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I am submitting herewith a thesis written by John H. Tullock entitled "Endosymbiotic Bacteria in Amoeba proteus strain xD: Characterization and Comparison of Microorganisms Associated with Symbiotic and Non-symbiotic Amoebae." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Zoology.

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ENDOSYMBIOTIC BACTERIA IN AMOEBA PROTEUS STRAIN xD: CHARACTERIZATION AND COMPARISON OF MICROORGANISMS ASSOCIATED WITH SYMBIOTIC AND NON-SYMBIOTIC AMOEBAE

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ABSTRACT

A strain of Amoeba proteus (xD) containing bacterial endosymbionts (x-bacteria) was isolated from laboratory cultures of A. proteus strain D, in 1966. The bacteria are Gram-negative rods contained within vesicles in the amoeba cytoplasm and are required for normal functioning of the amoeba nucleus. The bacteria are able to infect nonsymbiotic amoebae and can become required cytoplasmic components in about 200 macrosymbiont generations. Symbiotic amoebae cannot grow at temperatures above 26° C, a characteristic mediated by the endosymbionts.

The purpose of this study was to further characterize x-bacteria, and to ascertain the possible relationships of x-bacteria to more typical, free-living, Gram-negative rods.

Antisera to x-bacteria were prepared and evaluated by agglutination and indirect fluorescent antibody (IFA) tests. Ten strains of bacteria isolated from amoeba cultures were tested by the IFA method to determine possible cross-reactivity with x-bacteria. Attempts to culture x-bacteria in vitro were made out. The numbers and kinds of free-living bacteria present in D and xD cultures were compared by bacteriological techniques. Bacteria with two different electron densities

within xD amoeba vesicles were compared by statistical methods.

The major findings of the study were:

1) The x-bacteria stimulate antibody production in rabbits, as demonstrated by IFA tests;

2) Antibody to x-bacteria is not produced in rabbits or rats is not detectable by agglutination tests. Immunizing doses were administered which have been shown to result in high antibody titers to antigens prepared from other Gram-negative bacteria;

3) Bacteria of the genus Xanthomonas were found to be associated only with xD amoebae. While isolates of Xanthomonas are not immunologically cross-reactive with x-bacteria, the two organisms do seem to have some characteristics in common.

It was concluded that x-bacteria are less antigenic than other Gram-negative rods. Some unique feature of the x-bacteria cell wall may be responsible for this phenomenon, and could explain the ability of x-bacteria to avoid digestion by amoebae as well.

The x-bacteria could not be cultured in vitro. However, based on studies of organisms present in amoeba culture dishes, the bacterial species ancestral to x-bacteria is believed to be an organism very much like Xanthomonas.

Statistical comparison of the mean lengths of dark and light bacteria seen within xD vesicles suggests

that two distinct types of bacteria are present in the amoeba cytoplasm. The implications of this finding are discussed in terms of what is known about bacterial endosymbionts of Paramecium aurelia.

A possible mechanism for the establishment of the endosymbiosis between x-bacteria and xD amoebae is proposed.

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CHAPTER I

INTRODUCTION

Stable endosymbiotic relationships between dissimilar types of living organisms often achieve such a degree of intimacy that some investigators have postulated microbial precursors to certain structures presently referred to as "intracellular organelles." Mitochondria, chloroplasts, and the centriole-flagellum-mitotic spindle complex, all clasically considered to be of endogenous origin in the eucaryotic cytoplasm, may be visualized as having an exogenous origin; that is, to have evolved from endosymbiotic procaryotic ancestors.

These ancestral forms are pictured as having entered into symbiotic associations with hypothetical "proto-eucaryote" cells (41, 42, 43). Subsequent refinements of such relationships would have led to the interdependence of the associants to an increasingly greater degree. In theory, then, the "host" came to rely upon the "symbiont" (as a source of ATP, for example), while the "symbiont" came to relegate more and more of its metabolic functions (the replication of most of its genome, for example) to the "host". With time, the eucaryotic cell and its organelles evolved to their modern form. "Host" and "symbiont" can no longer be distinguished, and are considered to be a single system.

While there is evidence to support this hypothesis, and to which I shall shortly allude, much more must be learned about the nature of intracellular organelles, about symbionts themselves, and about the development of stable intracellular symbioses, before the hypothesis is accepted (64).

Various terms have been employed to describe the relationship between the organisms in the numerous examples of symbiosis presently known. Throughout this thesis, the organism usually denoted as "host" will be referred to as "macrosymbiont," while the term "microsymbiont(s)" will refer to the other associant(s) in the relationship. The term "endosymbiosis" will be used when referring to the relationship itself.

The development of a persistent, stable, and interdependent intracellular association with a living eucaryotic cell requires of the potential microsymbiont the solution of various problems of compatibility between it and the macrosymbiont. These include: avoidance of digestion by, or ejection from, the macrosymbiont; development of synchronous reproduction with the macrosymbiont; development of mechanisms to insure for exchange of materials (e.g., metabolites) with the macrosymbiont (63).

Further, if the development of intracellular organelles from microsymbionts is postulated, the ecological conditions which brought about the occurrence of the original

endosymbiosis, as well as the conditions which resulted in the relationship's being maintained, must also be understood.

There are a number of instances of stable endosymbioses which lend support to the theory of origin of organelles from endosymbiotic microorganisms, as outlined above. It is through the study of these systems that knowledge contributing to a complete understanding of the origin of eucaryotic cells will be obtained.

One widely known example of endosymbiosis is the association of zoochlorellae and zooxanthellae with certain protozoa, sponges, and cnidarians (44). These relationships have been well studied. The investigations have contributed much to our knowledge of the interactions between symbiotic partners. In marine cnidaria zooxanthellae (generally assigned the species designation Symbiodinium microadriaticum) interact with the macrosymbiont by:

1) Transferring reduced organic carbon and nitrogen compounds to the macrosymbiont;

2) Accelerating the rate of calcium carbonate deposition in reef-building corals.

Zooxanthellae have also been found to utilize urea, ammonia, uric acid, and nucleosides heterotrophically and may thus augment nutrient cycling in the cnidarian (61, 44).

In Hydra, substances have been shown to move from zoochlorellae to the cnidarian (44). The algae are

of value to Hydra, as starving macrosymbionts survive longer than non-symbiotic controls. Food may be transferred from microsymbiont to macrosymbiont, perhaps in the form of maltose. This disaccharide is rarely used by algae.

Zoochlorellae isolated from sponges and from Paramecium bursaria can be cultured in vitro. Those from Hydra cannot (44). Further, infection of Hydra by isolated algae is highly strain specific.

Symbiotic procaryotes are found in a variety of eucaryote cell types. Species representing some 12 orders of insects have been found to harbor intracellular bacteria (59). Some of the bacteria are associated with the gonads of the macrosymbiont and are involved in embryonic development in these insects. Specialized structures, mycetomes, are produced, within which the bacteria are contained in cells referred to as mycetocytes. The relationship is believed to be analogous to the development of root nodules in legumes as a result of invasion by nitrogen-fixing bacteria. The specific function of insect microsymbionts is unknown, although several possible functions have been suggested. In aphids, the bacteria may participate in the digestion of sugars present in the plant juices on which the insect feeds. Lice may be able to survive on blood because their symbionts produce vitamins normally absent from this specialized diet. Of 14 insect species

for which information was given, 11 of the microsymbionts were reported to be Gram-negative, and the majority of these were rod-shaped (59).

Perhaps the best-studied relationship between a protozoan and bacteria is that between Paramecium aurelia and particles designated kappa, lambda, mu, sigma, gamma, pi, tau, delta, nu, and alpha (4, 5, 49). These particles have the appearance of typical bacteria and are enclosed in membrane-bounded vesicles or found free in the cytoplasm or within the macronucleus of the host. All except gamma are Gram-negative (5). Kappa particles, which have been the objects of the most experimental study, are believed to be under the control of the host genome (49). Lambda particles are known to benefit Paramecium by supplying folic acid, thus enabling the protozoan to survive in a medium free from this compound (57). While most of these particles are known to divide by binary fission, there is no clearly defined nuclear region (5). In this respect, the particles differ from free-living bacteria.

Some of the endosymbionts are responsible for conferring upon the particle-bearing Paramecium the ability to kill certain susceptible strains of P. aurelia (4, 49). At least two of the particles, lambda and mu, can be cultivated outside the protozoan cell, requiring a complex medium for growth (72). Mu particles have been shown to contain diaminopimelic acid, which is characteristic of the cell walls of free-living bacteria, but is not found in other microsymbionts.

Crithidia oncopelti, a small flagellate parasite of the intestine of milkweed bugs, has been shown to contain endosymbionts (64). Two rod-shaped bodies, typically found in the most posterior portion of the protozoan cytoplasm, are similar to bacteria ultrastructurally (except for the absence of a bacterial cell wall). The symbionts are enclosed in a double unit membrane, apparently comprised of the plasma membrame of the bacterium and a membrane produced by the host (8). The cytoplasm of the symbionts is granular in appearance with DNA-like fibrillae (8, 65).

While comparisons of the nutritional requirements of symbiotic and nonsymbiotic Crithidia have shown that the microsymbionts enable the protozoan to dispense with exogenous hemin and other substances, the endosymbiosis is not obligatory, as macrosymbionts can be freed of bacteria by treatment with chloramphenicol (CAP) (9, 10, 11, 12). Surface membrane carbohydrate residues are altered in symbiotic Crithidia. This phenomenon is mediated by the microsymbionts (20). Unlike most protozoa, Crithidia is able to synthesize the purine skeleton. It has been suggested that the bacteria are responsible for this ability (24).

Grell (27) cites a number of references to Protozoa which have endo- or exosymbiotic bacteria. These include Euplotes patella, Oxytrichia bifaria, and Halteria

grandinella. In Paramoeba eilhardi, the bacteria are always associated with the nucleus.

Myxotricha paradoxa, part of the gut flora of certain termites, possesses exosymbiotic spirochaetes which endow the protozoan with motility (L. Margulis, personal communication). Margulis has found this "motility symbiosis" in other termite gut inhabitants and believes the association to be analogous to the ancestral relationship which gave rise to the typical flagellated eucaryotic cells commonly observed today.

Euplotes aediculatus is host to a bacterium which is required for cell division (29). Proca-Ciobanu et al. (50) reported bacterial microsymbionts in the cytoplasm of Acanthamoeba castellani.

In the organism Pelomyxa palustris there are at least two, and possibly more, types of bacterial symbionts (13). An understanding of the relationship of Pelomyxa to its symbionts and, in turn, of the host-symbiont complex to the external environment, provides convincing evidence to support the hypothesis that contemporary eucaryotic mitochondria were derived from endosymbiotic bacteria.

Daniels, Breyer and Kudo (18) and Daniels and Breyer (16) carried out ultrastructural studies on P. palustris, using transmission electron microscopy, and found two species of rod-shaped bacteria in the cytoplasm, each enclosed singly within its own membrane. Even after

examination of scores of amoebae, however, they were unable to find any structure which could be called a mitochondrion.

Pelomyxa palustris is a large, free-living amoeba of variable size. The organism is typically multinucleate and several different types of nuclei have been described (18). Mitosis has not been observed, although a variation of mitosis may occur. It appears that the nuclear material does not condense into structures recognizable as chromosomes although a variety of changes in the nucleus may be observed.

P. palustris possesses many of the cytoplasmic structures seen in other large, free-living amoebae (e.g., Amoeba, Chaos) but Golgi apparatus and mitochondria are absent (17). In addition, and unlike other amoebae, P. palustris often contains numerous silica granules, which cause problems for investigators interested in the ultrastructure of the organism as the silica interferes with the preparation of thin sections for electron microscopy. This problem was overcome when a silica-free strain was discovered (18).

In addition to its other peculiarities, P. palustris is unique among the large, free-living amoebae in that it is anaerobic (40). This organism is thus adapted to life in the bottom sediments of lakes and ponds where low oxygen tensions prevail. Indeed, such habitats are where the organism is found, generally among decaying vegetation,

and in areas of the water mass which are sufficiently shallow to permit the growth of algae upon which the amoeba feeds. The organism is found in several locations in Europe and the United States (18). In this country, for example, the organism has been collected from Falmouth, Massachusetts (34); the northeast corner of Congers Lake, Congers, New York (40); and other sites. The organism was discovered in Germany by Greef.

P. palustris is generally regarded as herbivorous, although a variety of debris, both of animal and plant origin, are ingested (40). Living, motile organisms are not captured unless by chance (18). Hence, the organism apparently moves slowly along in the bottom litter, indiscriminately ingesting particles of organic debris which happen to lie in its path. That P. palustris can survive and reproduce on vegetable food alone is shown by Kudo's (40) success in cultivating this amoeba on chopped Spirogyra as its sole food. As the organism is rather difficult to maintain in culture, however, there is little information as to its specific nutritional requirements. Such studies must await the successful axenic culture of P. palustris which has thus far not been accomplished with even the more typical amoebae, such as Amoeba proteus (28).

In addition to the microsymbionts with which I shall shortly deal, and the silica grains mentioned earlier, glycogen bodies have been reported (17). The possible role

of the latter in the life cycle of the organism will be discussed below.

Chapman-Andresen (13) has reviewed the information known about bacterial endosymbionts of P. palustris. At least two types of these bacteria are present in strains found worldwide. One is a large, Gram-negative rod, 0.8 x 3 μm in size, often with a characteristic indented appearance and often found in short chains. This organism has been named Cladothrix pelomyxae. The other is Gram-positive, a slender rod with pointed ends. One Danish strain of amoeba has three types of bacteria. In addition to the two mentioned above, a Gram-negative rod with rounded ends is seen. The proportions of these organisms in the cytoplasm vary with oxygen tension and cell type.

It should be pointed out that P. palustris exists in three primary cell types as well as intermediate types. The characteristics of each of the primary types are listed below (13):

1) "Light" cells are lively, well-oriented, and possess a pronounced uroid. They contain few silica grains, many glycogen bodies, and many of the smaller Gram-negative, rod-shaped bacteria;

2) "Grey" cells are sluggish and less well-oriented, with many quartz grains, few glycogen bodies, and many of the large, Gram-negative bacteria;

3) "Black" cells are small (volume 0.1 ul), uniform in shape, with small vacuoles, many small silica grains, and many large bacteria.

Each of these types may represent a different stage in the life cycle of the amoeba.

Further complicating the picture of Pelomyxa palustris and its microsymbionts is the changing pattern of bacteria present in the cytoplasm under various oxygen tensions (13). Three to 5% of the bacteria are Gram-negative rods with rounded ends, with the lower percentage recorded at high O_2 tensions. This organism may simply dwell within the amoeba. However, there are marked differences in the percentages of the other two bacterial types in amoebae cultured under high and low oxygen tensions. Under anaerobic conditions the organisms are present in approximately equal numbers (48% large, Gram negative; 47% small, Gram positive). Under aerobic conditions, however, the proportion shifts to 70% small, 27% large. Clearly, the large form is better adapted to P. palustris' physiological state under low O_2 tensions.

The bacteria have been designated Bn (large, Gram-negative, associated with the nucleus) and B (small, Gram-positive, with pointed ends, found in the cytoplasm), respectively (17). I shall refer to the Gram-negative, small rod as "R." The information regarding the various

percentages of these bacteria under different conditions are summarized in Table 1.

As previously mentioned, several types of nuclei are observed in P. palustris. Daniels et al. (18) distinguished six such types and interpreted these as different stages in the life cycle of the amoeba. There are characteristic variations in the intracellular bacterial population associated with each of these types. The bacteria associated with each stage are summarized in Table 2. Small, bacteria-free nuclei have been interpreted as young.

Seasonal conditions also influence the appearance of specimens of P. palustris collected (18). Large numbers of very small amoebae are found in early spring. Collections made in November also contain mostly small amoebae. More typical, large specimens are found during late spring and summer.

Encystment has occasionally been reported in P. palustris (13). Cysts are formed in autumn. The cyst consists of two walls with food debris in between, and possesses a central core of autolysosomes. Small, Gram-negative bacterial colonies are present in the inner wall space of the cyst, and large and small Gram-positive bacteria in palisades are found just beneath the inner cyst wall. Some evidence of sporulation in the larger bacteria has been observed.

TABLE 1

PERCENTAGES OF BACTERIAL TYPES IN *Pelomyxa palustris* UNDER VARIOUS CONDITIONS

CONDITION	BACTERIAL TYPES (%) Bn	B	R
Cell Type			
Light	24	68	8
Grey	43	46	12
Black	48	38	15
O_2 Tension			
High	27	70	3
Low	48	47	5

Source: Chapman-Andresen, C. (1971) Biology of the Large Amoebae. *Annual Review of Microbiology* 25:27-48.

TABLE 2

BACTERIA IN *Pelomyxa palustris*
ASSOCIATED WITH LIFE CYCLE STAGES

STAGE	CELL SIZE	NUCLEATION	BACTERIA NUCLEAR	BACTERIA CYTOPLASMIC
I	200 μm-1 mm	multinucleate	Bn	B, Bn
II	*	multinucleate	Bn	B, Bn
III	100-300 μm	uninucleate	Bn[1]	B[2]
IV	*	less than 100	*	B[2]
V	*	multinucleate	*	B
VI	*	uninucleate	*	B,Bn

*Not reported.

[1]Unusually large and altered in appearance.

[2]Atypical in appearance.

Source: Daniels, E. W., E.P. Breyer and R.R. Kudo (1966) *Pelomyxa palustris* Greef II: Its ultrastructure. *Zeitschrift fur Zellforschung und Mikroskopische Anatomie* 73:367-383.

All the observations thus far made on Pelomyxa palustris and its microsymbionts may be interpreted to mean that these organisms form an association which may provide a selective advantage to the amoeba. The protozoan is able to adapt to changing conditions within its habitat. A summary of the information available (13) about the life cycle of this amoeba will call attention to the significance of the endosymbiosis in the relationship of the amoeba to its environment. Apparently, the "light" cells feed and store glycogen. These cells vary greatly in size, and small ones are most common in early spring. "Grey" cells are most likely to be dividing amoebae. "Black" cells are those preparing for encystment. If the data available concerning P. palustris and its bacteria are tabulated and correlated with what is known about the conditions in a lake or pond at various times of year, the following is found:

1) In early spring, cool temperatures and higher levels of dissolved O_2 prevail in the lake. At this time, newly encysted amoebae (light cells) are accumulating glycogen reserves and actively feeding. Most of the intracellular bacteria are small, Gram-positive rods with pointed ends (B);

2) In late spring and summer, as the lake becomes warmer and oxygen tensions consequently fall, the complex events associated with cell division begin to occur. Most amoebae are in the grey stage. Large, Gram-negative, perinuclear (Bn) bacteria and small, Gram-positive (B) bacteria are present in approximately equal numbers;

3) By late summer or early fall, when the lake is warmest and O_2 levels lowest, the black cell type is predominant. Bn bacteria are present in the highest proportion;

4) As the temperature begins to drop with the approach of winter, encystment occurs, and the amoeba awaits the return of spring in this form. Much more work needs to be done before this suggested life cycle can be confirmed as fact, but the hypothesis does fit in with presently available data.

It has been said that the bacteria in P. palustris may function as mitochondria (13). I suggest that there are two energy-producing systems, one aerobic and the other anaerobic, within this amoeba. B bacteria may function as mitochondria under aerobic conditions, while Bn bacteria may function as mitochondria under anaerobic conditions. Alternatively, both types of bacteria may fulfill some of the energy requirements of the host cell at all times. Bn bacteria could produce energy for the specialized needs of nuclear division (e.g., DNA replication). The otherwise unexplained association of Bn with the amoeba nucleus suggests such a role for this bacterial type.

One might question why the utilization of two bacterial types in this manner should be favored evolutionarily over a simpler scheme involving only one. It should be remembered that efficiency of energy production by aerobic

respiration requires sufficient oxygen for oxidative phosphorylation. Lowered O_2 levels in the lake during the summer, coupled with the increased energy demands resulting from nuclear division might have favored the inclusion of an anaerobe in the amoeba's "collection" of microsymbionts. Data concerning the relationship between temperature and nuclear division in Pelomyxa palustris might reveal how nuclear division could have become associated with the seasonal warming of the amoeba's environment.

Several strains of Amoeba proteus, other than xD, contain bacteria. The content varies from strain to strain, and both spiral and flagellated bacteria are seen (13, 53).

These symbionts differ in some respects from those reported from ciliated and flagellated protozoa, e.g., they cannot be removed by antibiotic treatments without detriment to the host (36).

Among the symbionts of Amoeba are DNA-containing bodies. These are contained individually within vacuoles surrounded by a host-derived membrame. They carry out independent nucleic acid synthesis and contain structures resembling ribosomes (51, 73). The bodies average 0.5 μm in diameter (73) and their numbers within the host varies from 11,000 in predivision amoebae to 5,400 in daughter cells (52). Micrurgical studies have shown that the structural components of the microsymbionts are under the control of the macrosymbiont genome (34).

One strain of A. proteus contains bacteria which cannot be removed by repeated washing and starvation (53). As these amoebae are not known to grow under axenic or monoxenic conditions of culture (44), it has been suggested that organisms in the culture medium, as well as the endosymbionts, are involved in some specific association with the Amoeba (1).

Bacteria found in the cytoplasm of Amoeba species are typically contained within vesicles, a finding which suggests that phagocytosis is the means by which the microsymbionts are acquired by the host (53).

Jeon and Lorch (38) reported bacteria in the cytoplasm of A. proteus strain D in 1966. At that time the bacteria were considered to be parasitic for several reasons:

1) Infected amoebae grew more slowly, with mean generation time of five days at 18° C, as compared with two days for uninfected amoebae;

2) Under conditions which yielded greater than 90% clones of uninfected cells, infected amoebae rarely yielded viable clones;

3) Infected amoebae were smaller in size than uninfected amoebae;

4) More than 50% of the infected cells were dead after starvation for seven days, while only 5% of control cells died under the same conditions;

5) Infected cells were more fragile than uninfected cells;

6) As compared with uninfected amoebae, infected cells showed a decrease in the number of intracellular crystals, becoming yellowish in appearance.

After one year of infection, the infected amoebae (designated "strain xD") exhibited the following characteristics (with respect to strain D from which they were derived):

1) Growth rate was slower, with much variation in individual generation time;

2) Sensitivity to starvation was increased;

3) Vesicles of varying size (up to 40 μm in diameter) were present in the cytoplasm, containing one to many bacteria;

4) The total number of bacteria within a given cell varied between 60,000 and 150,000.

The bacteria are Gram-negative and are seen via electron microscopy to occur in two forms of differing electron densities (1, 38).

After five years of infection, the relationship between xD amoebae and their intracellular bacteria (designated "x-bacteria") developed into a stable endosymbiosis. The mean generation time of xD amoebae was the same as that of the uninfected cells. Micrurgical studies showed that the xD nucleus had become dependent on the

symbionts for normal functioning, and that no harmful effects upon D amoebae were exhibited following injection of xD cytoplasm (32).

Freesh (23) examined xD amoebae in 1974 and found greater similarity to the D strain from which they were derived than when the infection was first observed eight years earlier. She concluded:

1) D and xD are morphologically indistinguishable;

2) xD is more difficult to culture in large numbers;

3) There are minimal differences in growth rates of the two strains.

She also noted that xD exhibited a reduced sensitivity to starvation as compared to 1967, and the number of bacteria per amoeba had fallen from 60,000 - 150,000 in 1967 to 15,000-41,500 in 1975. The x-bacteria were found impossible to culture in vitro. One colony which resembled x-bacteria was obtained on nutrient agar supplemented with amoeba lysate. This colony failed to grow when replated, however.

Further micrurgical studies confirmed that the xD nucleus was dependent upon the presence of the symbionts (36). Furthermore, amoebae could not be freed of their symbionts by treatment with chloramphenicol (CAP). In the presence of this antibiotic, the bacteria degenerated along with the host mitochondria. After one week in CAP-containing

medium, the number of symbionts decreased while the fibrous intravesicular matrix in which the bacteria were contained increased in volume. The bacteria showed overall deformation and expansion of the nuclear region following two weeks of CAP treatment. A few amoebae recovered when returned to culture medium free from CAP, but none of these was devoid of symbionts. CAP was thought to render the symbiont cell wall soluble in 2N sodium hydroxide, a property characteristic of the DNA-containing bodies, but not of symbionts which were not exposed to CAP.

Symbionts were able to survive in other strains of amoebae (37).

To check the possibility that the symbionts were utilized by amoebae as nutritional supplements, acid phosphatase activity of the bacterial vesicles was studied (36, 37). Enzyme activity was not detected, even after amoebae were starved for a week or more.

The xD strain of A. proteus is presently characterized as containing approximately 40,000 Gram-negative, rod-shaped bacteria per cell. The bacteria are enclosed in vesicles surrounded by a double membrane and ranging up to several micrometers in diameter. Within these vesicles, the bacteria are surrounded by a fibrous matrix derived from channeling structures originating in the host cytoplasm (1).

The host genome is dependent upon the presence of these bacteria for normal functioning, and the symbionts, while able to grow in other strains of amoebae, cannot be cultured outside the host cell (23). Studies of the relationship by experimental infection of nonsymbiotic D amoebae have shown that the bacteria are initially harmful, resulting in low clonability (1). This effect is dependent on the nature of the food organism supplied to the experimental cultures.

The growth rate of the bacteria within the amoeba cytoplasm was found to be 0.165, with a mean generation time of 1.82 days. It is believed that amoebae actively control the symbiont population in connection with which the symbionts or vesicles are excreted by the amoeba cell. The xD amoebae do not grow under sterile conditions, suggesting that they are dependent on other organisms in the external environment as well as the endosymbionts. Quantitative, but not qualitative, differences were found between the protein banding patterns observed following polyacrylamide gel electrophoresis of xD and D amoebae (1). These observations are not considered to reflect differences in the protein patterns of the two strains, however. In addition, the amoebae have acquired the trait of temperature sensitivity, apparently mediated by the microsymbionts (35). After fewer than 200 host cell generations following experimental infection of

D amoebae with x-bacteria, the amoebae are unable to survive at temperatures above 26° C.

Thus, the occurrence of stable endosymbioses between bacteria and protozoa is a relatively common phenomenon. Further, certain of these systems may be taken to represent modern analogs of the endosymbiotic acquisition of flagella (Myxotricha paradoxa) and mitochondria (Pelomyxa palustris) (13).

Endosymbiotic photosynthetic procaryotes (blue-green algae) are present within vesicles of certain cells of the subepithelium of two species of echiuroid worms (39). Bacteria are also present, contained in vesicles, but separate from the algae. The alga can be cultured in vitro and a metabolic relationship between it and the host is suspected.

There are examples of protozoa which contain eucaryotic algae in symbiotic association, such as Paramecium bursaria, mentioned earlier (3, 44).

That isolated chloroplasts can survive and carry on photosynthesis in cells other than those from which they were derived is shown by the finding that many, if not all, saccoglossan molluscs (e.g., Tridachia crispata) are able, in some as yet unknown manner, to isolate the chloroplasts from the algal cells on which they feed and transfer them intact to specialized structures, called cerata, which protrude like rows of ruffles from the dorsal surface of the animal (27, 70). Here the chloroplasts survive for 24 hours to 30 days or more, and produce more

intermediate metabolites than when in the plant. In some cases, these microsymbionts may supply glycolic acid for mucus synthesis by the macrosymbiont. Interestingly, some plastids appear able to maintain normal chlorophyll turnover despite their need for at least two nuclear genes to carry out this function in the plant cell.

The recent discovery (22) of a procaryotic alga containing chlorophyll types previously thought to be characteristic only of eucaryotic algae, provides a measure of hope that an endosymbiotic analog of the hypothetical proto-chloroplast may yet be discovered.

Thus, endosymbiotic microorganisms are found within the cells of a variety of species. In the majority of cases, little is known about the mechanisms of integration of macro- and microsymbiont activities; however, these systems have numerous features in common. Among these are:

1) Nonphotosynthetic microsymbionts are typically Gram-negative, rod-shaped bacteria, contained within vesicles in the macrosymbiont;

2) With some exceptions, the symbionts are surrounded by two membranes, one derived from the macrosymtiont and the other representing the plasma membrane of the microsymbiont;

3) In some cases (e.g., Pelomyxa palustris and xD amoebae) there appears to be a connection between the activities of the symbiotic complex and specific environmental conditions.

Generally, little is known about the history of the association between the symbiotic partners. The case of xD amoebae and x-bacteria is, in fact, the only such association about which this information is presently available. In this connection, xD amoebae are excellent subjects for study leading to an understanding of the nature of such endosymbioses. The development of dependence of the amoebae upon the acquired cytoplasmic bacteria has occurred within an observable span of time. Furthermore, the existence of DNA-containing bodies dependent upon the amoeba genome and the presence of mitochondria within the xD cytoplasm gives rise to a system which can be plausibly considered to reflect three stages in the development of intracellular organelles from an endosymbiont within a single host/symbiont complex.

The observed dependence of these amoebae upon other organisms in the culture medium offers the possibility of investigating the nature of environmental conditions attendant upon the development of such stable endosymbioses. Thus, further characterization of the relationship between xD amoebae, x-bacteria and the organisms present in their environment is significant in enhancing understanding of the development of endosymbiotic associations between bacteria and protozoa.

In the present study, several methods were employed to elucidate the nature of the relationship of

x-bacteria to the free-living bacteria present in xD and D amoeba cultures. Antisera to x-bacteria were prepared and isolates of bacteria from amoeba cultures were screened for cross-reactivity with these antisera. The microbial flora of xD and D cultures were compared by bacteriological techniques. Attempts to cultivate x-bacteria in vitro were made. The nature of some of the carbohydrate residues of the x-bacteria cell surface were studied by means of lectin agglutination tests. The ability of x-bacteria to produce cytochrome oxidase was investigated. Statistical comparisons were made between x-bacteria of different electron densities to determine if these differences in electron density could be correlated with differences in the average length of the two bacterial types.

As stated earlier, an understanding of the role of endosymbiotic relationships between procaryotic and eucaryotic cells may be fundamental to our knowledge of the means by which the early evolution of eucaryotes took place (67). Such endosymbioses are of frequent occurrence and hold in common many characteristics, suggesting that basically similar mechanisms may have led to the development of a variety of associations with corresponding variation in potential for further evolutionary change. As Cudmore (15) has stated:

> The hospitality of the protists is phenomenal and unending. They have been kinder than almost any country has to its immigrants. Their hospitality has brought them success.

With all of these acquired talents, they were able to turn into multicellular animals and plants, able to construct the complexities of a flower or a nervous system. They had the energy for motion, the structure of the giant redwoods.

CHAPTER II

MATERIALS AND METHODS

A. Culture of amoebae and food organisms.

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, an infected variant strain, was first noticed in D amoeba cultures in 1966.

Stock solutions of modified Chalkley's medium were prepared as follows. Solution A consisted of NaCl 5.84 gm, KCl 0.75 gm, $NaHCO_3$ 0.84 gm, and $Na_2HPO_4 \cdot 7H_2O$ 0.38 gm in one liter of glass-distilled water. Solution B contained $CaCl_2 \cdot 2H_2O$ 73.5 gm in one liter of glass-distilled water. Working solution was prepared by adding 5 ml of solution A and 0.1 ml of solution B to one liter of glass-distilled water.

Both strains were grown with Tetrahymena as the food organism in Falcon plastic dishes (60 x 15 mm) or glass baking dishes (32x22x4 cm inner dimension) at 22° C. All dishes were covered. Each baking dish normally contained about 3×10^5 to 4.5×10^5 cells in about 500 ml of medium. The medium was changed and amoebae fed three times a week (Monday, Wednesday, Friday) after removing debris by aspiration. The culture dishes were changed once a week.

Tetrahymena pyriformis were cultured axenically in the medium described by Goldstein and Ko (25). A stock solution of salts and vitamins was prepared and kept frozen at -20° C. Twenty grams of proteose peptone and 2 gm of liver concentrate (Sigma) were added to 10 ml of the frozen salt and vitamin mixture, and brought up to 500 ml with glass-distilled water. The medium was distributed in 100 ml aliquots in 500-ml flasks and autoclaved for 15 minutes at 121° C.

The organisms were grown on a shaker for two days at room temperature. Before harvest, Chalkley's solution equal to one-half volume of the medium was added and the mixture allowed to stand for 10 minutes to minimize osmotic shock. Tetrahymena were harvested by centrifugation for three minutes at 230 x g and washed twice in Chalkley's medium. The organisms were suspended in Chalkley's solution to a density of about 10^5 Tetrahymena per ml.

B. Preparation of Crude and Purified x-bacteria.

Amoebae (xD) were harvested by centrifugation for one minute at 170 x g and washed two times in Chalkley's solution. The washed pellet was suspended in 0.5 ml 0.15 M NaCl and sonicated in an ice bath at 20 percent power on the Fisher Model 300 sonic dismembrator in six 5-second bursts with 5 second rests between bursts. Ten milliliters of 0.15 M NaCl were added, and the sonicate was centrifuged for 5 minutes at 1000 x g. The pellet was resonicated and recentrifuged.

Supernatant fractions were pooled and centrifuged for 15 minutes at 22,000 x g, and the resulting pellet was washed once and then suspended in 1.0 ml 0.15 M NaCl for injection into animals.

All procedures were done at 0°-4° C. Amoebae were harvested by centrifugation for 60 seconds at 170 x g, and washed five times in Chalkley's solution. The volume of amoeba pellet was noted. At a relative centrifugal force of 170 x g for one minute, 1.0 ml packed amoebae pellet contains approximately 3.8×10^5 amoebae. Since one amoeba contains about 4×10^4 x-bacteria, 1.0 ml packed amoebae should yield approximately 1.5×10^{10} bacteria. After noting the volume, cells were placed at -20° C and kept frozen until ready for use. Generally, x-bacteria were prepared the day after harvest of amoebae.

To each ml of pellet 1.0 ml ice-cold phosphate-buffered saline (PBS, 0.15 M NaCl in 0.01 M phosphate buffer, pH 6.8) was added and the amoebae suspended by vigorous stirring, avoiding the formation of foam. The suspension was centrifuged for 10 minutes at 1000 x g and the supernatant fraction decanted and reserved. About 1.0 ml PBS was added to the pellet and the resulting suspension sonicated in an ice bath with a Fisher Model 300 sonic dismembrator for 20 seconds at 25% power. Five to 10 ml PBS were added, the sonicate was centrifuged for 10 minutes at 1000 x g, and the supernatant fraction pooled with that

from the first centrifugation. The pellet was resuspended in 0.5-1.0 ml PBS, and the procedure repeated, as above, until satisfactory recovery of x-bacteria was achieved. This was determined by counting the number of bacteria in the pooled supernatant fractions by means of a Petroff-Hausser bacteria counter under the phase contrast microscope at 400 X magnification. The pooled fractions were then centrifuged for five minutes at 1000 x g and the entire sonication/centrifugation procedure was repeated five more times.

Pooled supernatant fractions from the second series of sonications described above were centrifuged for 15 minutes at 22,000 x g. Five ml of PBS were added to the pelleted x-bacteria and the suspension was sonicated for 2-3 seconds at 25 percent power. In order to avoid loss of x-bacteria, approximately 1.0 ml of the saline was added prior to sonication and the remaining 4.0 ml were used to rinse material from the probe of the sonicator back into the centrifuge tube. (At no time in the procedure should the x-bacteria suspension be transferred from tube to tube. This will result in a substantial loss of bacteria, as they remain in traces of moisture clinging to the inside walls of the glassware.) After resuspension, the bacteria were washed two more times by centrifugation in PBS. To the final pellet was added 4.9 ml PBS and 0.1 ml 5 percent (w/v) Triton X-100. The bacteria were dispersed by gentle shaking and the suspension was stored for thirty minutes at 4° C.

Bacteria were freed of Triton X-100 by washing three times in PBS, then resuspended in PBS to the desired final concentration. The highest yield of x-bacteria obtained by this method was 50% or approximately 8×10^9 x-bacteria/ml packed xD amoebae. The calculated value for 100% yield is 1.6×10^{10} x-bacteria/ml xD amoebae.

C. Immunological Techniques.

Antigens. The first antigen preparation is referred to as "untreated antigen." The desired amount of crude x-bacteria fraction was mixed with an equal volume of Freund's complete adjuvant immediately prior to injection subcutaneously in the mid-scapular region of the back. The x-bacteria suspension was stored in sterile vaccine bottles without preservative at -20° C.

"Untreated antigen II" was prepared as follows: Amoebae were harvested by centrifugation for one minute at 170 x g, and washed five times in Chalkley's solution. The pellet was frozen and the cells then were ruptured by addition of 2.0 ml PBS and passing back and forth through a Pasteur pipette. The supension was centrifuged for five minutes at 1000 x g, and the supernatant fraction collected. One ml of PBS was added to the pellet and the suspension sonicated for 20 seconds at 20% power to free the bacteria. One ml PBS was added and the suspension centrifuged for five minutes at 1000 x g. The supernatant fraction was collected and pooled

and the sonication/centrifugation procedure repeated four times. Pooled supernatant fractions contained approximately 8×10^9 x-bacteria in 9.0 ml PBS. This material was centrifuged for one hour at 22,000 x g, and the pellet resuspended in 2.0 ml PBS containing 0.3% formaldehyde as preservative. The antigen was stored in sterile vaccine bottles at 4° C until use. This antigen was administered by injection into the medial ear vein. Freund's adjuvant was not used.

"Heat-treated antigen" was prepared by holding a tube containing purified x-bacteria in a boiling water bath for 2½ hours. Formaldehyde was added to a final concentration of 0.3% (v/v) prior to storage at 4° C. This antigen was administered by injection into the medial ear vein. Freund's adjuvant was not used.

Immunization of animals. Female New Zealand White rabbits weighing 2-3 kg were bled for control serum by nicking the marginal ear vein with a scalpel and collecting the blood in a centrifuge tube held on ice. The blood was allowed to clot overnight at 4° C and serum collected by centrifugation for 30 minutes at 1,000 x g. Serum was frozen in 0.2 ml aliquots in heat-sealed ampoules and stored without preservative at -40° C.

The immunization schedule employed for each antigen is given in Table 3.

TABLE 3

IMMUNIZATION SCHEDULES FOR RABBITS

EXPERIMENT	ANTIGEN	DAY	NO. OF BACTERIA ADMINISTERED	ADJUVANT USED
1	Untreated	0	3×10^5	Yes
		2	4.5×10^5	
		5	1.1×10^6	
		8	1.2×10^8	
		11	1.2×10^8	
		14	1.5×10^8	
2	Heat-treated	0	5.0×10^8	No
		4	5.0×10^8	
		16	5.0×10^8	
3	Untreated-II	0	5.0×10^8	No
		4	1.0×10^9	
		8	1.0×10^9	
		12	2.0×10^9	

Immune sera were collected, prepared, and stored as for control sera, described above.

Due to the failure of the serum obtained after the first series of injections to evidence any agglutinating antibody to the untreated x-bacteria preparation, a second series of injections was begun, using heat-treated antigen, 7 months after the beginning of the first series of injections. Since the intention was to prepare antibody to the x-bacteria by whatever means available, the same rabbits were employed for the second attempt as for the first.

The second series of injections was administered according to the schedule outlined in Table 3 (p. 34). Serum was collected, prepared, and stored as outlined for control serum, except that rabbits were bled by inserting a needle into the marginal ear vein, rather than by nicking the vessel with a scalpel.

Because no agglutinating antibody could be detected in serum prepared from rabbits, two female white Holtzman rats, aged four months, were immunized by a single intraperitoneal injection with 4 ml/kg heat-treated antigen containing 10^9 bacteria per ml. To stimulate antibody production, each rat was given 0.3 ml Freund's complete adjuvant subcutaneously in the mid-scapular region of the back at the same time when antigen injections were given. A third rat received only the injection of Freund's adjuvant. All three animals were bled by decapitation six days later and immune sera were pooled. Sera were collected, prepared, and stored as for rabbit sera.

Agglutination tests. Agglutination tests were carried out using reciprocal dilutions of antigen and serum in a series of six-well depression slides. Two control wells, one containing antigen and PBS only, as a control for autoagglutination, and one containing only serum and PBS, were also set up. Dilutions of antigen and antibody were made in PBS. Doubling dilutions from 1:1 to 1:8 were employed for serum, which was tested against undiluted antigen, and decimal dilutions of antigen from 1:1 to 1:100 were tested against undiluted serum. In three separate trials, the depression plates were incubated 15 minutes at 37° C, 30 minutes at 22° C, and 18 hours at 37° C, respectively. The first test was carried out using untreated antigen, the second using heat-treated antigen, and the third with untreated antigen II. Results were evaluated by visual observation and under phase contrast.

Indirect fluorescent antibody (IFA) tests. Because of the failure of agglutination tests to reveal the presence of specific antibody to x-bacteria, indirect fluorescent antibody staining was carried out to determine if antibody to x-bacteria could be detected by this method. This technique is routinely used for the localization of antigens in tissues and sera where the antigen under study is present in only small amounts and many interfering antigens may be present (70). It was thus felt that the IFA test might be better suited to the detection of antibody to x-bacteria than the agglutination

test. The tests were carried out on smears of heat-treated antigen, untreated antigen II, and on sectioned xD amoebae. Smears were prepared by allowing a loopful of antigen to air dry at room temperature on a microscope slide.

Preparation of amoeba sections for IFA tests. Cells were harvested by centrifugation for one minute at 170 x g, and washed three times in Chalkley's solution. After fixation in 2 percent (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2½ hours at 4° C, cells were washed two times in phosphate buffer and one time in glass-distilled water. Dehydration was carried out in a graded ethanol series. The cells were cleared in toluene and infiltrated with melted Tissuemat (m.p. 56°-58° C) prior to embedding in Tissuemat. Seven micrometer sections were cut on a rotary microtome, and mounted with albumen fixative on glass slides. After deparaffinization in xylene, the sections were run up to water through a graded alcohol series.

Staining. Smears or sections were flooded with normal sheep serum and incubated 30 minutes at room temperature. Slides were washed five times in PBS. Control or immune rabbit serum was then applied and the slides incubated one hour at room temperature. Slides were again washed five times with PBS. Fluorescein isothiocyanate-labeled sheep anti-rabbit IgG (Source: Nutritional Biochemicals, Inc.) was diluted 1:5 with PBS and applied to each slide. Slides

were incubated 30 minutes at room temperature. (All incubations were carried out in a humid chamber to prevent the slides from drying out.) The slides were washed four times in PBS and once in glass-distilled water, and blotted dry. The stained smears or sections were examined under a microscope with ultraviolet illumination. Preparations showing more brilliant fluorescence than controls were recorded as positive.

D. Bacteriological Techniques.

Media. Tryptic soy broth (TSB), nutrient agar (NA), brain heart infusion (BHI) broth, PPLO broth, tryptose broth, thioglycollate broth, phenol red broth base, Sabouraud dextrose broth, yeast extract, lactose, glucose, proteose peptone and agar were obtained from Difco Laboratories, Inc., Detroit, Michigan (19). Yeast extract was used to supplement TSB at a concentration of 0.5% (w/v). Lactose and glucose were used with phenol red broth base at 0.5% (w/v). Proteose peptone was used alone at 0.1% (w/v). Agar was employed unsupplemented at 0.8% (w/v) and at 0.1% to 1.0% (w/v) in other media, depending on the degree of firmness desired. Acetate agar was prepared as described by Blair et al. (7).

All dry media were rehydrated with Chalkley's solution rather than distilled water to avoid any possible osmotic shock to the x-bacteria. Media were autoclaved for

20 minutes at 121° C (under 15-pounds pressure) prior to use. Aseptic technique was followed throughout.

Other media were prepared using lysates of Amoeba proteus strain D and Tetrahymena pyriformis, alone or in combination at the concentrations given in Table 4.

Amoeba lysate was prepared as follows: amoebae were harvested from mass culture dishes by centrifugation for one minute at 170 x g, and washed three times in Chalkley's solution. The final volume of pellet was noted. The cells were placed at -20° C, then thawed in 9 volumes of cold Chalkley's solution, sonicated (six 5-second bursts at 30% power on the Fisher Model 300 Sonic Dismembrator, with 5-second rests between bursts), and the lysate was centrifuged for twenty minutes at 22,000 x g. The lysate was sterilized by passing through a 0.45-um Millipore filter and added aseptically to 1.0% agar in Chalkley's solution , cooled from prior autoclaving, at the concentrations shown in Table 4.

Tetrahymena were harvested by centrifugation for 10 minutes at 1000 x g. They were washed five times by centrifugation and sterile lysate was prepared as for amoeba lysate. One percent agar in Chalkley's solution, cooled from prior autoclaving, was supplemented with lysate as give in Table 4.

TABLE 4

BACTERIOLOGICAL MEDIA TESTED FOR THE GROWTH OF x-BACTERIA

MEDIUM	COMPOSITION
Acetate Agar	--[1]
Agar, unsupplemented	0.8% (w/v) in Chalkley's
Amoeba Lysate Agar, 10%	Amoeba lysate 10.0% (v/v), agar 1.0% (w/v)
Amoeba Lysate Agar, 50%	Amoeba lysate 50% (v/v), agar 0.1% (w/v)
Amoeba/*Tetrahymena* Agar	Amoeba lysate 1.0% (v/v), *Tetrahymena* lysate 10%, (v/v), agar 1.0% (w/v)
Brain Heart Infusion (BHI) Broth	--[2]
BHI Agar	BHI Broth + 1.0% agar
Nutrient Agar (NA)	--[2]
PPLO Broth Base	--[2]
Phenol Red Broth Base	Sugar supplements 0.5% (w/v)
Proteose Peptone	0.1% (w/v) in Chalkley's
Sabourand's Dextrose Agar	--[2]
"Soldo's Medium"	--[3]
Tetrahymena Lysate Agar	*Tetrahymena* lysate 10.0% (v/v), agar 1.0% (w/v)
Thioglycollate Broth	--[2]
Tryptic Soy Broth (TSB + YE)	supplemented with 0.5% yeast extract
Tryptic Soy Agar (TSA + YE)	TSB + 1.0% agar, supplemented with 0.5% yeast extract
Tryptose Broth	--[2]

1 See text (p. 39).

2 Difco Laboratories, Inc., Detroit, Michigan.

3 See text (p. 41).

The medium of Soldo et al. (58), with palmitic, stearic, linoleic, linolenic and oleic acids substituting for TEM-4T and guanosine monophosphate and uridine monophosphate substituting for yeast nucleic acid, was prepared as described by those authors.

Preparation of inocula. Several treatments were employed for the removal of contaminating bacteria from xD amoebae for use as inocula. Freezing did not affect the viability of the symbionts, as measured by their ability to infect D amoebae (2).

Treatments included: starvation; repeated washing in sterile Chalkley's solution; repeated washing in sterile yeast extract; culture of amoebae in Chalkley's solution, supplemented with chloramphenicol, tetracycline, or a combination of both antibiotics; serial dilution of the xD amoeba lysate prior to inoculation; various combinations of these procedures. The most effective technique was found to be repeated washing of intact amoebae in sterile Chalkley's solution, followed by serial dilution of lysate.

Experiments indicated that each amoeba cell carried with it an average of three contaminating bacteria following five washings in Chalkey's solution. Further washings did not eliminate these contaminants. However, since each xD amoeba carried about 4×10^4 x-bacteria, and only three contaminants, the contaminants remaining after washing could

be effectively eliminated by diluting the lysed amoebae 10^4 times and inoculating two or more tubes of the medium to be tested. Approximately 10^3 amoebae were lysed for each inoculum preparation. Therefore, about 4×10^3 x-bacteria were introduced into each tube or plate of medium tested, while fewer than one contaminant, on the average, would have been introduced per culture.

In Vitro culture conditions. All cultures were incubated at 20° C for 14 days and examined daily for bacterial growth. Tubes showing growth were subcultured to TSB + YE to ascertain whether the growth observed was due to x-bacteria or contaminants. Tubes showing no growth on the 14th day were examined under the phase-contrast microscope (400 X) for the possible presence of small numbers of bacteria.

Response of x-bacteria to lectins. Lectins were obtained from Sigma Chemical Company, Chicago, Illinois. A suspension of x-bacteria, prepared as for heat-treated antigen and containing 10^9 bacteria/ml was mixed with a solution containing 1000 µg/ml of either soybean agglutinin (SBA), wheatgerm agglutinin (WGA) or concanavalin A (Con A) to final lectin concentrations of 500, 250, 125, 50, 25, 10, and 0 (control) micrograms/ml. Dilutions of lectins were made in 0.01 M PBS, pH 6.75, at twice the final concentration desired and mixed with equal amounts of bacterial suspension in six-well depression slides. Duplicate tests

were run using x-bacteria fixed for one hour at 4° C in 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The preparations were incubated on a shaker for 30 minutes at room temperature, and observed for agglutination by visual inspection and examination under phase contrast.

Oxidase test of x-bacteria. Approximately 0.05 ml of a suspension containing approximately 2 x 10^8 x-bacteria/ml PBS was applied to an oxidase test strip (General Diagnostics Division, Warner-Chilcott Co., Morris Plains, N.J.). After 10 seconds, the strip was observed for a blue color reaction indicating the presence of cytochrome oxidase.

The test was repeated by mixing 0.05 ml of the same suspension with an equal amount of 1% (w/v) p-aminodimethylaniline oxalate (DMA) solution. With this reagent, a rose-purple color indicated oxidase production.

Isolation of bacteria from amoeba cultures. Since the acquisition of symbiotic bacteria by xD amoebae occurred under conditions of laboratory culture, the bacterial flora of D and xD cultures were studied and compared. Large, free-living amoebae are always associated with bacteria in one way or another, as these amoebae cannot be cultured axenically (28).

TSA with 0.5 percent yeast extract was used as primary isolation medium for plate counts and for maintenance of stock cultures. All isolates were picked from single colonies and restreaked on TSA to insure isolation of organisms in pure culture. From the second set of plates, inocula from individual colonies were transferred to TSA slants or to test media. Cultures were incubated for two to seven days, depending on the nature of the experiment and the growth rate of the cultured organisms. Cultures were incubated at 20° C, except in cases where the ability of the organisms to grow at different temperatures was being studied. Gram stains were performed routinely on all cultures.

For plate counts samples of amoeba culture media were serially diluted and 0.1 ml of each dilution plated by spreading with a sterilized glass rod bent to an "L" shape. After incubation, colonies were counted and the number of organisms per ml of amoeba medium was calculated.

To determine if the kinds of bacteria present in amoeba cultures were influenced in some manner by the strain of amoeba present, healthy Falcon dish cultures of D and xD amoebae were harvested in sterile tubes by centrifugation for 10 seconds at 1000 x g, and the supernatant fractions distributed into four sterile Falcon dishes, two

for each strain of amoebae. The pelleted amoebae were then washed five times in sterile Chalkley's solution and inoculated into the dishes. Half the inoculum from each strain was introduced into medium in which the opposite strain had been cultured and half returned to its original medium. The cultures were then given usual care for three weeks. At the end of that time, 0.1 ml from each dish was plated onto TSA and the bacteria isolated and characterized.

To ascertain what organisms were normally present in xD and D cultures, 0.1 ml aliquots from mass cultures and Falcon dish cultures were plated on TSB and the bacteria isolated and characterized. Since, in initial experiments, no difference was found in the types of organisms obtained from mass cultures as opposed to Falcon dish cultures, work was carried out with material from mass cultures alone. Inocula always contained amoeba cells, which lysed on the agar surface within 24 hours, freeing any organisms present on or in the amoeba cell. Thus, bacteria from both inside and outside the amoeba were isolated.

In the course of attempted culture of x-bacteria in vitro, it became apparent that some bacteria could not be separated from amoeba despite repeated washings. This has been the experience of other workers (45).

To determine the number of bacteria remaining on the amoebae after washing, 100 xD and 100 D amoebae were picked up with a capillary pipette, washed five times and lysed in 1.0 ml sterile Chalkley's solution by repeated freezing and thawing. The lysate was diluted and plated on TSA. Bacterial colonies were counted after one week.

In a similar experiment, approximately 500 cells of each strain were washed, lysed, and plated. Isolated colonies were picked for characterization. One organism obtained in this experiment, a Xanthomonas species, was found to be present only in xD amoeba cultures. To determine if some growth enhancing substance is produced by xD amoebae, acetate agar was prepared containing 10.0% xD lysate, 10.0% D lysate or no supplement. Each tube was inoculated with Xanthomonas, incubated four days at 20° C, and observed for growth.

F. Statistical Comparison of Bacteria of Different Electron Densities Within xD Amoeba Vesicles.

Within the xD amoeba vesicles, bacteria of two different electron densities are seen (1, 38). A vesicle containing both types of cells is shown in Figure 1. These may be designated as "dark" (D) and "light" (L) cells.

It was decided to determine if these two types of bacteria differ in other respects. A number of electron micrographs of xD vesicles were examined. Measurements of the lengths of D and L bacteria visible in these micrographs

were compared by the statistical method of analysis of variance (ANOVA).

Measurements were made from prints and the actual lengths of the bacteria were calculated from the known magnification of negatives. Care was taken to measure only those cells which had been sectioned longitudinally. Such cells could easily be recognized because both ends appeared round and uniform (Figure 1: "D" and "L"). Obliquely sectioned cells had one rounded end and one end somewhat pointed in appearance (Figure 1: "A"). Cells sectioned transversely appeared as circles in the micrographs (Figure 1: "B"). The vacuolated appearance of dark cells is an artifact.

Twenty-seven negatives were examined and measurements of 41 cells of each type were made.

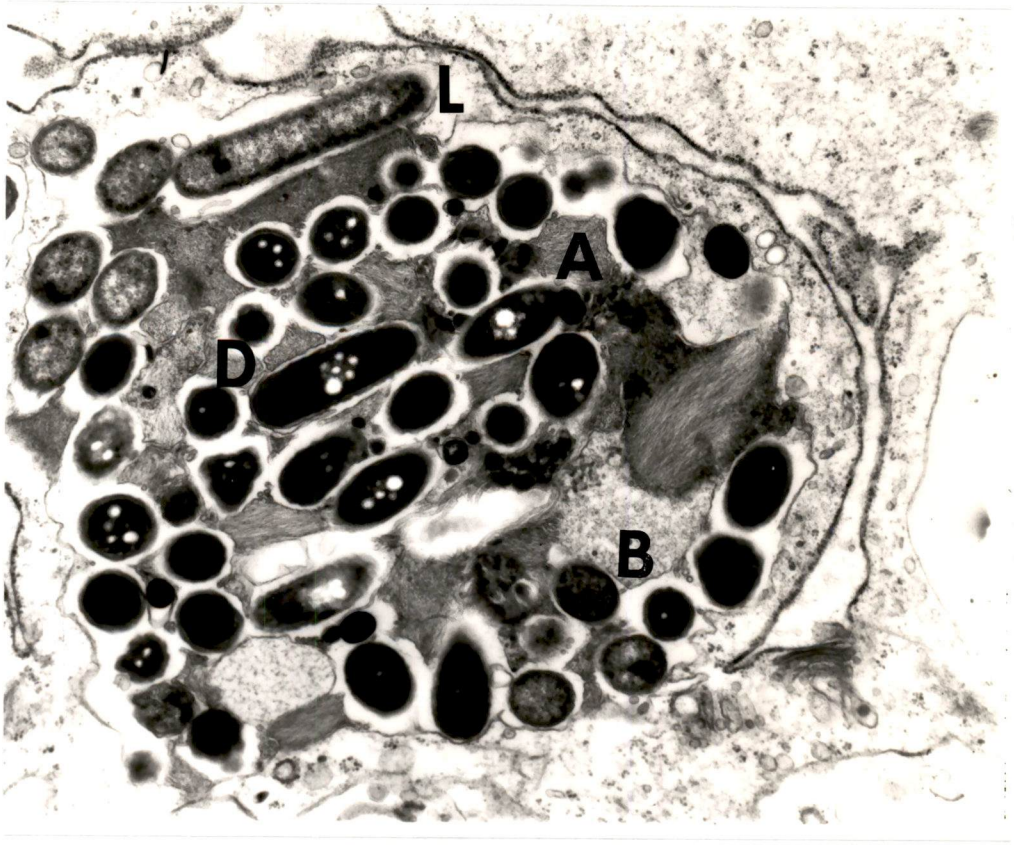


Figure 1. Electron micrograph of an xD amoeba vesicle. Magnification: 18,200 times. Both light (L) and dark (D) bacteria are visible. Bacteria sectioned obliquely (A) or transversely (B) were not included in the statistical study. Photograph courtesy of T.I. Ahn.

CHAPTER III

RESULTS

A. Immunological Studies.

The results of agglutination tests with sera prepared against x-bacteria are reported in Table 5. Neither rabbits nor rats produced agglutinating antibody to any of the antigen preparations of x-bacteria. In the case of rat sera, inspection revealed agglutinated material in all wells except controls. Phase-contrast examination showed that the aggregates were not composed of x-bacteria, which could be seen floating freely in the surrounding fluid.

Results of the indirect fluorescent antibody (IFA) tests indicated that antibody to x-bacteria was present in the rabbit sera. The results of these tests are reported in Table 6. Typical negative and positive reactions are presented in Figures 2 and 3, respectively. Control serum was applied to the smear in Figure 2 and immune serum to the smear in Figure 3. Following incubation for one hour at room temperature, the smears were washed and exposed to fluorescein-conjugated sheep anti-rabbit IgG for 30 minutes. The smears were washed again and photographed on Kodak High Speed Ektachrome Film (ASA 200) under ultraviolet light at 400 X magnification. Exposure time was two minutes. Clearly, more of the labeled antibody was bound to the smears treated with immune serum. The antigen preparation used was untreated antigen II. The results indicate that antibody to x-bacteria was

TABLE 5

RESULTS OF AGGLUTINATION TESTS
OF ANTISERA TO x-BACTERIA

ANIMAL IMMUNIZED	TRIAL	ANTIGEN	INCUBATION	AGGLUTINATION TITER
Rabbit	1	Untreated	37°C,15 min	0
Rabbit	2	Untreated	22°C, 4 hr	0
Rabbit	3	Heat-treated	37°C,18 hr	0
Rabbit	4	Untreated II	37°C,18 hr	0
Rat	1	Heat-treated	37°C,18 hr	0

TABLE 6

FLUORESCENT ANTIBODY BINDING
BY x-BACTERIA AND FREE-LIVING BACTERIA
TREATED WITH IMMUNE RABBIT SERUM

EXPT.	IMMUNIZING ANTIGEN	TEST MATERIAL	FLUORESCENCE
1	Heat-treated	Heat-treated	+
2	Heat-treated	Sectioned xD Amoebae	+
3	Heat-treated	*Pseudomonas fluorescens*	-
		P. diminuta strain 1	-
		P. diminuta strain 2	-
		P. diminuta strain 3	-
		Pseudomonas sp.	-
		Xanthomonas strain 1	-
		Xanthomonas strain 2	-
		Xanthomonas strain 3	-
		Acinetobacter sp.	-
4	Untreated-II	Untreated antigen II	+
		Xanthomonas strain 4	-

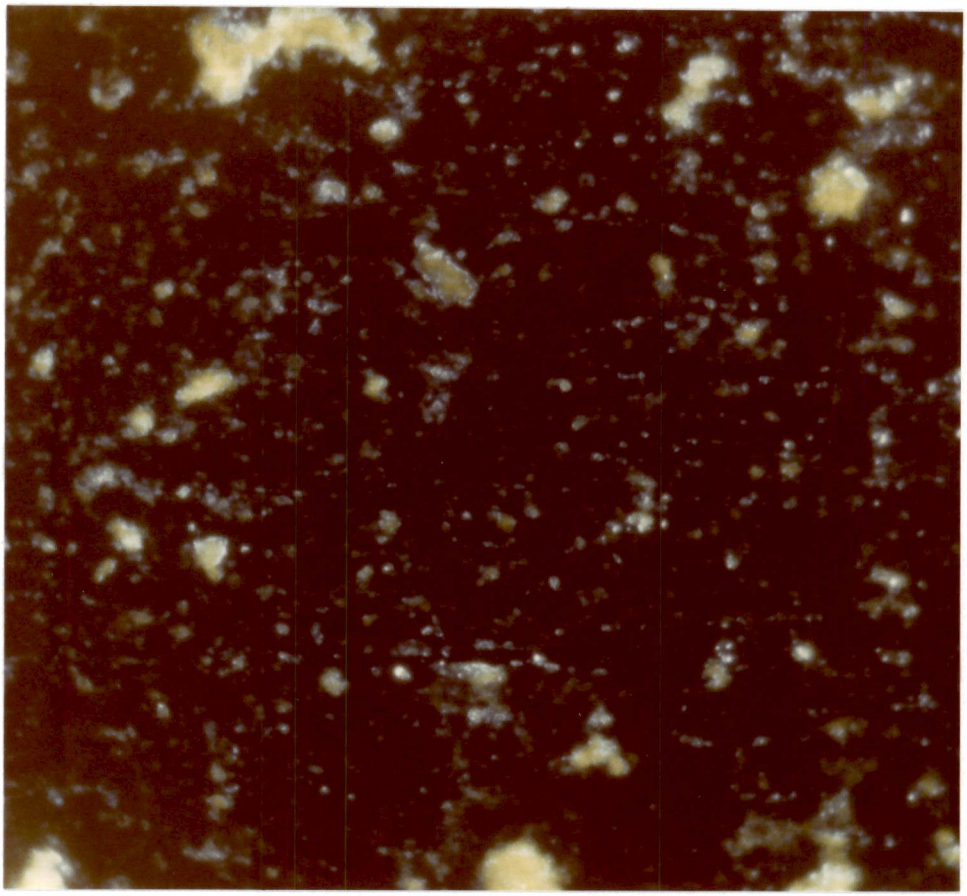


Figure 2. Micrograph of x-bacteria smear treated with control rabbit serum and stained with fluorescein-conjugated sheep anti-rabbit IgG. Magnification 1600 times. Photographed with ultraviolet illumination.

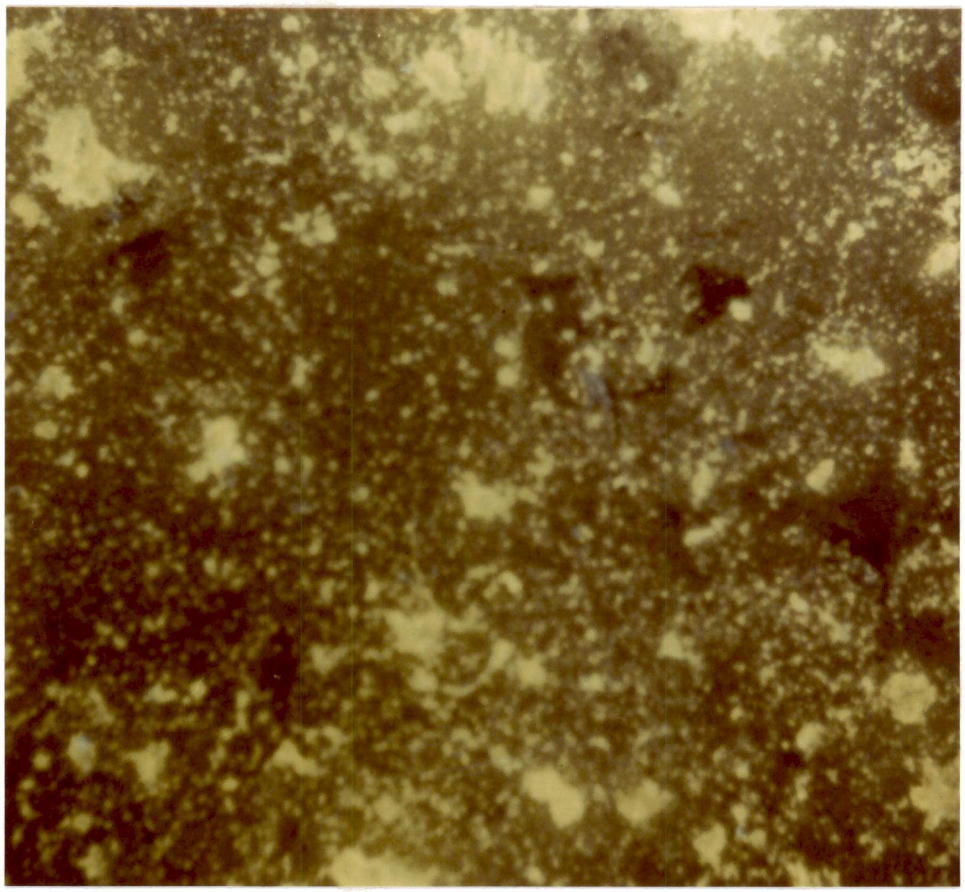


Figure 3. Micrograph of x-bacteria smear treated with immune rabbit serum and stained with fluorescein-conjugated sheep anti-rabbit IgG. Magnification 1600 times. Photographed with ultraviolet illumination.

produced by the rabbits. Antibody titers were insufficient to be detected by an agglutination test. Fluorescent antibody tests using the rat sera were not done.

Since antibody to x-bacteria was detected in rabbit sera by means of the IFA technique, organisms isolated from amoeba culture dishes were tested with immune sera in order to determine if any were cross-reactive. Presumably, a cross reactive organism would be related to x-bacteria, and such information might shed light on the nature of the ancestral type of bacteria from which x-bacteria were derived. The symbiotic association between x-bacteria and xD amoebae began in a laboratory culture of D amoebae. Thus the ancestral symbiont must have been present in the culture prior to the association.

A total of nine organisms, all of which were similar to x-bacteria in Gram-stain, size, and morphology, were tested for cross-reactivity by the IFA method. None of these organisms, when treated with immune serum prepared against x-bacteria, reacted with the labeled sheep anti-rabbit IgG. One organism did give a positive test initially, but this result could not be repeated on a second trial.

One organism was found to be associated only with xD amoeba cultures. This organism was identified as Xanthomonas. When tested for immunological cross-reactivity with x-bacteria by the IFA test, the results were negative.

Apparently none of the free-living bacteria present in cultures of D or xD amoebae were immunologically related to x-bacteria as determined by the indirect fluorescent antibody test.

B. In Vitro Culture.

All media used for attempted in vitro culture of x-bacteria are listed in Table 4 (p. 40). None supported the growth of x-bacteria. To check the possibility that x-bacteria might require low oxygen tensions for growth, one set of 1% amoeba lysate plates was incubated under nitrogen, also with negative results. In the case of liquid media, a sufficient volume was provided to insure that anaerobic conditions prevailed near the bottom of the culture. Growth was never observed in this region of the tube.

All attempts to culture x-bacteria were made at 20° C. xD amoebae grew at 20° C under routine laboratory conditions. Although x-bacteria seem to have some psychrophile-like characteristics (35), there was no reason to suspect that they would grow in vitro at some temperature lower than 20° C. Incubation temperature was, therefore, not a variable in any of the in vitro culture attempts.

C. Microflora of Amoeba Cultures.

Information about three aspects of the microbial flora of D and xD cultures was obtained. The number of

bacteria present in D and xD dishes was estimated. The types of bacteria present in each dish were compared. Bacteria remaining in association with D and xD amoeba cells after repeated washings were isolated and characterized.

Estimates of the number of bacteria/ml in amoeba cultures were obtained by plate counts. The number of bacterial colonies per plate is a function of :

1) the number of viable cells present in the inoculum

2) the ability of the culture medium used to support the growth of the organisms under study, and

3) the kinds of bacteria present in the sample. Tryptic soy agar supplemented with yeast extract is adequate for the culture of most free-living Gram-negative rods. Because of their similarity to x-bacteria, such organisms were of primary interest in this study. However, filamentous, appendaged, and other "atypical" bacteria, along with some yeasts and molds, also grow on tryptic soy broth supplemented with yeast extract.

Counts from mass cultures and Falcon dish cultures of both washed and unwashed D and xD amoebae are reported in Table 7. Counts calculated for individual, washed amoebae are also reported. No difference was found in the number of organisms present in cultures of the two strains.

Each mass culture contains about 500 ml of medium, and yields, on the average, 1.0 ml packed amoebae. One ml

TABLE 7

PLATE COUNT ESTIMATES OF NUMBER OF BACTERIA IN AMOEBA CULTURES

SOURCE OF INNOCULUM/TREATMENT	BACTERIA/ml xD	D
Mass culture/unwashed	1.35×10^6	1.7×10^6
Falcon dish/unwashed	1.4×10^6	1.6×10^6
Falcon dish/washed 1X	3.19×10^2	3.88×10^2
Single cells/washed 5X[1]	3	3

[1]Counts reported as bacteria/cell

packed amoebae contains about 3.8×10^5 cells. From the information in Table 7, the percentage of the total bacterial population which is associated with amoebae could be calculated. The total number of bacteria in an average mass culture of amoebae was thus approximately 7.5×10^7. The number of bacteria associated with amoebae was 1.14×10^6, or about 1.5%.

Approximately 98.5% of free-living bacteria were removed from amoebae by washing five times in sterile Chalkley's solution. The remainder were located on, or within, the amoebae themselves.

Thus, part of the bacterial population in an amoeba culture was associated with amoeba cells, rather than dispersed in the surrounding medium. This finding agreed with that of other workers (1, 45).

To determine if different bacteria were associated with different strains of amoebae, cells of each amoeba strain were washed five times in sterile Chalkley's solution and returned to culture in medium which had previously been used for culture of the opposite strain. After three weeks of routine care, medium from these "switched-medium" cultures was plated on TSA + YE. Bacteria thus isolated were compared with isolates from control amoeba cultures. Control cultures contained amoebae which had been washed, but were returned to their original medium, i.e., "unswitched medium." The results

this experiment, reported in Table 8, showed that one organism, Xanthomonas, was found in xD cultures, but not in D cultures. This bacterial species was associated with xD cells, as it was isolated only from cultures in which xD cells, and not xD medium alone, were present.

When medium and amoebae from routinely maintained D and xD cultures were plated on TSA + YE, Xanthomonas was again isolated only from xD cultures. In one experiment, another organism, Pseudomonas fluorescens was isolated from xD cultures only. This was the only isolate of P. fluorescens obtained in the study. Since P. fluorescens is a common inhabitant of soil and water environments (30), its presence in xD cultures is not considered significant.

Organisms isolated from D cultures, xD cultures, or both, are listed in Table 9. The tests which were performed to characterize the isolated bacteria and the responses of the Gram-negative rods tested are shown in Table 10. A brief description of other organisms isolated is included in this table.

The finding that Xanthomonas was consistently present in xD amoeba cultures and could not be removed from the amoebae by repeated washing led me to seek the reason for these observations. One possibility which was considered was that xD amoebae, but not D amoebae, produced some factor required by Xanthomonas for growth. To check this possibility, a mineral medium with acetate as the sole carbon source (7) was prepared. Lysate from

TABLE 8

ORGANISMS ISOLATED FROM "SWITCHED-MEDIUM" CULTURES

xD CELLS/D MEDIUM	D CELLS/xD MEDIUM	xD	D
Xanthomonas	Pseudomonas sp. 1	Xanthomonas	Pseudomonas sp. 1
Pseudomonas sp. 1	Acinetobacter	Pseudomonas sp. 1	Pseudomonas sp. 2
Pseudomonas sp. 3		Pseudomonas sp. 3	Organism A
		Acinetobacter	Organism B
		Organism B	

TABLE 9

DISTRIBUTION OF BACTERIAL ISOLATES FROM AMOEBA CULTURES

D CULTURES ONLY	xD CULTURES ONLY	BOTH STRAINS
Hyphomicrobium	*Xanthomonas* sp.	*Pseudomonas diminuta*
Organism A	*P. fluorscens*	*Pseudomonas* sp. 1
Organism F		*Pseudomonas* sp. 2
Organism H		*Pseudomonas* sp. 3
Organism I		*Acinetobacter* sp.
		Organism B
		Organism E

TABLE 10

ORGANISMS ISOLATED FROM AMOEBA CULTURES

NAME	LAC	PIGMENT	OXIDASE	GLUCOSE	ACETATE	4°C	41°C
Gram-negative rods:							
Pseudomonas fluorescens	-	fluorescent	+	-	+	+	-
P. diminuta	-	-	+	-	-	+	-
Pseudomonas sp. 1	N.D.[1]	-	+	-	+	+	-
Pseudomonas sp. 2	N.D.	yellow	+	-	+	+	-
Pseudomonas sp. 3	N.D.	-	+[2]	-	+	+	-
Xanthomonas	N.D.	yellow	-[2]	-	-	-	-
Acinetobacter calcoaceticus	-	-	-	-	+	+	-

TABLE 10 (continued)

NAME	LAC	PIGMENT	OXIDASE	GLUCOSE	ACETATE	4°C	41°C
Other Organisms:[3]							
Hyphomicrobium	Gram-negative filaments with bulbous ends, some branches						
A	Gram-positive prosthecate organism forming white colonies						
B	Yeast						
E	Gram-negative slender rods, often forming filaments						
F	Gram-negative large rods with vacuolated appearance						
H	Coccoid to rod-shaped Gram-negative cells						
I	Gram-negative curved rods						
J	Mold						

1 Not done.

2 One isolate weakly positive.

3 Biochemical tests not performed on these isolates.

D amoebae was added to a portion of the acetate medium. xD lysate was added to another portion. A third portion of the acetate medium received neither lysate. Five tubes of each preparation were inoculated with Xanthomonas and incubated. It was found that Xanthomonas grew well in media supplemented with amoeba lysate regardless of the strain of amoeba from which the lysate was prepared. A summary of these results is given in Table 11.

An interesting finding in this study was that several more types of "atypical" bacteria (those other than Gram-negative rods) were isolated from D cultures than from xD cultures. Other than simply noting their presence, no work was done with these atypical isolates.

Neither fixed nor unfixed x-bacteria were agglutinated by any of the three lectins at the concentrations tested. In the Con A test, all wells (except control) contained an amorphous white substance that, upon examination under phase contrast, was seen not to be composed of the bacteria, which remained free in the surrounding medium. Fixed cells did not exhibit this reaction.

The presence of cytochrome oxidase in x-bacteria could not be demonstrated. No color chance indicating a positive reaction was obtained, either with oxidase test strips or p-aminodimethyl-aniline oxalate (DMA). Approximately 10^7 x-bacteria were used for each test. The concentration of cytochrome oxidase, if present, may not have

TABLE 11

ABILITY OF *Xanthomonas* TO GROW ON MEDIA SUPPLEMENTED WITH AMOEBA LYSATES

MEDIUM	GROWTH
Acetate medium, unsupplemented	-
Acetate medium + 10% xD lysate	+
Acetate medium + 10% D lysate	+

been sufficient to produce an observable color change. However, x-bacteria were considered to be oxidase negative.

D. Morphology of Endosymbionts.

Dark (D) and light (L) bacteria are seen in xD vesicles when sectioned amoebae are studied by electron microscopy (1, 38). Mean length of dark cells was found to be 0.96 ± 0.32 (S.D.) μm. Light cells were found to have a mean length of 1.32 ± 0.53 (S.D.) μm. Analysis of variance calculations revealed these means to be significantly different ($P = 0.95$). The results are summarized in Table 12.

TABLE 12

COMPARISON OF MEAN LENGTHS OF BACTERIA OF DIFFERENT ELECTRON DENSITIES IN xD VESICLES

LENGTH IN MICROMETERS (MEAN ± S.D.)	
DARK CELLS	LIGHT CELLS
0.96 ± 0/32	1.32 ± 0.53

ANALYSIS OF VARIANCE DATA:

	Degrees of Freedom	Sum of Squares	Mean Square	F
BETWEEN GROUPS	1	2.7	2.7	12.49*
AMONG GROUPS	80	17.3	0.22	
TOTAL	81	20.0		

*Significant at the 0.05 level.

CHAPTER IV

DISCUSSION

In both rabbits and rats, x-bacteria failed to stimulate specific antibody production detectable by agglutination. This is surprising as each of these animals is known to respond well to bacterial antigens prepared and administered as in this study (23, 70).

Rabbits, for example, produce agglutinating antibody titers of 1:320 to 1:640 when immunized with Salmonella O antigen preparations. Results were obtained when 0.5, 1.0, 2.0, and 3.0 ml of a suspension of heat-treated Salmonella cells (10^9/ml) were administered intravenously or intraperitoneally at 4-day intervals. Rabbits were bled six days after the last injection.

Rats responded with a 1:430 titer of agglutinating antibody to a single intravenous injection of Salmonella antigen preparation administered at 4 ml/kg (23). The animals were bled six days after the injection.

It can be seen from Table 3 (p. 34) that 2×10^9 x-bacteria was the largest single dose of antigen administered to any rabbit. This is fewer than the 3×10^9 Salmonella administered in the experiments cited above (23). Perhaps x-bacteria were administered in too small a dose to elicit the production of agglutinating antibody in rabbits. Agglutinating antibody might be produced in rabbits by

using antigen preparations containing 10^{10} or more x-bacteria/ml.

A single mass culture of xD amoebae yielded approximately 8 x 10^9 purified x-bacteria when prepared by the method described earlier (p. 29). Crude preparations were found to contain consistently lower numbers of bacteria than those prepared in more purified form. Ten dishes of xD amoebae for each one used in this study would be required to achieve a 10-fold increase in each dose of antigen.

Rats failed to produce agglutinating antibody to x-bacteria administered at the same dosage which elicited a 1:430 titer of antibody to Salmonella (23).

All immune rabbit sera prepared did contain antibody to x-bacteria, as shown by IFA tests (see Table 5, p. 50). This observation, together with the results obtained from experiments with rats, leads to the suggestion that x-bacteria are less antigenic than Salmonella in these animals.

Antiserum to x-bacteria was found not to cross-react with any of the free-living Gram-negative rods tested by the IFA method. There are two possible explanations for this observation:

1) x-bacteria are not related to the organisms tested,

2) in the process of becoming symbiotic with xD amoebae, x-bacteria have undergone some modification of the

outer cell surface which has resulted in a change in antigenic determinants, or

3) the precursor to x-bacteria is no longer present in amoeba cultures.

A modification of the bacterial cell surface could explain not only the failure of the cross-reactivity tests, but also would account for the absence of sacharide ligands detectable by the lectin agglutination tests, the lesser antigenicity of x-bacteria when compared with Salmonella, and, perhaps, the ability of the symbiont to escape digestion within the amoeba cytoplasm. All these phenomena could be related to a cell surface change, possibly brought about by mutation, which enables the bacteria to survive and grow within the amoeba.

In a discussion of the symbiotic association of bacteria of the genus Rhizobium with leguminous plants, Nutman (48) has pointed out that the gene products of host and symbiont become functionally interdependent. Unique structures are produced, although the genetic apparatus of the two organisms are "separate, discreet, and probably immiscible."

Likewise, some interaction between x-bacteria and xD amoebae may have rendered the bacterial cell surface so different from that of its ancestors that immunological cross-reactivity is not observed.

Glucosamine and galactosamine, as well as their acetylated derivatives, are widespread in bacterial lipopolysaccharides (LPS) (71). The absence of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine from the x-bacteria cell wall, as determined by lectin agglutination tests, is further reason to suggest that the symbiont possesses an atypical cell wall.

Con A does not agglutinate x-bacteria. Thus, α-D-methyl-mannopyrannoside (MeMAN) is also absent from the symbiont's cell surface. An amorphous material present in the x-bacteria suspension was agglutinated by Con A. This observation could mean that MeMAN-containing material was dissociated from the bacteria during purification, or that traces of amoeba material containing MeMAN were present in the preparation. Cells fixed with glutaraldehyde did not give this reaction, perhaps because the fixative reacted with MeMAN molecules to make them unavailable for lectin binding. Thus, it cannot be conclusively stated that α-D-methyl-manno-pyranoside is absent from the x-bacteria cell surface.

On the basis of immunological studies and lectin agglutination tests, it seems plausible to assert that x-bacteria possess an atypical cell surface. That the bacteria are not digested by amoebae has been shown (36, 37). The ability to avoid digestion may be related to the atypical structure of the x-bacterial cell wall; i.e.,

the bacteria ancestral to x-bacteria may have possessed the ability to avoid digestion by the amoeba, or may have acquired this ability through mutation. If this is what did, in fact, happen, a series of events can be postulated whereby the macro- and microsymbionts became mutually dependent.

The LPS of the bacterial cell wall is responsible for the O-antigen specificity of Salmonella and other bacteria (71). The LPS is the primary component of the cell envelope and has the structure diagrammed in Figure 4. Changes in the saccharide composition of the O-specific side chain results in changes in immunological specificity and lectin binding.

I shall call a bacterium which has acquired the ability to escape digestion by amoebae a "potential microsymbiont." The entry of a potential microsymbiont into an amoeba by phagocytosis would constitute the first event in the development of the endosymbiosis. Not being digested, the potential microsymbiont would be in an environment rich in nutrients as a result of the amoeba's metabolic activities. It would multiply at the expense of the amoeba as a typical parasite might do.

Present cultures of xD amoebae are derived from clones established and maintained in Dr. Jeon's laboratory for over 10 years. Many hundreds of generations of xD amoebae have been cultured since the initial infection, and only viable clones containing bacteria have been maintained. Selection pressures for the establishment of a stable



Figure 4. Diagram of a typical bacterial lipopolysaccharide. Adapted from S.T. Wilkinson (1977) Composition and structure of bacterial lipopolysaccharides. In I.W. Sutherland (1977) Surface Carbohydrates of the Prokaryotic Cell. Academic Press, New York. 472 pp.

endosymbiosis have thus been artificially enhanced. Under natural conditions, the potential microsymbiont while able to avoid digestion by the amoebae, might have been unable to form a stable association. Amoebae would have died from the infection before enough generations had passed for the necessary regulatory mechanisms to become operative.

The inability to culture symbionts free from living amoeba cells, together with the observation that the symbionts originally multiplied within the amoebae, even to the point of killing the protozoan, suggests that amoebae are producing some growth factor required by the symbionts for survival. The symbiont population could be regulated by the amoeba through control of the synthesis of such a growth factor, although recent work indicates that amoebae may in some degree control the bacterial population by extrusion of bacterial vesicles to the external environment via exocytosis (1). Possibly both mechanisms of control are operative.

xD nuclei are dependent on the presence of the symbionts for proper functioning (32, 37). The mechanism of this dependence is unknown (36).

It is plausible that the symbionts may serve as "processing centers" for some substance which the amoeba requires, but, owing to a loss of some critical enzyme, is unable to synthesize. Consider a metabolic pathway in the amoeba which proceeds from some substrate,

A, through a series a intermediate compounds, B, C, and D, to a product, E. A simple mutation could result in the loss of the ability to convert, say, B to C, without affecting the rest of the system. I shall refer to an amoeba with such a mutation as a "potential macrosymbiont." A potential macrosymbiont might obtain the intermediate C from ingested bacteria until a potential microsymbiont was phagocytosed.

Potential microsymbionts would deplete the supply of intermediate B, diverting it to their own needs and multiplying at the expense of the amoeba. However, with time and the selective influence of experimental manipulation, strains of amoebae able to regulate the growth of potential microsymbionts by controlling the supply of intermediate B could develop. In a process analogous to natural selection, amoebae able to survive with bacteria in their cytoplasm would predominate in the cultures. Those without this ability would die out. In this manner, clones of symbiotic xD amoebae could become established.

This hypothesis requires the accumulation of much more experimental evidence concerning the metabolic activities of both xD amoebae and x-bacteria. It does fit with presently available data about xD amoebae and x-bacteria, and with current knowledge concerning the metabolic activities of bacteria and amoebae in general (cf. 33, 30).

The hypothesis outlined above requires the assumption of the occurence of two mutations. One of these is assumed to account for the ability of x-bacteria to avoid digestion by xD amoebae. The other is assumed to explain a metabolic deficiency in amoebae which could have led to their dependence on the microsymbionts. The probability of two such random events (mutations) occurring so as to result in the symbiotic relationship is not as low as one might expect. In the well-studied symbiosis between bacteria of the genus Rhizobium and various leguminous plants, events which would seem unlikely occur with regularity (48). Only a few rhizobia enter a given plant and become nitrogen-fixing bacteroids associated with the root nodules. The association between bacteria and plant is species specific. Several enzymes and cofactors are required for successful entry of the bacteria into the root. Fixed nitrogen enters the plant metabolism and energy, in the form of photosynthate, is transferred from plant to bacteria. All this is associated with a complex of environmental factors such as carbon dioxide levels in the atmosphere; mineral, sunlight, and water availability; and biological factors such as the presence of fungi, insects, and other microsymbionts. Yet the process occurs again and again in a wide variety of natural and artificial situations.

None of the media tested would support the growth of the symbionts, including that of Soldo et al. (58), which

was used successfully (72) for the in vitro culture of symbionts of Paramecium aurelia.

This work extends that of Freesh (22) and supports her conclusion that x-bacteria may require contact with living amoebae for growth, since amoeba lysate should contain any growth factors which the symbionts might require.

The failure of the media tested to support the growth of x-bacteria is not surprising in view of the fact that, while numerous examples of symbiotic associations are known, few of the microsymbionts are readily cultured free from the macrosymbiont. Only 17 of 70 strains of Symbiodinium microadriaticum isolated from different host species can be grown axenically in a defined medium (54). Only two the the 10 or more types of symbiotic bacteria from P. aurelia have been cultured in vitro (72).

Intracellular bacteria from a number of species of insects and ticks were found difficult or impossible to culture in vitro (59). Actinomycete endosymbionts from species in five orders of non-leguminous plants cannot be cultured in vitro, but can be grown on cultured cells of the host plant (6). Attempts have not been made, to my knowledge, to culture the microsymbionts of Pelomyxa palustris, Myxotricha paradoxa, Crithidia oncopelti, or other symbiont-containing protozoa (cf. 27).

There are no obvious differences between xD and D amoebae which would render lysate prepared from the latter

less effective in promoting the growth of x-bacteria, since D amoebae can be converted to xD upon infection with isolated symbionts (2). The observed failure of x-bacteria to grow in media containing amoeba lysate cannot, therefore, be due to the fact that D lysate was used instead of xD lysate.

The x-bacteria are typically surrounded by a matrix of fibrous material within the xD amoeba vesicles (37). Since this material is apparently produced by the amoebae (1), its presence may be required by the bacteria for growth. As yet, no method for providing this matrix in an in vitro system is available.

The presence of a matrix surrounding the x-bacteria within amoeba vesicles is interesting in view of the fact that this same phenomenon occurs in other cases of endosymbiosis. For example, actinomycetes in symbiosis with nonleguminous plants are contained in vesicles produced by the host and imbedded in a matrix of polysaccharide material (6).

As pointed out in Chapter I, environmental factors may be important in our understanding of the means by which endosymbioses develop under natural conditions. In the system under study, the environment of xD amoebae, and the D amoebae from which they were derived, is the laboratory culture dish. It is significant that only one genus of Gram-negative, rod-shaped bacteria, Xanthomonas, was found to be associated exclusively with symbiont-containing amoebae.

Xanthomonas is apparently contained inside the xD cell, as it is not removed by repeated washing of the amoebae and is not found in cultures of D amoebae grown in medium previously used to culture xD amoebae.

Xanthomonas grows equally well in mineral media supplemented with lysates from D or xD amoebae, but does not grow in unsupplemented mineral media. These bacteria are known to require glutamic acid, methionine, and nicotinic acid (30). These compounds are apparently present in both D and xD lysates.

Earlier in this discussion, it was suggested that x-bacteria may be able to avoid digestion by xD amoebae as a result of some unique feature of the LPS of their cell wall. In this connection, it is interesting to note that LPS from Xanthomonas is regarded as "most unusual" in composition, as compared to other Gram-negative rods (71). Xanthomonas produces cytochrome oxidase weakly, if at all (30). It is similar to x-bacteria in this respect.

An immunological relationship between Xanthomonas and x-bacteria could not be demonstrated by the techniques employed in this study. However, differences in the cell wall LPS O-specific side chains of Xanthomonas and x-bacteria could account for this observation. Such differences would not necessarily imply that the two organisms were of different genera, merely that they were of different strains.

Information is available on the composition of the Xanthomonas cell wall (73). Investigation of the structure of the x-bacteria cell wall, by means of appropriate lectin agglutination tests, would enable comparisons of the symbiont cell wall with that of Xanthomonas to be made.

The guanine plus cytosine (G + C) content of x-bacteria DNA should be determined. The percent G + C for many bacterial species is known. For Xanthomonas the reported value is 63.5-69.2 moles percent (30).

It cannot be concluded from available data that a Xanthomonas species was ancestral to x-bacteria. The evidence does suggest that the two may be related. That x-bacteria were derived from an organism very much like Xanthomonas is certainly possible, although further work will be needed before this possibility can be confirmed.

Statistical comparisons of light (L) and dark (D) bacteria indicate that the L and D cells differ significantly in length, L cells being longer. Both types of bacteria are seen in the process of cell division (T.I. Ahn, personal communication). It is felt, therefore, that D and L cells represent two different populations of bacteria, rather than two stages in the life cycle of one population. Both types of bacteria have been observed in xD amoebae since the endosymbiosis was first detected (36).

There are three possible interpretations for these observations:

1) D and L cells represent two strains of x-bacteria;

2) D and L are different species of bacteria, acquired by the amoebae at different times;

3) D and L are different species of bacteria, simultaneously acquired by xD amoebae.

Further work will be necessary before a choice between these three alternatives can be made. I favor the first choice.

Kappa symbionts of Paramecium aurelia are known to exist in "bright" and "nonbright" phases (49). Bright kappas are distinguished by the presence of refractile structures referred to as "R bodies." Nonbright kappas are infective and frequently seen in division. Bright kappas are not infective and are only easily seen in the dividing state. Furthermore, only bright kappas are associated with mate-killing activity (toxin production) by the Paramecium. These findings, along with evidence that phage-like structures are associated with the R bodies, has led to the conclusion that bright kappas are infected with a virus responsible for both the presence of R-bodies and toxin production. The recent finding that plasmid DNA is present in x-bacteria (J. Han, personal communication) suggests that the genetic makeup of this endosymbiotic system may be quite complex.

There is no method presently available for the separation of dark and light x-bacteria. Should such separation become possible, experiments to determine whether plasmid DNA is present in only one, or both, types of bacteria should be carried out. The ability of each type to infect D amoebae should also be studied.

One experiment which could be carried out with present techniques would be to determine the proportion of D and L bacteria present in experimentally infected D amoebae, as a function of the time elapsed from exposure of amoebae to the x-bacteria preparation. Such a study should reveal whether one type is derived from the other.

CHAPTER V

SUMMARY

Several conclusions can be drawn from the data obtained in this study.

Immunological techniques revealed that injections of x-bacteria stimulate the production of antibody in rabbits. Antibody can be detected by immunofluorescent methods, but not by agglutination. Agglutinating antibody was not detected in sera from rats immunized with x-bacteria. Thus, x-bacteria are considered less antigenic than Salmonella. The latter stimulate high antibody titers in both rabbits and rats when administered under conditions like those used in this study.

The x-bacteria cannot be cultured in vitro on any of the media tested. One of these media has been used successfully to cultivate symbiotic bacteria from Paramecium aurelia. It was concluded that x-bacteria require living amoebae for survival, as lysate from amoebae failed to support the growth of the symbionts. Intact amoeba vesicles might be sufficient for growth of x-bacteria in vitro. However, there is no available method of supplying such vesicles in an in vitro system.

Con A, wheatgerm agglutinin, and soybean agglutinin do not agglutinate x-bacteria. The specific saccharides with which these lectins react are thus considered to be absent from x-bacteria cell walls.

Cytochrome oxidase was not detected in x-bacteria preparations.

One organism, Xanthomonas, was found to be associated with xD amoebae, but not with D amoebae. Xanthomonas could not be removed from xD amoebae by repeated washing. Lysates from D and xD amoebae were both found to enable Xanthomonas to grow in a mineral medium supplemented with these lysates. The bacteria do not grow in unsupplemented mineral media.

No immunological relationship between Xanthomonas and x-bacteria could be found. However, strain differences could account for this observation. It was concluded that the ancestral bacteria from which x-bacteria were derived may have been quite similar to Xanthomonas.

Further work should be undertaken to determine if x-bacteria are similar to Xanthomonas in terms of cell wall composition and percent G + C in their DNA.

Bacteria of two different electron densities, as seen in xD amoeba sections observed by electron microscopy, were found to differ significantly in mean length. The dark and light bacteria may be two strains of one species, or may be two separate species, of x-bacteria. A choice between these alternatives cannot be made at this time.

Through this study, the characterization of symbiotic bacteria from the xD strain of Amoeba proteus

has been extended. It is hoped that our understanding of the nature of this system, and of the phenomenon of symbiosis in general has thus been enhanced.

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