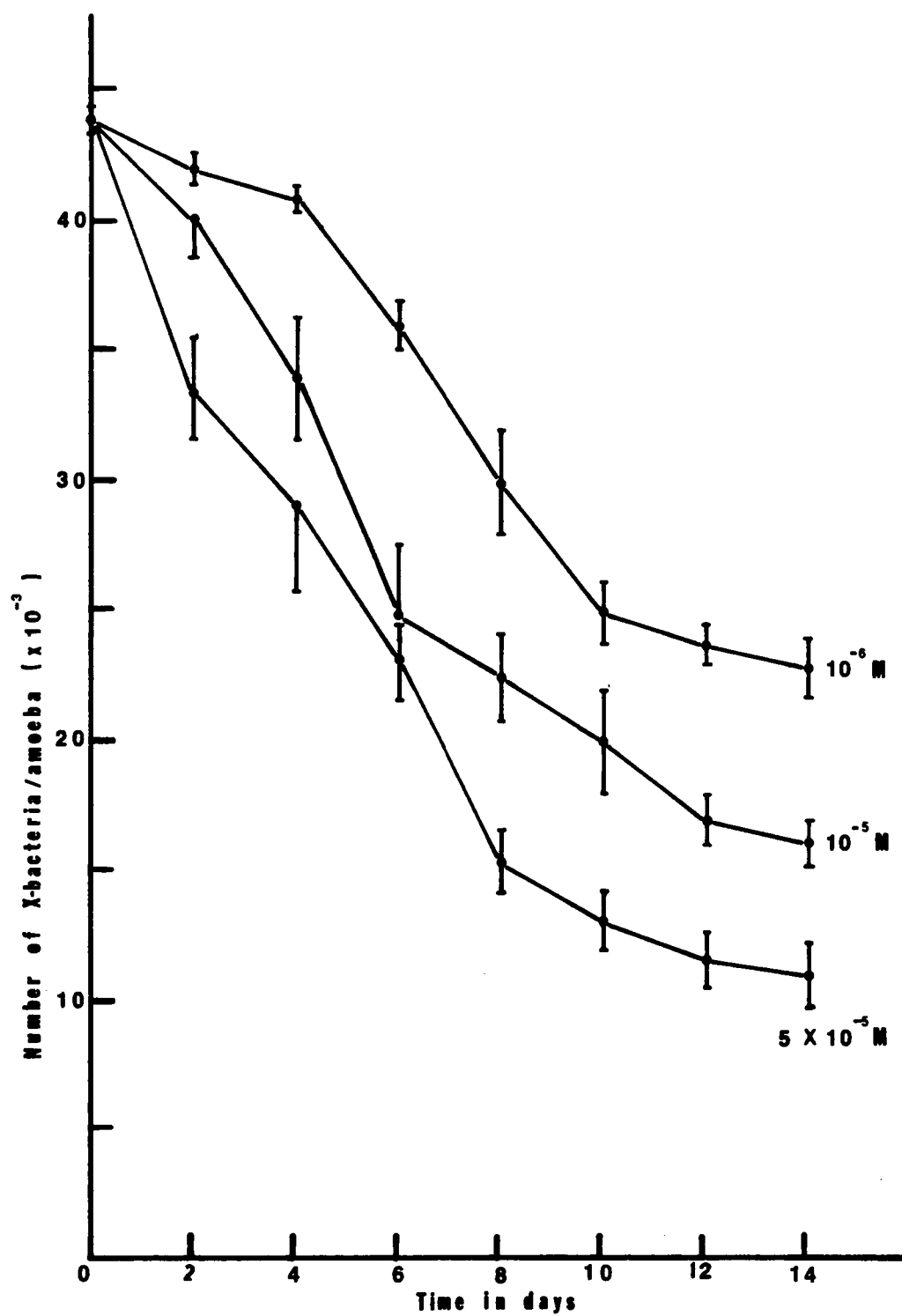


Figure 4. The average number of X-bacteria per amoeba in a Falcon dish culture during treatment with ethidium bromide.

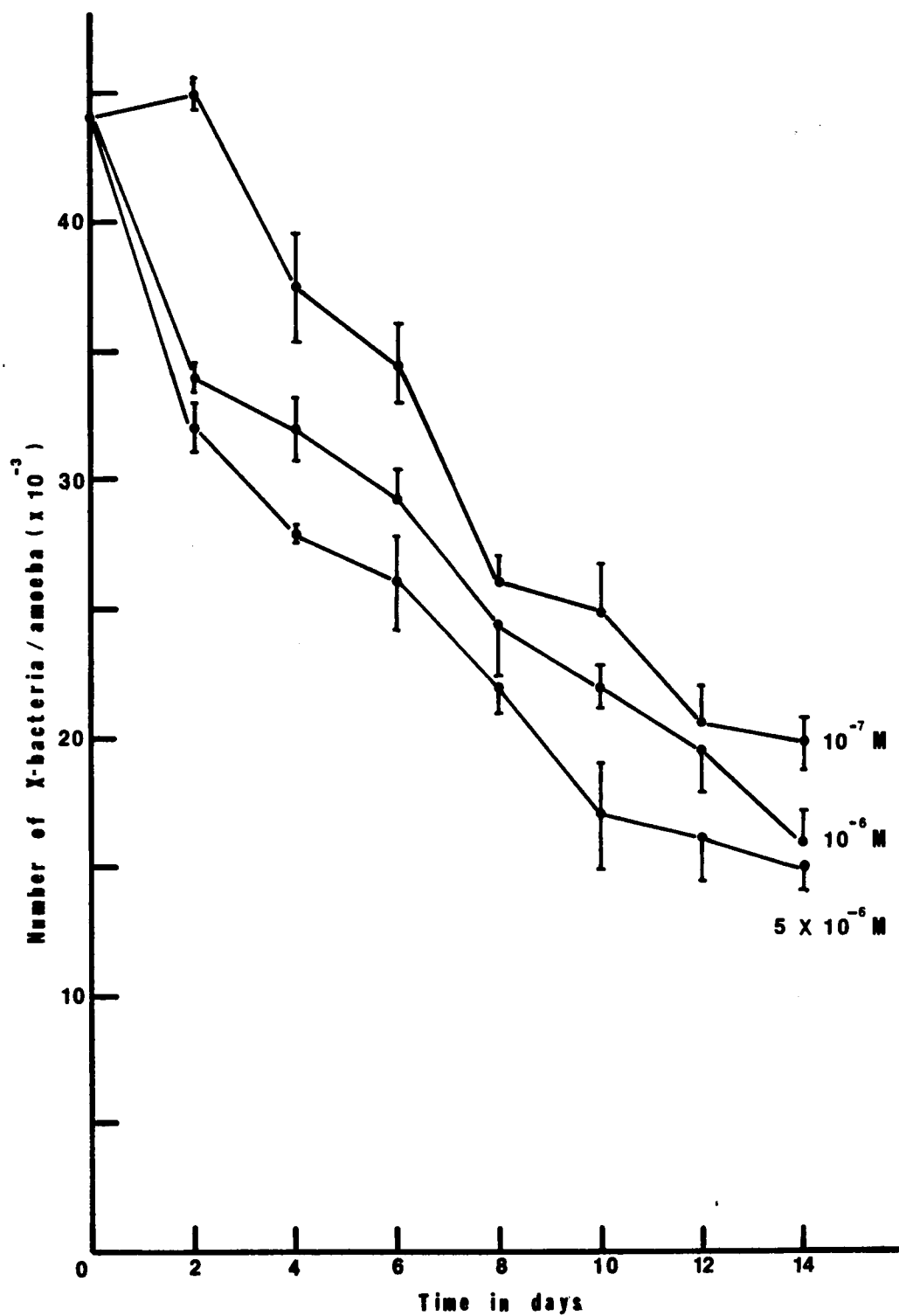


Figure 5. The average number of X-bacteria per amoeba in a Falcon dish culture during treatment with acridine orange.

host-symbiont relationship during the initial stages of infection, non-symbiotic D amoebae were infected with X-bacteria isolated from symbiotic amoebae. D amoebae were infected with E. coli and plasmid-cured symbiotic bacteria at the same time to confirm whether the establishment of symbiosis in amoebae was due to the presence of plasmid DNA. Immediately after 2 hours of incubation with the bacteria suspension, about 15 amoebae were picked up and examined under the phase-contrast microscope. The cytoplasm of each amoeba was full of small and large vesicles containing aggregated X-bacteria, but no symbiotic vacuoles were detected at the same time. When infected amoebae were ruptured and examined under a phase-contrast microscope, X-bacteria-containing vesicles were found to have irregular shapes, which were different from the regular and round-shaped symbiotic vacuoles.

Each amoeba ingested 20,000 - 45,000 X-bacteria by induced infection. Newly infected amoebae lost more than 50 % of initially ingested X-bacteria within 24 hours, while the number of vesicles containing X-bacteria decreased sharply (Fig. 6). From the 2nd day on, small symbiotic vacuoles containing 1 - 15 X-bacteria were detected, while the size and number of symbiotic vacuoles increased gradually. On the 5th day, large vesicles containing X-bacteria were hardly detectable, but the symbiotic vacuoles containing 5 - 30 X-bacteria were found and amoebae had only about 10 % (average 3,300 per amoeba) of the maximally ingested X-bacteria. After 5 days of infection, the number of X-bacteria increased and reached the maximum carrying capacity (about

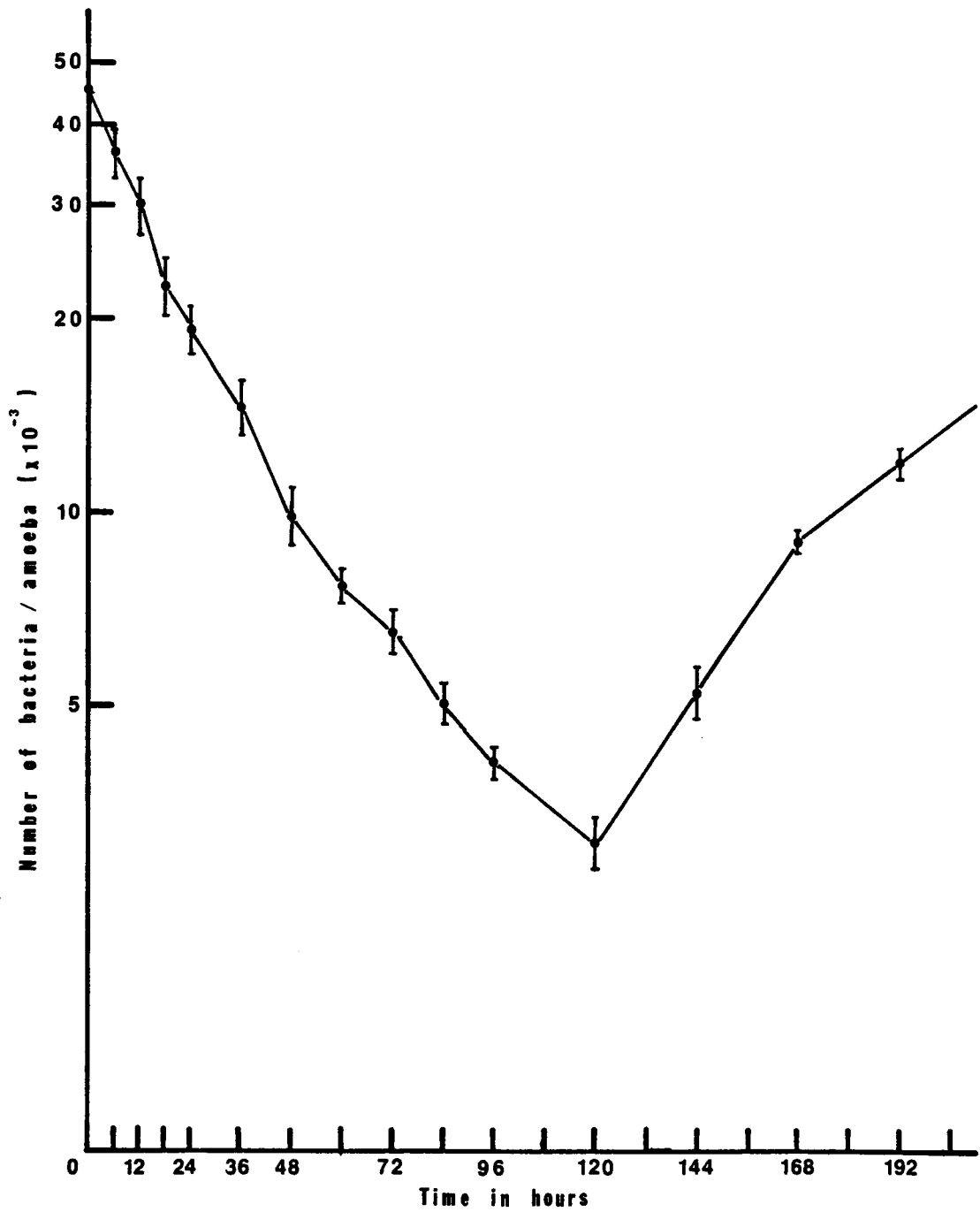


Figure 6. The average number of X-bacteria per amoeba after induced infection.

42,000) on the 14th day.

When amoebae were infected with E. coli, the number of ingested bacteria decreased in a similar trend as that of X-bacteria during the first 24 hours of infection. However, E. coli disappeared from the amoeba cytoplasm rapidly after 36 hours of infection. The major difference as compared with X-bacteria was that no E. coli were detected in the amoeba cytoplasm 72 hours after infection (Fig. 7). This indicated that all E. coli were digested by this time.

D. Effect of Plasmid-Curing Agents on Infectivity of Symbionts

In order to investigate the effect of plasmid-curing agents on the infectivity of endosymbiotic bacteria, symbiotic bacteria were treated with ethidium bromide or acridine orange in vivo and in vitro, introduced into D amoebae, and checked their survival in the amoebae.

In vivo. As the results show, X-bacteria isolated from the control group were able to infect and establish their population in the D amoebae (Fig. 6). However, X-bacteria isolated from amoeba culture in the medium with 10^{-5} M ethidium bromide or 10^{-6} M acridine orange for 7 and 14 days lost their infectivity (Figs. 8 and 9). When bacteria were treated with curing agents for 7 days in vivo, they completely disappeared from the amoeba cytoplasm within 60 hours of experimental infection. Particularly, the bacteria isolated from symbiotic amoebae grown for 14 days in curing agents decreased rapidly in number inside amoebae and disappeared completely during the first 36 hours of induced

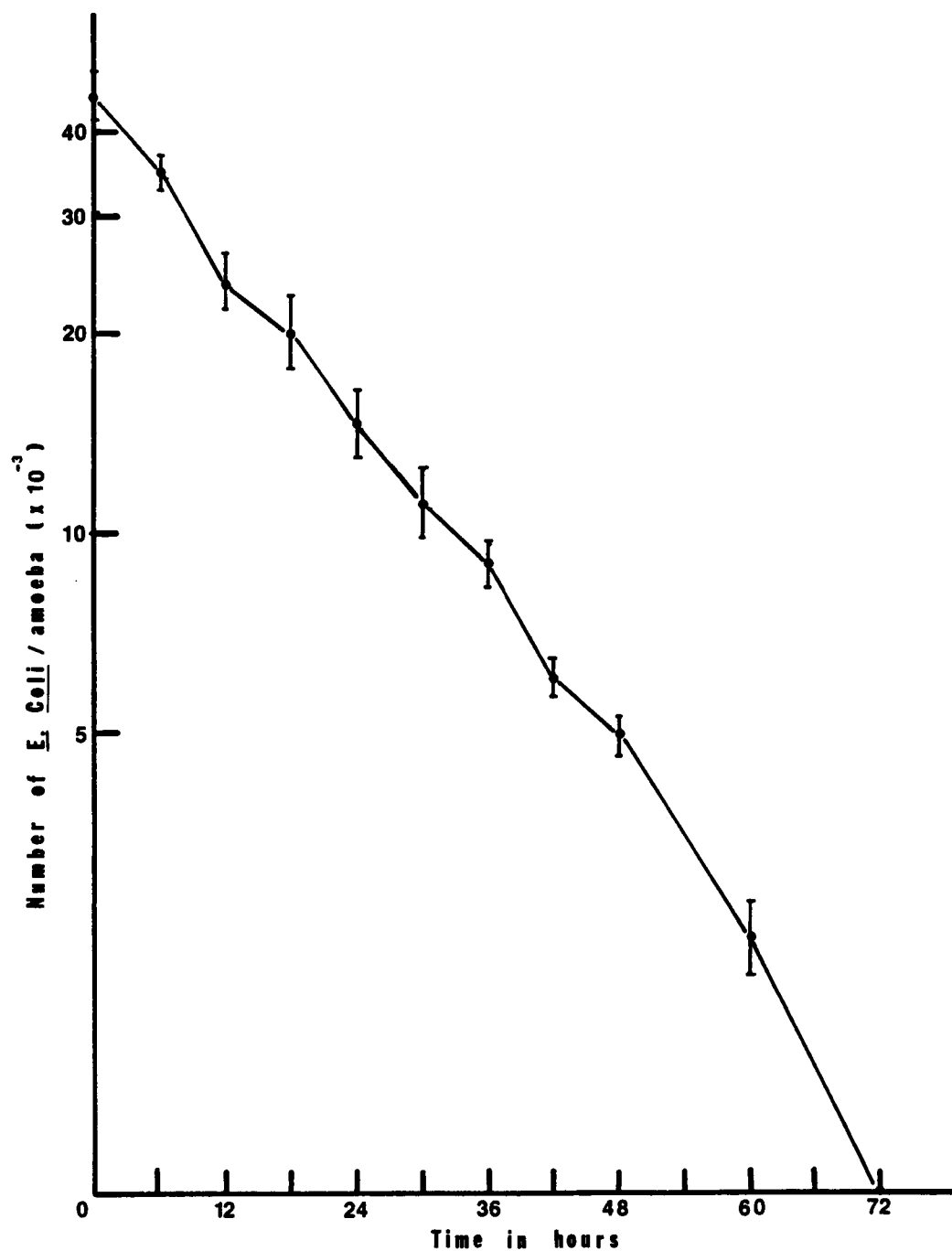


Figure 7. The average number of *E. coli* per amoeba after induced infection.

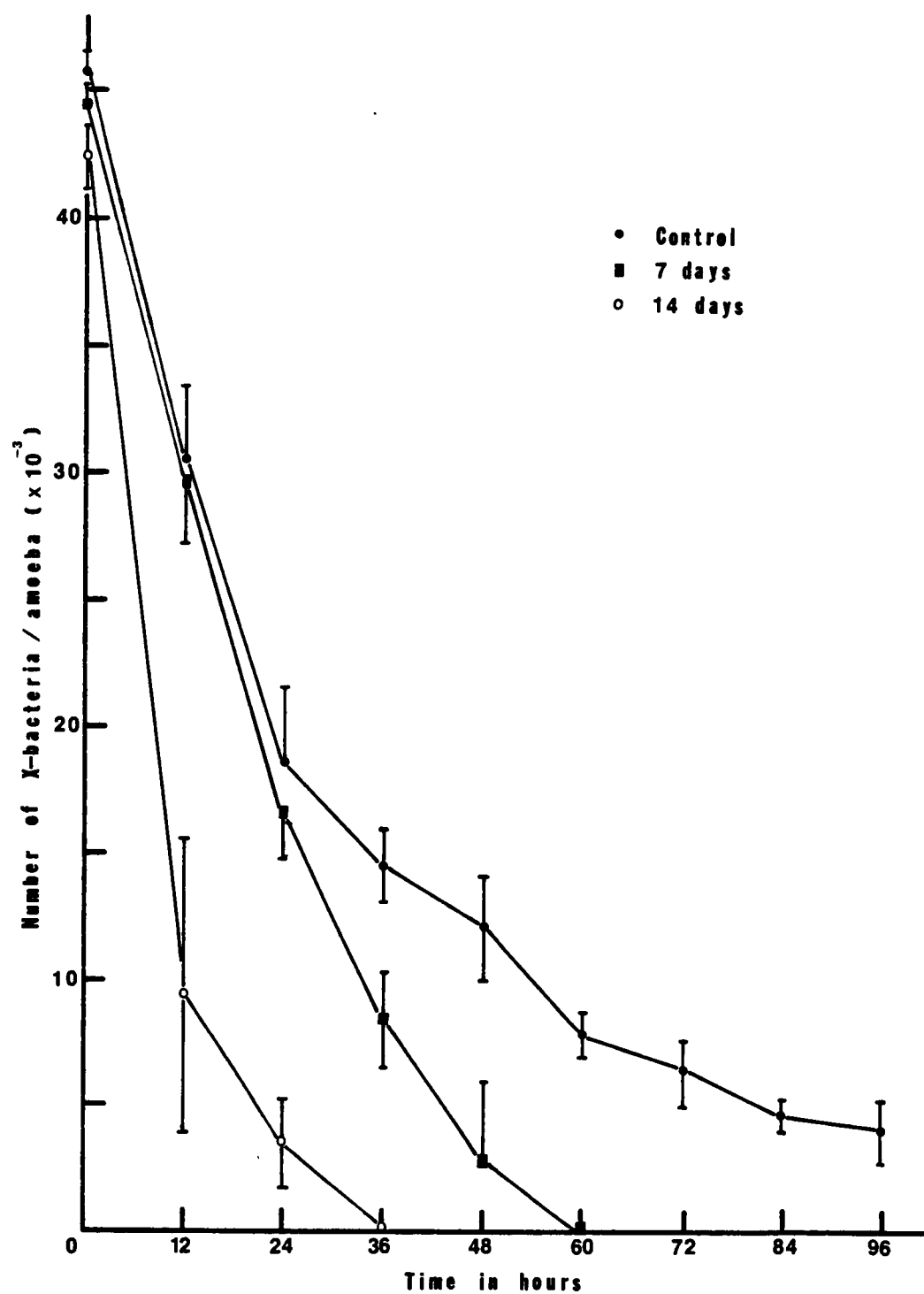


Figure 8. The average number of plasmid-cured X-bacteria per amoeba after induced infection. Plasmid had been cured in vivo for 7 or 14 days with 10^{-5} M ethidium bromide.

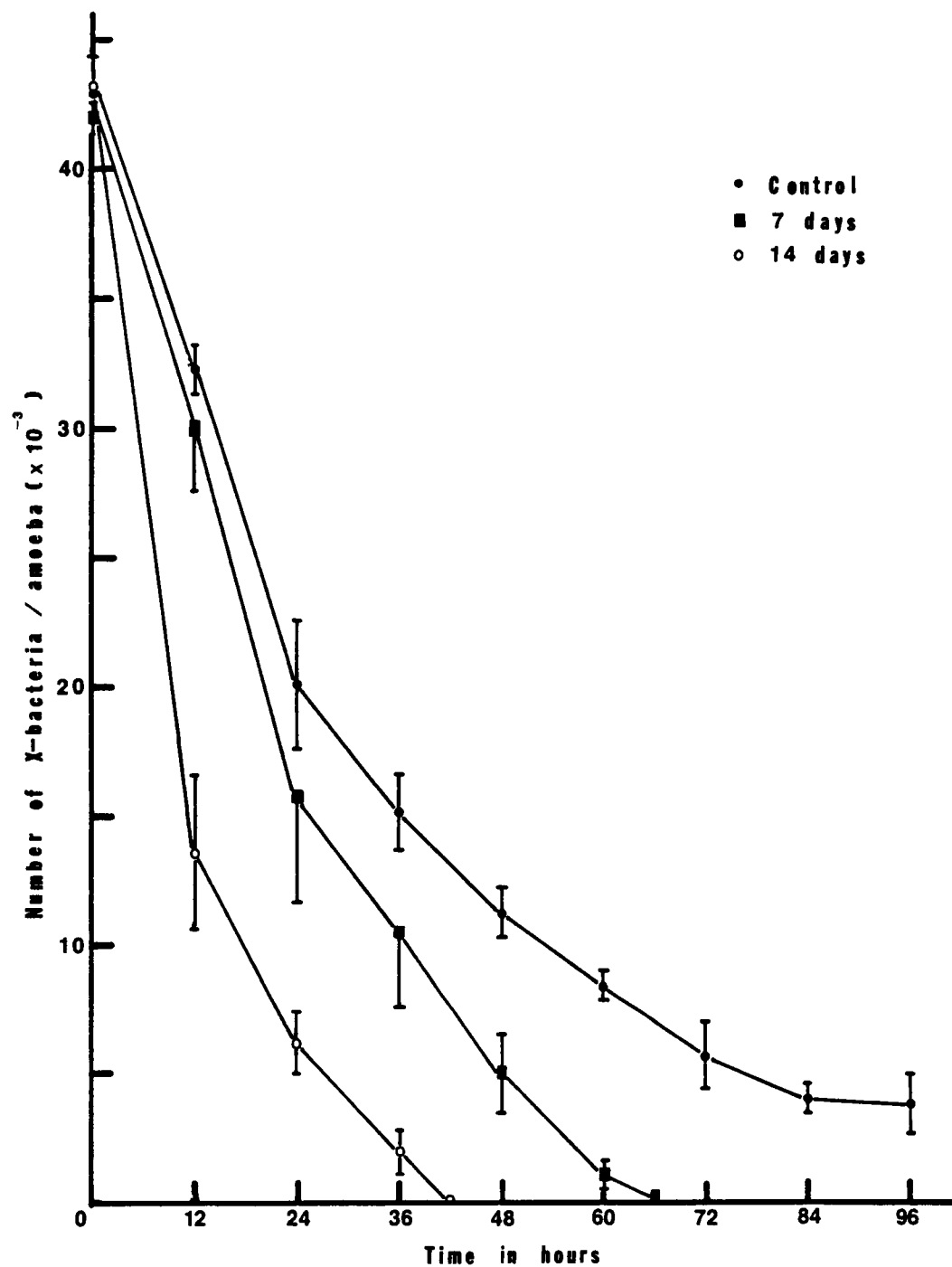


Figure 9. The average number of plasmid-cured X-bacteria per amoeba after induced infection. Plasmid had been cured in vivo for 7 or 14 days with 10^{-6} M acridine orange.

infection and failed to establish symbiotic bacterial population (Fig 8).

In vitro. X-Bacteria isolated from amoebae cultured at normal condition were incubated in ethidium bromide (10^{-5} M) or acridine orange (10^{-6} M) for 3, 9, and 12 hours at 4 C with shaking. When X-bacteria had been incubated for 3 hours, they did not disappear from the cytoplasm of infected D amoebae after 96 hours. On the other hand, it had been treated for 12 hours, no X-bacteria were detected after 84 hours (Figs. 10, 11 and 12). X-Bacteria incubated in acridine orange disappeared earlier than those incubated in ethidium bromide. When the concentration of curing agents was higher than that routinely used in this study, amoebae lysed immediately after induced infection. The reasons for this lysis are not clear, but it seemed to be due to differential cytotoxicity of curing agents, because the centrifuged X-bacterial pellet after incubation became red or orange colored, possibly due to the incorporation of curing agents into bacteria. As a result, highly concentrated curing agents in bacteria could disrupt the intracellular environment of amoebae.

E. Effect of Plasmid-Curing Agents on Plasmid DNAs of Symbionts

To verify the presence or absence of plasmid DNAs in the symbiotic bacteria after treatment with plasmid-curing agents, isolated DNA from plasmid-cured X-bacteria was subjected to 0.5 % agarose-gel electrophoresis with plasmid DNA markers of known molecular weights

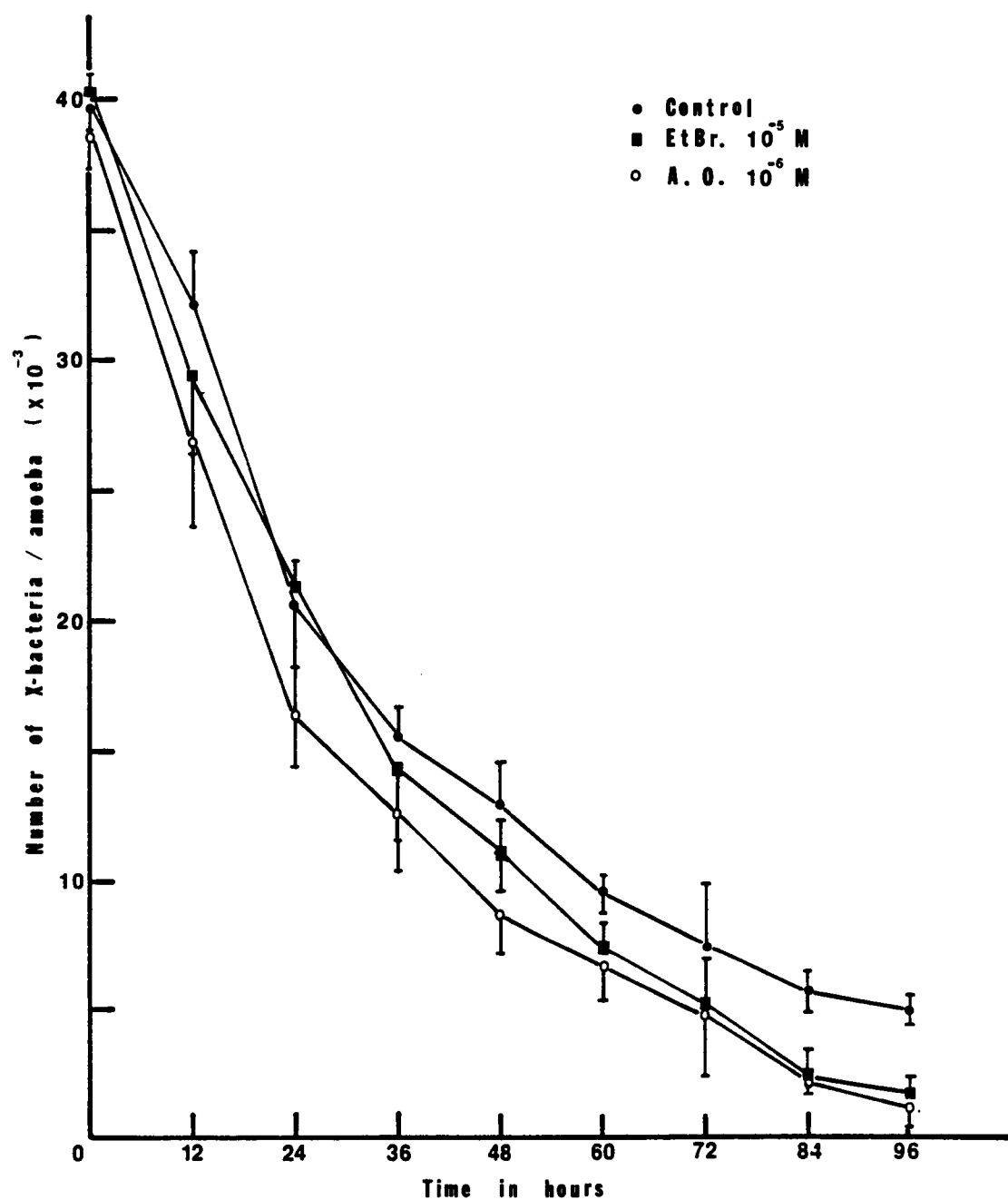


Figure 10. The average number of plasmid-cured X-bacteria per amoeba after induced infection. X-bacteria plasmid had been cured in vitro for 3 hours with 10^{-5} M ethidium bromide (EtBr) or 10^{-6} M acridine orange (A.O.).

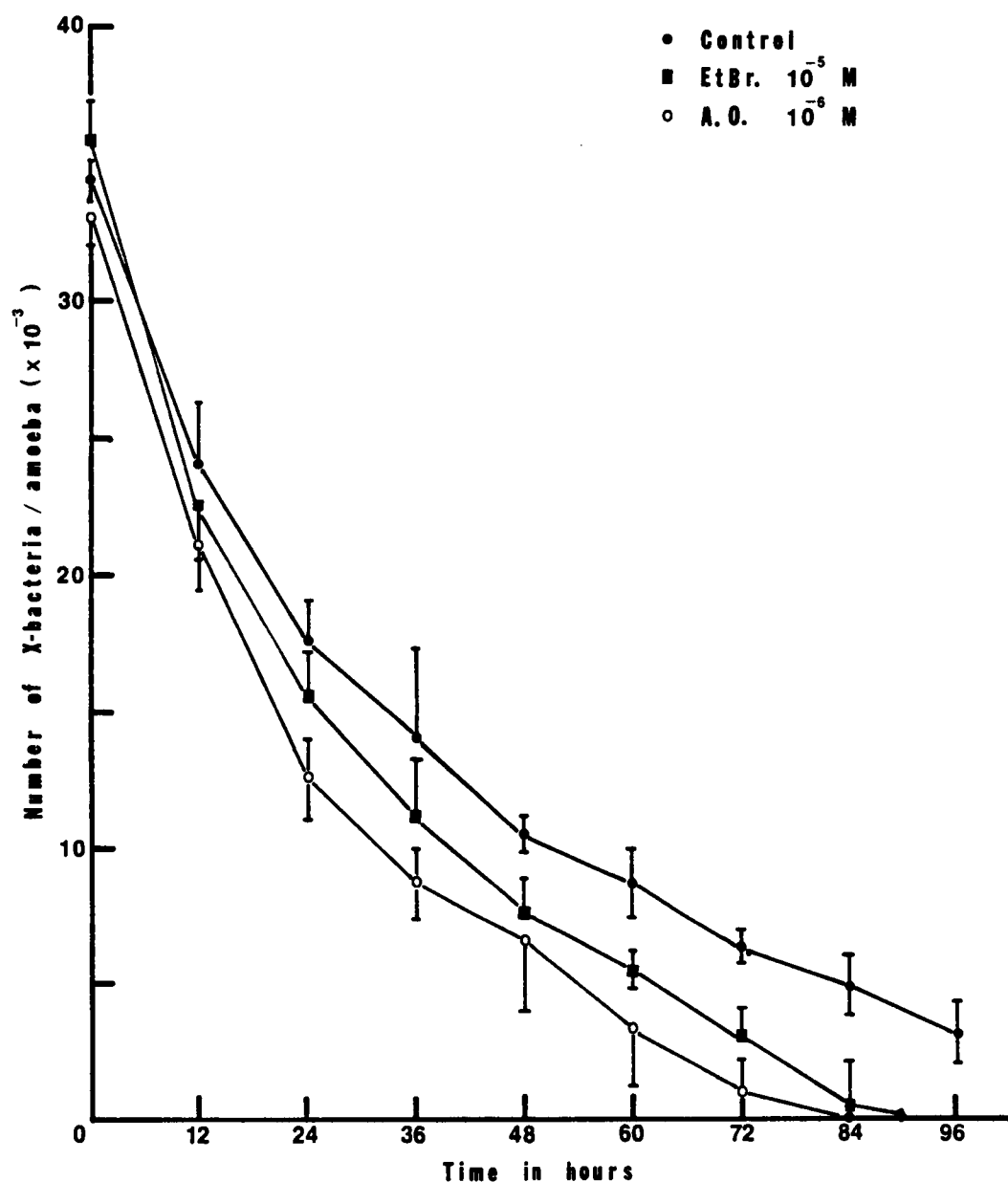


Figure 11. The average number of plasmid-cured X-bacteria per amoeba after induced infection. X-bacteria plasmid had been cured in vitro for 9 hours with 10^{-5} M ethidium bromide (EtBr) or 10^{-6} M acridine orange (A.O.).

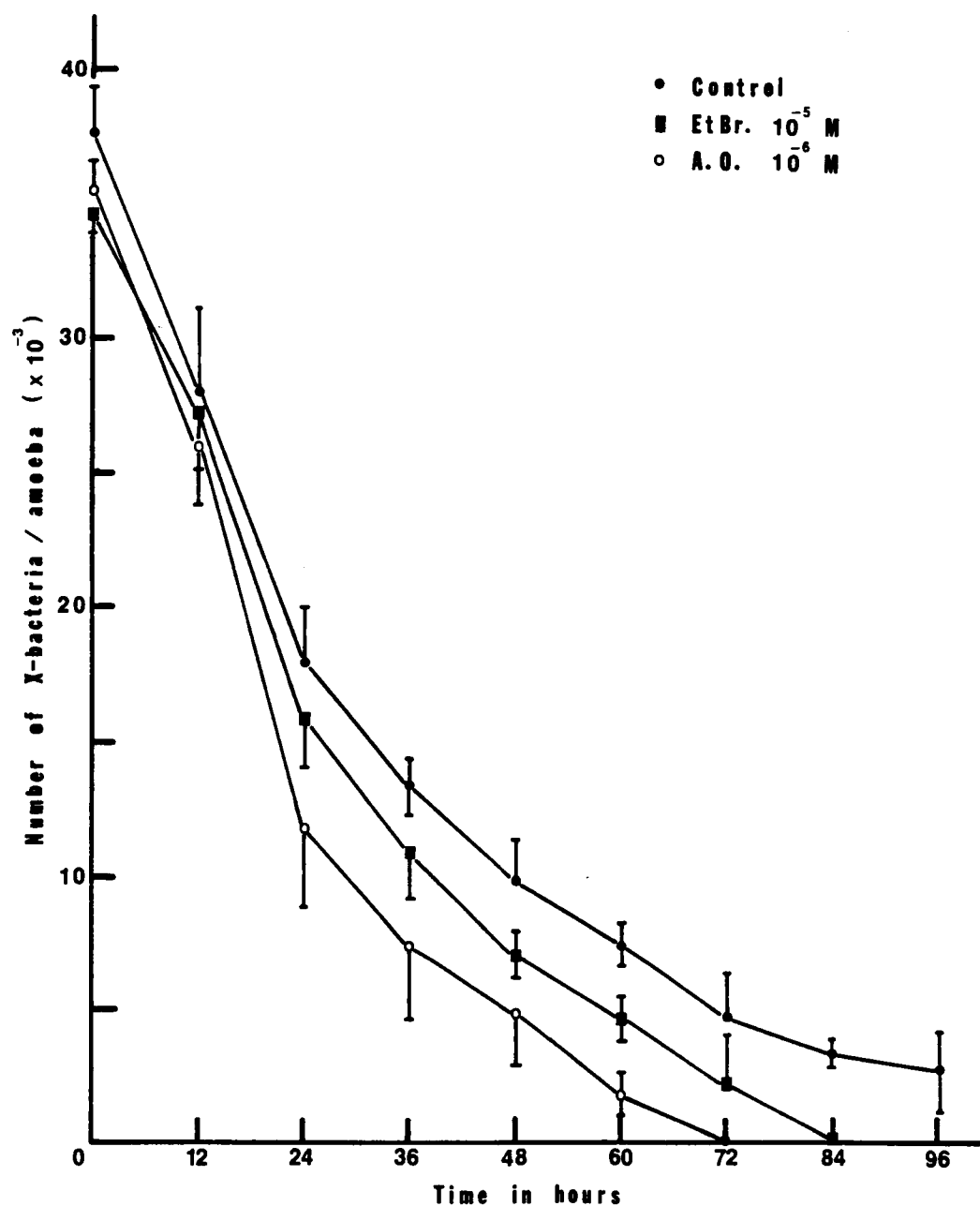
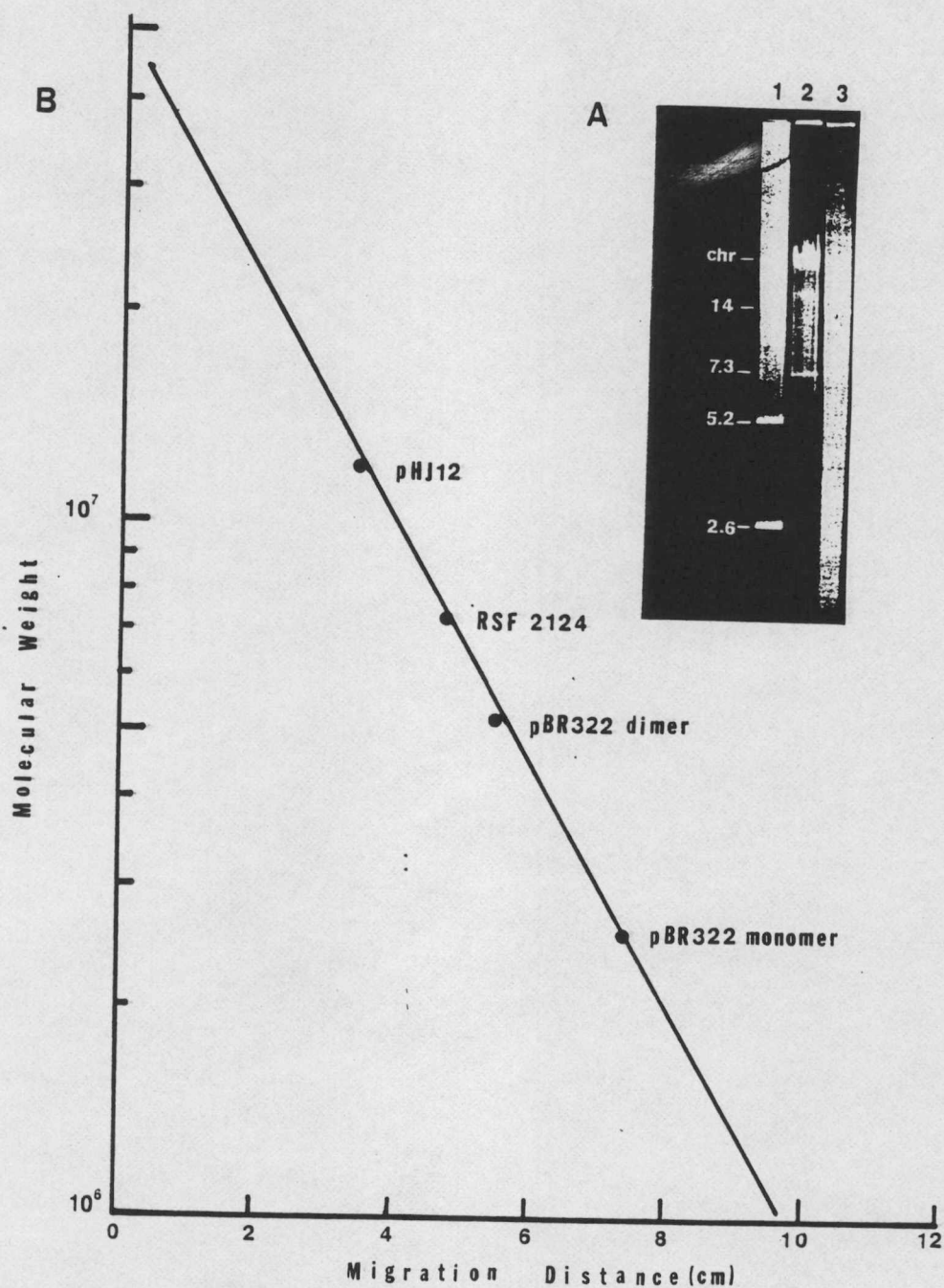


Figure 12. The average number of plasmid-cured X-bacteria per amoeba after induced infection. X-bacteria plasmid had been cured in vitro for 12 hours with 10^{-5} M ethidium bromide (EtBr) or 10^{-6} M acridine orange.

from E. coli. The electrophoretic pattern of these plasmid DNAs on agarose gel is shown in Fig. 13-A. 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 (Fig. 13-B).

Fig. 14 shows the electrophoretic patterns of plasmid DNAs isolated from X-bacteria of symbiont-bearing amoebae cultured under different conditions. Each lane represents the plasmid DNAs obtained from 1×10^{10} X-bacteria. It appeared that the amount of the plasmid DNAs of X-bacteria from amoebae grown in the medium with plasmid-curing agents decreased as the lengths of culture periods increased. No plasmid DNA bands were detected from X-bacteria isolated from xD amoebae grown in plasmid-curing agents longer than 7 days.



- A. Electrophoretic pattern of plasmid DNAs.
 Lane 1: Top band, pBR322 dimer (M.W. 5.2×10^6)
 Lower band, pBR322 monomer (M.W. 2.6×10^6)
 Lane 2: Top band, chromosomal DNA
 Lower band, RSF2124 (M.W. 7.3×10^6)
 Lane 3: pHJ12 (M.W. 14×10^6)
- B. Molecular weight calibration curve. The molecular weights of plasmid DNAs were plotted against migration distance.

Figure 13. Electrophoretic pattern of plasmid DNAs and molecular weight calibration curve.

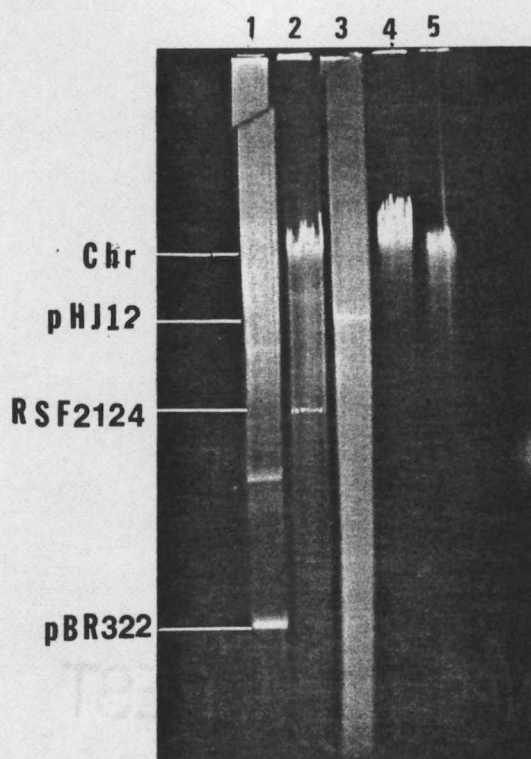


Figure 14. Electrophoretic pattern of plasmid DNAs isolated from *E. coli* and X-bacteria of xD amoebae grown under different conditions.

- Lane 1: pBR322 as a molecular weight standard.
- Lane 2: RSF2124 as a molecular weight standard.
- Lane 3: Plasmid DNA of X-bacteria isolated from the amoebae grown in normal Chalkley's solution.
- Lane 4: Plasmid DNA of X-bacteria treated with 10^{-5} M ethidium bromide for 7 days in vivo.
- Lane 5: Plasmid DNA of X-bacteria treated with 10^{-6} M acridine orange for 7 days in vivo.

CHAPTER IV

DISCUSSION

The present study was designed to determine the possible roles of plasmid DNAs in the symbiotic X-bacteria within amoebae. The results showed that treatment of symbiotic bacteria with either acridine orange or ethidium bromide leads to the loss of their plasmids, which are thought to be responsible for encoding protein component(s) that gives the symbiotic bacterial cell wall resistance to the lysosomal enzymes of the host and/or prevents symbiotic vesicles from fusing with lysosomes of hosts. The loss of plasmid DNAs correlated with the loss of infectivity of the symbiotic bacteria to symbiont-free amoebae.

When the symbiotic amoebae were grown in mass culture in a medium containing either acridine orange (10^{-6} M) or ethidium bromide (10^{-5} M), half of the original symbiont population was lost within a few days. After that, the average number of symbiotic bacteria per amoeba stayed at 40% of the original population during the later culture period. The reasons of the sharp decline of symbiotic bacterial population during early period of culture in plasmid curing agents are not clear. However, one possible explanation can be the degeneration of X-bacteria inside symbiotic vacuoles which was caused by digestion after fusion with lysosomes. Acridine orange and ethidium bromide are known intercalative dyes which are suggested to disrupt the replication machinery of plasmid DNAs by binding to the double helix structure of the covalently closed circular DNA (Glass, 1982). The plasmids in

X-bacteria were destroyed or removed during the culture period in the presence of these dyes. As a result, the symbiotic bacteria might have become unable to synthesize the protein component that was speculated to prevent the fusion between symbiotic vacuoles and lysosomes, which in turn caused the digestion of symbiotic bacteria. The above view is supported by an experiment in which plasmids of the symbiotic bacteria were removed by culturing symbiotic amoebae at 26 °C (Park, 1983). In this case, the loss of plasmid from symbiotic bacteria was explained in terms of the suppressed replication of plasmids at higher temperature. After a prolonged culture of symbiotic amoebae at 26 °C, the size of symbiotic vacuoles and the average number of bacteria per symbiotic vacuole decreased, accompanied by the decrease in the average number of X-bacteria per amoeba. In addition, some of the X-bacteria degenerated inside symbiotic vacuoles because of fusion with lysosomes.

How then are the 40 % of symbiotic bacteria still maintained in the host cytoplasm during the later period of culture of symbiotic amoebae in a medium with plasmid-curing agents? One possible explanation is that the concentrations of plasmid-curing agents routinely used in the experiments were not high enough to remove symbiotic bacteria completely from the host cytoplasm. As a result, a more or less balanced state between digestion and multiplication could be maintained in the population of symbiotic bacteria.

As the results of induced infection show, infected D amoebae lost 50 % of the maximally ingested X-bacteria within 24 hours and 90 % of them by day 5. There might be two possible explanations for this sharp

decrease in the number of ingested X-bacteria, digestion by amoebae and/or ejection of X-bacteria via exocytosis. Symbiotic bacteria are known to be resistant to the lysosomal enzymes of the host, possibly by a unique bacterial cell-wall protein encoded by plasmid DNAs (Han and Jeon, 1980). However, the plasmid DNA may be spontaneously cured or the cell walls damaged chemically and/or physically. This could lead to digestion of X-bacteria during the initial stages of infection, when they are in the phagocytic vacuoles together with other ingested food organisms. Other indirect evidence to support the above view is the connection between the copy number of plasmid per symbiotic bacterium, which appears to be less than 1 (Han, 1978), and the infectivity of isolated X-bacteria. Not all of the isolated X-bacteria were capable of infecting amoebae, instead, only a small fraction of bacteria was able to infect amoebae while a considerable number of bacteria seemed to be destroyed by amoebae. The fraction of bacteria which was digested by amoebae could be those that might have lost their plasmids by spontaneous curing or segregants of the X-bacteria which had lost plasmid DNAs. A long time after the establishment of intracellular symbiosis, segregation of X-bacteria into symbiotic vacuoles could provide X-bacteria with a chance for spontaneous loss of plasmid DNAs. Among such mixed populations a fraction of X-bacteria which still had plasmid DNA could infect other amoebae and establish new symbiosis. This idea is further supported by Clowe's suggestion that plasmids larger than 35×10^6 daltons are generally under stringent control and have less than one copy per cell (Clowes, 1972). Considering the small

copy number of the plasmids, it could be believed that the X-bacteria digested during the initial stages of infection might be those that did not have plasmids.

The ejection of X-bacteria by means of exocytosis could be another possible explanation for the decreasing number of X-bacteria during the initial stages of infection. After two hours of incubation in X-bacteria suspension, amoebae were washed thoroughly with fresh medium to remove bacteria. Dishes were also changed in order to remove the remainder of X-bacteria as much as possible. After a few hours the bottom of the dish was covered with a thin film of small particles. These particles appeared to be composed of a large number of aggregated X-bacteria under the phase-contrast microscope. It is not clear whether digestion or exocytosis plays a major role in reducing the number of ingested X-bacteria. However, it seems that exocytosis is important during the initial 24 hours after infection, whereas, digestion is more important during the later period of infection. This idea is supported by the result of infectivity test with E. coli to D amoebae. During the first 24 hours there was little difference in the decreasing trend of the average number of ingested E. coli as compared with that of X-bacteria. However, after 24 hours, the ingested E. coli disappeared from the host cytoplasm more rapidly than did X-bacteria. E. coli were completely removed from the amoeba cytoplasm after 72 hours, a time when about 6,500 X-bacteria per amoeba still remained.

The results of induced infection in the control group showed that the initially ingested number of X-bacteria per amoeba was much smaller

than that found in the early stage of the spontaneous infection (60,000 - 150,000) (Jeon and Lorch, 1967). It also appeared that the number of ingested bacteria reaches the maximum after about 2 hours of incubation of nonsymbiotic amoebae in a bacteria suspension, and it declines after that time. The presence of a limit of ingestion might be explained in terms of the average capacity of food intake for each individual amoeba, i.e., 12 latex beads per amoeba (Ahn and Jeon, 1982). It is also interesting to note that amoebae showed unhealthy symptoms of overfeeding when they ingested over 35,000 bacteria per cell, that is, the nonsymbiotic amoebae that had ingested over 35,000 X-bacteria rounded up and did not produce pseudopods for a few days. Then they became slender and thin in shape, detached from the bottom of the dishes, and died. Over 60 % of nonsymbiotic amoebae died if they ingested more than 35,000 X-bacteria; when nonsymbiotic amoebae ingested fewer than 20,000 X-bacteria, they were healthier than those described above and were converted to symbiotic amoebae. A stable symbiotic relationship was established between surviving amoebae and newly infecting bacteria within a short period of time. These results agree with those of an earlier study (Ahn and Jeon, 1979). Even though the reasons are not known, it appears that the growth rate of X-bacteria seemed to be controlled by their relative number per amoeba.

A host's control over a symbiotic population is expected to be operative in any symbiotic association and at least four mechanisms have been proposed by others to account for the observed control; expulsion of symbionts (Steele, 1976), digestion (Weis, 1976), regulation of

symbiont growth (McAuley, 1981) and synchronization of cell division (Muscatine and Pool, 1979). Amoebae that had ingested over 35,000 bacteria lost about 90 % of their ingested bacteria by the 5th day of infection. After day 5, they became healthy and the number of X-bacteria reached the maximum carrying capacity (42,000 per amoeba) on day 14.

Kim (1983) showed similar infection kinetics. Amoebae that had ingested an average of over 30,000 X-bacteria lost 95 % of the ingested bacteria by the 4th day of infection. After day 4, amoebae became healthy, and the number of X-bacteria per amoeba reached the maximum number on day 16. X-Bacteria in a newly infected amoeba divided 9.1 times from day 4 to day 16 after infection. Ahn and Jeon (1979) also reported that each newly infected D amoeba divided 5.88 times and X-bacteria divided 12.3 times during the first 15 days of infection. Once the maximum carrying number (about 42,000 per amoeba) was reached, the growth rate of X-bacteria seemed to slow down to maintain an equilibrium.

When D amoebae were infected with plasmid-cured X-bacteria, they disappeared completely from the amoeba cytoplasm within 96 hours. The longer the period of treatment with plasmid-curing agents, the shorter the time that needed for complete disappearance of ingested bacteria. In particular, X-bacteria treated with plasmid-curing agents for 14 days in vivo disappeared from the infected amoeba cytoplasm within 36 hours. It appeared that X-bacteria treated with plasmid-curing agents in vivo were less resistant to digestion than those treated in vitro. Why is

the plasmid-curing in vivo more effective than that in vitro? Presumably, the replicative machinery of X-bacterial plasmids was perturbed more effectively in vivo than in vitro. As a result, the autonomously replicating plasmid DNAs whose doubling rates are equal to the cell-doubling rate under normal condition would be removed completely in the presence of plasmid-curing agents for longer periods in vivo. Even though the precise mechanisms of plasmid-curing agents are not understood at this time, it has been suggested that such influences on plasmid replication is by preferentially inhibiting plasmids, relative to initiation of chromosomal replication (Clowes, 1972). Therefore, under the longer period of culture of symbiotic amoebae in the plasmid-curing agents, the plasmids would be diluted out throughout the symbiotic bacterial population. Similar cases of the loss of bacterial plasmid DNAs caused by the various curing agents are known, e.g., ethidium bromide, acridine dyes, antibiotics, and SDS, (Clowes, 1972; Bouanchaud et al., 1968).

How does the loss of plasmid cause the loss of infectivity of X-bacteria? The answer to this question could be a clue to understanding the possible role of plasmid DNAs in establishment of symbiosis between amoebae and bacteria. A direct characterization of the correct plasmid-determined function of X-bacteria in symbiosis is extremely difficult at this point, but at least one function of the plasmids may be explained from the results of this study; the protection of symbiotic bacteria from digestion by hosts. Due to this function, X-bacteria may succeed in the establishment of symbiosis by

protecting themselves from digestion during the 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 vacuole membranes during the sustained multiplication of both hosts and symbionts (Jeon, 1983). These functions may result from the presence of unknown, plasmid-encoded polypeptides which may render the X-bacterial cell wall resistant to host lysosomal enzymes or prevent symbiotic vacuoles from fusion with host lysosomes, so that X-bacteria can establish and maintain their population in amoebae.

In a few cases, the metabolism of lipopolysaccharides and other components of bacterial cell wall has been shown to be controlled by plasmids (Hesslewood and Smith, 1974; Derylo et al, 1975). Recently Kopecko et al. (1980, 1981, and 1982) reported that a plasmid controls synthesis of the Shigella sonnei form 10-side chains. Susana et al. (1983) also reported that plasmid loss in cured E. coli strain RC424 cells resulted in the disappearance of the outer membrane components and in a concomittent change in the thickness of the peptidoglycan layer. 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 in legumes (Bishop et al., 1977).

From the results presented in this study, it could be confirmed that plasmid DNAs plays an important role in the establishment and/or maintenance of symbiosis between amoebae and bacteria.

A more direct way to prove the involvement of plasmid DNA in

intracellular symbiosis in this system may be to incorporate the X-bacteria plasmid DNA into other bacteria which are normally digested by amoeba lysosomal enzymes and see if they are able to infect and establish a new symbiosis with amoebae. However, due to the lack of known plasmid-determined phenotypic characteristics, it is almost impossible to screen the transformed bacteria which have X-bacteria plasmid in them. Another limit is the rather large size of the X-bacteria plasmid which reduces the chance of successful transformation (transformation efficiency). To overcome these problems, it is necessary to clone the restriction-enzyme digested X-bacteria plasmid DNA fragments into a potential cloning vector with known selective markers, such as antibiotics resistance. Then, the transformed bacteria can be screened by proper biological analyses. The screened transformants could be used for a resistance test by introducing them into D strain of amoeba by experimental infection. There are several lines of evidence for the obligatory symbiosis between X-bacteria and amoebae (Jeon and Jeon, 1976; Jeon and Hah, 1977). However, it has not been determined whether the dependence of symbiotic amoebae is genetically controlled by their symbionts. Therefore, another interesting subject to study using this system is the possible genetic interactions between the genome of the hosts and the symbionts. Biochemical and genetic studies of metabolism of the different cell envelope components and protein compositions are also required to provide an adequate explanation of the intrinsic nature of the cell wall resistance of the X-bacteria.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Ahmed, M. and Focht, D.D. 1973. Degradation of polychlorinated biphenyls by two species of Achromobacter. Can. J. Microbiol. 19:47-52.

2. Ahn, T.I. and Jeon, K.W. 1982. Structural and biochemical characteristics of the plasmalemma and vacuole membranes in amoebae. Exp. Cell Res. 139: 253-268.

3. Allen, J.M. 1969. Lysosomes in bacterial infection. In: Lysosomes in biology and pathology. Dingle, J.T., Fell, H.B. (eds.). Vol. 2, PP. 41-68. North Holland Pub. Co. London and Amsterdam.

4. Armstrong, J.A. and Hart, P.D. 1971. Response of cultured macrophages to Mycobacterium tuberculosis, with observation on fusion of lysosomes with phagosomes. J. Exp. Med. 134: 713-740.

5. Bannister, L.H. 1979. The interactions of intracellular protista and their host cells, with special reference to heterotrophic organisms. Proc. R. Soc. Lond. B 204: 141-163.

6. Beringer, J.E., Brewin, N., Johnston, A.W.B., Schulman, H.M. and Hopwood, D.A. 1979. The Rhizobium-legume symbiosis. Proc. R. Soc. Lond. B 204: 219-233.

7. Birnboim, H.C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.

8. Bishop, P.E., Dazzo, F.B. and Appelbaum, E.R. 1977. Intergeneric transfer of gene involved in the Rhizobium-legume symbiosis. Science 198: 938-940.

9. Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.L., Heyneker, H.L. and Boyer, H.W. 1977. Construction and characterization of new cloning vehicles. II. Multipurpose Cloning System. Gene 2: 95-113.

10. Bouanchaud, D.H., Scavizzi, M.R. and Chabbert, Y.A. 1968. Elimination by ethidium bromide of antibiotic resistance in Enterobacteriaceae and Staphylococci. J. Gen. Microbiol. 54: 417-425.
11. Broda, P. 1979. Plasmids. Freeman, San Francisco.
12. Casley-Smith, J.R. and Savanat, T. 1966. The formation of membranes around microorganisms and particles injected into amoebae: Support for the "reticulosome" concept. Aust. J. Exp. Biol. Med. Sci. 44: 111-112.
13. Chapman-Andresen, C. 1971. Biology of the large amoebae. Ann. Rev. Microbiol. 25: 27-48.
14. Chapman-Andresen, C. 1973. Endocytic processes, In: The biology of amoeba. Jeon, K.W. (ed.). pp: 319-418. Academic Press, New York.
15. Clark, A.J. and Warren, G.J. 1979. Conjugal transmission of plasmids. Ann. Rev. Genet. 13: 99-125.
16. Clowes, R.C. 1972. Molecular structure of bacterial plasmids. Bacteriol. Rev. 36: 361-405.
17. Cook, C.B., Pappas, P.W. and Rudolph, E.D. 1980. Cellular interaction in symbiosis and parasitism. In: Cook, C.B., Pappas, P.W., and Rudolph, E.D. (eds.). PP: 47-73. Ohio State Univ. Press, Columbus.
18. Costerton, J.W. 1977. Cell envelope as a barrier to antibiotics. In: Schlessinger, D. (ed.) Microbiology-1977. pp: 151-157. American Society for Microbiology, Washington, D.C.
19. Dayhoff, M.O., Schwartz, R.M. 1980. In: Endocytobiology: Proceedings of the international colloquium on endosymbiosis and cell research. Schwemmler, W. and Scherk, H.E.A. (eds.). pp: 63-83. Walter de Gruyter, Berlin.
20. Davies, J.K. and Reeves, P. 1975a. Genetics of resistance to colicins in Escherichia coli K 12: Cross-resistance among

colicins of group B. J. Bacteriol. 123: 96-101.

21. Davies, J.K. and Reeves, P. 1975b. Genetics of resistance to colicins in Escherichia coli K 12: Cross-resistance among colicins of group A. J. Bacteriol. 123: 102-117.
22. Davies, J. and Smith, D.I. 1978. Plasmid determined resistance to antimicrobial agents. Ann. Rev. Microbiol. 32: 469-518.
23. Derylo, M., Glowacka, M., Lorkiewicz, Z. and Russa, R. 1975. Plasmid-determined alterations of Salmonella typymurium lipopolysaccharides. Mol. gen. Genet. 140: 175-181.
24. Dilts, J.A. 1977. Chromosomal and extrachromosomal deoxyribonucleic acids from bacterial endosymbionts derived from stock 51 of Paramecium tetraurelia. J. Bacteriol. 129: 858-894.
25. Fangman, W.L. 1978. Separation of very large DNA molecules by gel electrophoresis. Nucleic Acids Res. 5: 653-665.
26. Friz, R.R. 1972. Interaction of L cells and Chlamydia psittaci: Entry of the parasite and host responses to its development. J. Bacteriol. 110: 706-721.
27. Glass, R.E. 1982. Gene function: E. Coli and heritable elements. Croom Helm, London.
28. Goldstein, L. and Ko, C. 1976. A method for mass culture of large free-living amoebae. Methods Cell Biol. 13: 239-246.
29. Guerinau, M. 1979. Plasmid DNA in yeast. In: Lemke, P.A. (ed.), Virus and plasmid in fungi. Marcel Dekker Inc., New York.
30. Guerry, O., Leblanc, D.J. and Falkow, S. 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. 116: 1064-1066.
31. Han, J.H. and Jeon, K.W. 1980. Isolation and partial

- charaterization of two plasmid deoxyribonucleic acids from endosymbiotic bacteria of Amoeba proteus. J. Bacteriol. 141: 1466-1469.
32. Hardy, K.G. 1975. Colicinogeny and related phenomena. Bacteriol. Rev. 39: 464-515.
 33. Hayes, W. 1968. The genetics of bacteria and their viruses. 2nd ed. Wiley, New York.
 34. Heckmann, K. 1975. Omikron, ein essentieller endosymbiont von Euplotes aediculatus. J. Protozool. 22: 97-104.
 35. Hesslewood, S.R. and Smith, J.T. 1974. Envelope alterations produced by R-factors in Proteus mirabilis. J. Gen. Microbiol. 85: 146-152.
 36. Hirota, Y. 1960. The effect of acridine dyes on mating type factors in Escherichia coli. Proc. Natl. Acad. Sci. U.S. 46: 57-64.
 37. Hopwood, D.A. 1978. Extrachromosomally determined antibiotic production. Ann. Rev. Microbiol. 32: 373-392.
 38. Horowitz, M.A. 1983. Symbiotic interaction between Legionella pneumophilla and human leukocytes. In: Jeon, K.W. (eds.), Intracellular symbiosis. Inter. Rev. Cytol. Suppl. 14: 307-327.
 39. Jeon, K.W. 1972. Development of cellular dependence on infective organisms: Micurgical studies in amoebae. Science 176: 1122-1123.
 40. Jeon, K.W. 1973. Intracellular symbionts as required cytoplasmic components in amoebae. Pro. IVth Inter. Congr. Protozool: 201.
 41. Jeon, K.W. 1983. Bacterial endosymbionts in amoebae. In: Jeon, K.W. (ed.). Intracellular symbiosis. Int. Rev. Cytol. Suppl. 14: 29-47.

42. Jeon, K.W. and Ahn, T.I. 1978. Temperature sensitivity: A cell character determined by obligate endosymbionts in amoebae. *Science* 202: 635-637.
43. Jeon, K.W. and Hah, J.C. 1977. Effect of chloramphenicol on bacterial endosymbionts in a strain of Amoeba proteus. *J. Protozool.* 24: 289-293.
44. Jeon, K.W. and Jeon, M.S. 1976. Endosymbiosis in amoebae: Recently established endosymbionts have become required cytoplasmic components. *J. Cell Physiol.* 89: 337-344.
45. Jeon, K.W. and Lorch, I.J. 1967. Unusual intracellular bacteria infection in large, free-living amoebae. *Exp. Cell Res.* 48: 236-240.
46. Johnston, R.B. and Lehmyer, J.E. 1977. The involvement of oxygen metabolites from phagocytic cells in bactericidal activity and inflammation. In: Michelson, A.M., McCord, J.M. and Fridsvich, I. (eds.). *Superoxide and superoxide mutase*.
47. Jones, J.C. and Hirsch, J.G. 1972. The interaction between Toxoplasma gondii and mammalian cells. II: The absence of lysosomal fusion with phagocytic vacuoles containing living parasites. *J. Exp. Med.* 136: 1173-1174.
48. Karakashian, M. and Karakashian, S.J. 1973. Intracellular digestion and symbiosis in Paramecium bursaria. *Exp. Cell Res.* 81: 111-119.
49. Karakashian, S.J. and Rudzinska, M.A. 1981. Inhibition of lysosomal fusion with symbiont-containing vacuoles in Paramecium bursaria. *Exp. Cell Res.* 131: 387-393.
50. Kim, H.B. 1983. Strain specific protein in symbiotic and non-symbiotic amoeba. Master's Thesis. University of Tennessee, Knoxville.
51. Klebanoff, S.J. 1975. Antimicrobial mechanism in neutrophilic polymorphonuclear leukocytes. *Sem. Hematol.* 12: 117-142.

52. Kopecko, D., Washington, B. and Formal, S. 1980. Genetic and Physical evidence for plasmid control of Schigella sonnei form I cell surface antigen. *Infect. Immun.* 29: 207-214.
53. Lau, R.H., Sapienza, C. and Doolittle, W.F. 1980. Plasmids in Cyanobacteria. In: *Plasmid and transposons*. Stuttard, C. and Rozee, K.R. (eds.), Academic Press, New York.
54. Margulis, L. 1970. *Origin of eukaryotic cells*. New Havens and London, Yale University Press.
55. Margulis, L. 1976. Genetic and evolutionary consequences of symbiosis. *Exp. Parasitol.* 39: 277-349.
56. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganism. *J. Mol. Biol.* 3: 208-278.
57. McAuley, P.J. 1981. Control of cell division of the intracellular chlorella symbionts in green hydra. *J. Cell Sci.* 47: 197-206.
58. McDonnel, M.W., Simon, M.N. and Studier, F.W. 1976. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *J. Mol. Biol.* 110: 119-146.
59. Meyers, J.A., Sauchez, D., Elwell, L.P. and Falkow, S. 1976. Simple agarose gel electrophoresis method for the identification and characterization of plasmid deoxyribonucleic acid. *J. Bacteriol.* 127: 1529-1537.
60. Morita, R.Y. 1975. Psychrophilic Bacteria. *Bacterol. Rev.* 39: 144-167.
61. Moulder, J.W. 1974. Intracellular parasitism: Life in extreme environment. *J. Infect. Dis.* 130: 300-306.
62. Muscatine, L. and Pool, R.R. 1979. Regulation of numbers of intracellular algae. *Proc. R. Soc. Lond. B* 204: 131-139.

63. Park, Y.M. 1983. Role of Plasmid DNA in the protection of endosymbiotic X-bacteria from digestion by amoebae. Master's Thesis. University of Tennessee, Knoxville.
64. Preer, J.R., Preer, L.B. and Jurand, A. 1974. Kappa and other endosymbionts in Paramecium aurelia. Bacteriol. Rev. 38: 113-163.
65. Quie, P. 1975. Pathology of bactericidal power of neutrophils. Sem. Hematol. 12: 143-160.
66. Reaney, O. 1976. Extrachromosomal elements as possible agents of adaptation and development. Bacteriol. Rev. 40: 552-590.
67. Richmond, M.H. and Smith, D.C. 1979. The cell as a habitat. Proc. R. Soc. Lond. B 204: 235-250.
68. Roth, L.E. and Daniels, E.W. 1961. Infective organisms in the cytoplasm of Amoeba proteus. J. Biophys. Biochem. 9: 317-323.
69. Sansonetti, P., Kopecko, D. and Formal, S. 1981. Shigella sonnei plasmid: Evidence that a large plasmid is necessary for virulence. Infec. Immun. 34: 75-83.
70. Sansonetti, P., Kopecko, D. and Formal, S. 1982. Involvement of plasmid in the invasive ability of Shigella flexneri. Infec. Immun. 35: 852-860.
71. Savanat, T. and Pavillard, E.R.J. 1964. The ability of Amoeba proteus to kill Salmonella enteritidis introduced by microinjection and the influence of opsonins on intracellular killing. Aus. J. Exp. Biol. Med. Sci. 42: 615-624.
72. Sbarra, A., Jacobs, A., Strauss, R.R., Paul, B.B. and Mitchell, G.W. 1971. The biochemical and antimicrobial activities of phagocytosing cells. Am. J. Clin. Nutr. 24: 272-281.
73. Schell, J., Montagu, M.V., Beuckeler, M.D., Block, M.D., Depicker, A., Wilde, M.D., Engler, G., Grnetello, C., Hernalsteens, J.P., Holsters, M., Seurinck, J., Silva, B., Vliet, F.V. and

- Villarroel, R. 1979. Interactions and DNA transfer between Agarobacterium tumefaciens, the Ti - plasmid and the plant host. Proc. R. Soc. Lond. B 204: 251-266.
74. Schwartz, R.M. and Dayhoff, M.O. 1978. Origins of prokaryotes, eukaryotes, mitochondria, and chloroplasts. Science 199: 395-405.
 75. Schwemmler, W. 1983. Analysis of possible gene transfer between an insect host and its bacteria-like endosymbionts. In: Jeon, K.W. (ed.), Intracellular symbiosis. Inter. Rev. Cytol. Suppl. 14: 247-266.
 76. Sharp, P.A., Sugden, B. and Sambrook., J. 1973. Detection of two restriction endonuclease activities in Haemophilus parainfluenzae using analytical agarose. Biochemistry 12: 3055.
 77. Smith, O.C. 1979. From the extracellular to intracellular: The establishment of a symbiosis. Proc. R. Soc. Lond. B 204: 111-130.
 78. So, M., Boyer, H.W., Betlach, M. and Falkow, S. 1976. Molecular cloning of an Escherichia coli plasmid determinant that encodes for the production of heat-stable enterotoxin. J. Bacteriol. 128: 463-471.
 79. Soldo, A.T. and Godoy, G.A. 1973. Observations on the production of folic acid by symbiont lambda particles of Paramecium aurelia stock 299. J. Protozool. 20: 502.
 80. Soldo, A.T. 1983. The biology of the xenosome, an intracellular symbionts. In: Jeon, K.W. (ed.), Intracellular symbiosis. Inter. Rev. Cytol. Suppl. 14: 79-109.
 81. Stanfield, S. and Helsinki, D.R. 1976. Small circular DNA in Drosophilla melanogaster. Cell 9: 333-345.
 82. Steele, R.D. 1976. Light intensity as a factor in the regulation of the density of symbiotic zooxanthella in Aiptasia tagetes (Coelentrata, Anthozoo). J. Zool. Lond. 179: 138-405.

83. Susana, B.R., Aldo, C., Jose, L.L.T., Nora, E.G. and Cesar, V. 1983. Involvement of a plasmid in Escherichia coli envelope alterations. J. Bacteriol. 155: 402-440.
84. Taylor, F.J.R. 1974. Implication of the serial endosymbiosis theory of the origin of eukaryotes. Taxon 23: 229-258.
85. Taylor, F.J.R. 1976. Autogenous theories for the origin of eukaryotes. Taxon 25: 377-390.
86. Taylor, F.J.R. 1979. Symbioticism revisited: A discussion of the evolutionary impact of intracellular symbiosis. Proc. R. Soc. Lond. B 204: 267-286.
87. Taylor, F.J.R. 1983. Some eco-evolutionary aspects of intracellular symbiosis. In: Jeon, K.W. (ed.), Intracellular symbiosis. Inter. Rev. Cytol. Suppl. 14: 1-28.
88. Verma, D.P.S. and Long, S. 1983. The molecular biology of Rhizobium-legume symbiosis. In: Jeon, K.W. (ed.), Intracellular symbiosis. Inter. Rev. Cytol. Suppl. 14: 211-245.
89. Weis, D.S. 1976. Digestion of added homologous algae by chlorella-bearing Paramecium bursaria. J. Protozool. 23: 527-529.
90. Whatley, J.M. 1976. Bacteria and nuclei in Pelomyxa palustris: Comments on the theory of serial endosymbiosis. New Phytol. 76: 111-120.
91. Wheelis, M.L. 1975. The genetics of dissimilatory pathway in Pseudomonas. Ann. Rev. Microbiol. 29: 505-524.
92. Wille, Jr., J.J., Weidner, E. and Steffens, W.L. 1981. Intranuclear parasitism of the ciliate Euplotes by a trypanosomatid flagellate. J. Protozool. 28: 223-227.
93. Wong, F.Y. and Wildman, S.G. 1972. Simple procedure for isolation of satellite DNAs from tobacco leaves in high yield and demonstration of minicircles. Biochim. Biophys. Acta 259: 5-12.

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