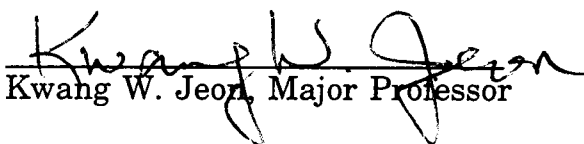
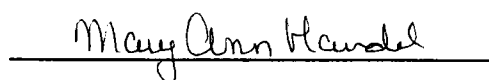


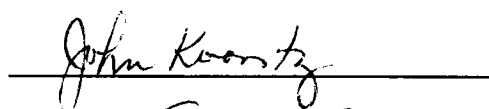
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I am submitting herewith a dissertation written by Keejun Kim entitled "Characterization of Lipopolysaccharides in Amoeba-Bacteria Symbiosis and Phylogenetic Studies of Bacterial Endosymbionts." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.


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We have read this dissertation
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Associate Vice Chancellor and
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**CHARACTERIZATION OF LIPOPOLYSACCHARIDES
IN AMOEBA-BACTERIA SYMBIOSIS
AND
PHYLOGENETIC STUDIES OF
BACTERIAL ENDOSYMBIONTS**

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

Kee Jun Kim
December 1996

ACKNOWLEDGEMENTS

I would like to express my appreciation to my major professor, Dr. Kwang W. Jeon for his encouragement and guidance. Without his support, patience and encouragement none of this would have been possible. I also thank my committee members, Dr. Mary Ann Handel, Dr. L. Evans Roth and Dr. John W. Koontz for their advice and many helpful suggestions. Special thanks are due to Dr. Anders Sonesson for helping me to analyze X-bacterial lipopolysaccharides while he was staying in United States.

I am grateful to Dr. Tae In Ahn, whose encouragement guided me to continue my graduate study in this laboratory, and to Dr. Eui Yul Choi, who helped me to initiate my study in this laboratory. I would also like to thank Sang Wook Oh, Siuk Yoo, Hong Jae Lee and Young Kim for their friendship.

I wish to express my gratitude to my family and my wife's family for their love, patience and support. Finally, I am most thankful to my wife, Yeon Seung Lee, for her love, sacrifice and encouragement and to my son, Taehon, who has always pleased me.

ABSTRACT

In the amoeba-bacteria symbiosis, unidentified Gram-negative bacterial endosymbionts, X-bacteria, reside within symbiosomes in the host cytoplasm and are known to avoid lysosome-symbiosome fusion for survival. However, the mechanism for the inhibition of lysosomal fusion is unknown, but several macromolecules were thought to be involved in the prevention of lysosomal fusion.

Among them, lipopolysaccharides (LPS) were characterized and their role was determined in this study. In order to characterize LPS of X-bacteria, the chemical composition was determined by using analytical methods. Eleven different kinds of sugars were identified in the polysaccharide part, including a rare sugar yersiniose A, which is found only in *Yersinia* sp. and *Legionella* sp. X-Bacterial LPS have a remarkably complex and distinct pattern of hydroxylated fatty acids, which is less common in that of Gram-negative bacteria. Glucosamine seems to be a sugar backbone connecting lipid A and polysaccharide parts.

For studying the role of LPS on symbiosome membranes, two kinds of monoclonal antibodies against X-bacterial LPS and lysosomal membrane proteins (LMP) were generated. Double immunostaining showed that some symbiosomes in xD amoebae microinjected with an anti-LPS antibody became stained with an anti-LMP antibody, implying that symbiosomes fused with lysosomes. Therefore LPS play a role in lysosome-symbiosome fusion prevention. LPS appeared on symbiosome membranes in newly infected D amoebae between 3 and 7 days after infection and reached a maximum level 21 days after infection. During a high-temperature treatment, the outer membranes containing LPS were released from X-bacteria 3 days after

treatment, and LPS were dispersed in symbiosomes by the 5th day. At the same time active vesicular fusions were observed around symbiosome membranes and X-bacteria were digested. LPS disappeared from symbiosomes 14 days after treatment. How LPS actively block lysosomal fusion and how LPS might be incorporated into symbiosome membranes are discussed.

Since the identity and origin of X-bacteria are unknown, phylogenetic studies were performed. The X-bacterial 16S ribosomal DNA was amplified and sequenced by a polymerase-chained reaction method. When compared with that of other bacteria, X-bacteria appeared to belong to the gamma subclass of *proteobacteria* and have the highest nucleotide-sequence identity (97.1%) with *Flavobacterium lutescens*, which is a member of *Pseudomonas* superfamily.

A novel 85-kDa protein was found in inclusions of symbiosomes. This protein was synthesized in X-bacteria and was present in inclusions of symbiosomes. 85-kDa protein began to appear in symbiosomes 3 days after infection and the amount of the protein was indistinguishable with that of established xD amoebae 21 days after infection. Interestingly, the appearance time of 85-kDa protein was the same as that of LPS. Therefore 85-kDa protein is thought to be involved in the establishment of symbiosis.

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CHAPTER I

INTRODUCTION

A. Background of Amoeba-Bacteria Symbiosis

In 1966, a large number of Gram-negative bacteria spontaneously infected the D strain of *Amoeba proteus* under laboratory conditions (Jeon and Lorch, 1967). Subsequently a symbiotic relationship between bacteria and amoebae became established within a few years (Jeon and Jeon, 1976), and has been investigated for nearly 30 years (Jeon, 1972, 1983, 1995a, 1995b).

The infecting bacteria were initially so virulent that infected amoebae showed harmful effects such as reduced cell size, decreased number of crystals, increased fragility, increased sensitivity to starvation and over-feeding, reduced growth rate, and poor ability of clone formation. However, the infecting bacteria appeared to have become less virulent in that the initially observed adverse effects on their host diminished (Jeon, 1983). Five years after the initial infection, infecting bacteria were transformed from harmful pathogens to indispensable symbionts and became required cell components (Jeon, 1972).

The symbiotic bacteria were designated as X-bacteria since their origin and identity were unknown, and the bacteria-bearing amoebae have been called the xD strain, compared to the original bacteria-free D strain (Jeon and Lorch, 1967). Currently, xD amoebae grow normally and harbor an average number of 42,000 symbionts per amoeba (Ahn and Jeon, 1979).

X-Bacteria are rod shaped and Gram-negative. They are enclosed in membrane-bounded vesicles of varying sizes called symbiosomes (Jeon, 1983; Roth et al., 1988). They are known to synthesize several macromolecules,

which are thought to be involved in the amoeba-bacteria symbiosis. A free-living counterpart of the X-bacteria has not been identified, but through the comparison of nucleotide sequences of the *groELx* gene, X-bacteria are considered to be most closely related to *Legionella* sp. (Ahn et al., 1991; 1994). It is worthy to note that amino acid sequences of heat-shock proteins (GroE homologues) were also used recently for classification of eubacteria (Viale and Arakaki, 1994). Besides X-bacteria, symbiosomes contain unknown inclusions as visualized by electron microscopic observation. Their role is unknown but they have fibrous structures and their size depends upon that of the symbiosomes.

The many characteristics of established xD amoebae and X-bacteria showed a mutual dependence between the two partners for survival (Jeon, 1972). First, X-bacteria grew normally inside their host xD amoebae, but they did not grow *in vitro*. Every attempt to culture them was unsuccessful, including the charcoal-yeast extract agar used for growing *Legionella* sp., which was suspected of being closely related to X-bacteria (Jeon, 1983). Second, when nuclei of xD amoebae were transferred into the cytoplasm of bacteria-free D amoebae, over 90% of the resulting cells were not viable and could be resuscitated only by microinjection of live X-bacteria into them (Jeon, 1972; Jeon and Jeon, 1976). This result suggested that the nuclei of xD amoebae had changed irreversibly under the influence of symbionts resulting in incompatibility with D amoeba cytoplasm (Lorch and Jeon, 1982). Third, incubation of xD amoebae at an elevated culture temperature of 26° C for 7 days made X-bacteria completely disappear and a sharp decrease in the amoeba population followed (Jeon and Ahn, 1978). This finding showed the dependence of host on symbionts. Additionally, the use of chloramphenicol (Jeon and Hah, 1977) and trimethoprim (Jeon, 1977) to remove X-bacteria selectively from xD amoebae

caused a decrease in the number of X-bacteria per amoeba and death of all host amoebae after the removal of all bacteria. On the basis of the above evidence, the relationship between xD amoeba and X-bacteria is considered to be an obligatory symbiosis (Jeon, 1983).

The D strain of amoebae can be induced to establish symbiosis with X-bacteria isolated from xD amoebae under laboratory conditions (Jeon and Ahn, 1979). Since amoebae are very phagocytic, the conventional phagocytosis is thought to be the mode of infection. Co-culturing of D and xD amoebae in the same dish causes most of amoebae in the dish to become xD amoebae containing X-bacteria. However, this method is less effective and takes a much longer period of time than induced phagocytosis. Other means known to be utilized by pathogens, e.g. active penetration into the host cells by making a hole in the host membranes (Moulder, 1985) or pathogen-facilitated phagocytosis shown in *Toxoplasma gondii* system (Sibley, 1995) have not been reported so far in amoeba-bacteria symbiosis. In addition, a protein factor that promotes phagocytosis such as an invasin of the enteropathogenic *Yersinia* sp. (Pepe, 1993) has not been found.

Induced phagocytosis of X-bacteria by D amoebae and microinjection of X-bacteria into the cytoplasm of D amoebae have been used for the experimental infection of amoebae. After isolated X-bacteria were coated with poly-L-lysine and phagocytosed by D amoebae, X-Bacteria were first found in phagosomes, which fused with the host lysosomes within a few minutes. Most of X-bacteria in phagolysosomes were then digested but some of them survived and became segregated in separated vesicles in which they multiplied (Ahn and Jeon, 1979). After segregation, vesicles containing X-bacteria prevented lysosomal fusion like those in established xD amoebae. The number of X-bacteria in infected D amoebae increased from the third day after infection and

reached that of symbionts in established xD amoebae within 3 weeks (Ahn and Jeon, 1979). When X-bacteria were introduced into the cytoplasm of bacteria-free D amoebae by microinjection, they became enclosed by newly formed vesicle membranes within a few hours, and such vesicles fused with lysosomes quickly. Some of the injected X-bacteria survived and then multiplied within newly formed vesicles (Jeon and Lorch, 1982). Therefore, all of the X-bacteria residing in amoebae are enclosed in symbiosomes and prevent lysosomal fusion with symbiosomes that surround them.

It is interesting to note that microbes surviving in other organisms such as *Chlorella* sp. and *Legionella* sp. are killed and digested in phagolysosomes of amoebae even though they have been known to survive inside the cells of green hydra and *Acanthamoeba* or macrophages, respectively (Jeon, 1994). Thus, it is reasonable to suspect that X-bacteria may produce factors rendering them able to survive in phagolysosomes, though only a few survived, and in the symbiosomes.

In order to survive in host cells after entry by exploiting the endocytic pathway, several species of intracellular symbiotic or pathogenic microorganisms avoid destruction by host cells (Moulder, 1985). The survival mechanisms utilized by them were classified in three categories. The first is to become resistant to lysosomal enzymes and reside inside phagolysosomes. *Coxiella burnetii* (Hackstard and Williams, 1981), *Leishmania donovani* (Chang and Dwyer, 1976; El-On et al., 1980) are known to live in phagolysosomes, which are acidified to below pH 5.0 through the vesicular proton-ATPase complexes and contain the late endosomal and lysosomal marker LAMP1 (Sturgill-Kszycki et al., 1994). The second is to escape from phagosomes to the cytosol of host cells. *Shigella flexneri* (Sansonetti et al., 1986), *Listeria monocytogenes* (Bielecki et al., 1990), and *Trypanosoma cruzi*

(Andrews, 1995) can be included in this category. *T. cruzi* were found to be free in the cytosol between 15 min and 2 hr after the invasion by disrupting the surrounding membranes with a secreted protein having a pore-forming activity at low pH (Andrews, 1995). The last is to modify surrounding vacuole membranes to prevent acidification of vacuoles and/or fusion with host lysosomes. Organisms such as *Mycobacterium* sp. (Armstrong and Hart, 1971; Sturgill-Kszycki et al., 1994), *Legionella pneumophila* (Horwitz, 1983), and *Toxoplasma gondii* (Jones and Hirsch, 1972; Joiner et al, 1990) have been known to use this kind of strategy. In the case of *Toxoplasma* infection, a prominent membranous network is formed around parasites within the surrounding vacuole, involving redistribution of the outer membrane proteins of the *Toxoplasma* cell (Sibley and Krahenbuhl, 1988).

During experimental infection of amoebae, X-bacteria seem to utilize two strategies for their survival; (1) resistance to lysosomal enzymes, and (2) modification of symbiosome membranes to avoid lysosome-symbiosome fusion. So far several macromolecules were identified and suspected to be involved in the survival of X-bacteria (Jeon, 1992).

About 10% of X-bacteria ingested by an amoeba avoid digestion in phagolysosomes (Jeon and Jeon, 1976; Ahn and Jeon, 1979; Han and Jeon, 1980). Isolated X-bacteria are also known to be resistant to lysing agents such as lysozyme and mild detergents (Han and Jeon, 1980). The reasons for their resistance are not known, but it has been suggested that the X-bacterial surface may contain resistant components encoded by chromosomal or plasmid genes. Two kinds of plasmid DNAs were found in X-bacteria, whose sizes are about 59 and 21 kb (Han and Jeon, 1980). Curing of these plasmids with ethidium bromide or acridine orange made X-bacteria susceptible to be

digested by amoebae and they failed to infect D amoebae (Han and Jeon, 1980). xD Amoebae treated with the curing agent maintain 60% of the average number of bacteria as that of untreated xD amoebae (Ahn and Ahn, 1987). These results suggest that plasmid DNAs are needed at least in the early stage for X-bacterial infection and their product(s) may change the composition of the bacterial surface, enabling X-bacteria to become resistant.

A 29-kDa protein (S29x) is synthesized by X-bacteria and exported to the amoeba cytoplasm continuously (Kim and Jeon, 1986). The S29x protein is one of the most abundant among symbiont-produced proteins, and was recently showed to be a peripheral protein (Jeon, 1995). Thus, it is suspected to be involved in the protection of X-bacteria, possibly in a role like that of the 27-kDa outer membrane protein (Com 1) found in *Coxiella burnetii* (Jeon, 1994; 1995b). However, the function and transporting mechanism carrying this protein into the host cytoplasm across bacterial membranes as well as symbiosome membranes have not been elucidated. The interesting property of the S29x protein is that it is transported efficiently across the symbiosome membrane without any known signal peptide or any distinct hydrophobic domain in the hydropathy plot. Moreover, the molecular mass of this protein is not altered during transport (Pak and Jeon, 1995).

Unlike phagosomes, symbiosomes containing X-bacteria do not fuse with host lysosomes even though both membranes originated from the same plasma membrane. This finding suggests that the symbiosome membrane must become different during its formation or maturation and that it may contain some component(s) that actively block the lysosomal fusion (Jeon, 1992a). Symbiont-produced macromolecules such as a 96-kDa protein and lipopolysaccharides (LPS) are suspected to be involved in lysosomal fusion prevention.

Indirect immunofluorescence staining using monoclonal antibodies (mAbs) against X-bacterial LPS revealed that the LPS were located on both the bacterial surfaces and symbiosome membranes (Choi and Jeon, 1992). This finding is a unique feature of the amoeba-bacteria symbiosis because there is no report showing the presence of pathogen-produced LPS on vesicle membranes surrounding them. In addition, microinjection of purified anti-LPS mAb into an individual xD amoeba showed that the antigenic determinant of X-bacterial LPS were presented on the cytoplasmic side of symbiosome membranes (Choi and Jeon, 1992). Together with the biological characteristics described later, the presence of LPS on symbiosome membranes, especially on the cytoplasmic side, strengthens the possibility that they are involved in preventing lysosome-symbiosome fusion. However, it is not known how the LPS are added to symbiosome membranes and how they might function to play a role in fusion blocking.

A 96-kDa protein is also produced by X-bacteria and is located on symbiosome membrane (Ahn et al., 1990). Due to its location on symbiosome membrane, this protein is also thought to be involved in fusion prevention like the LPS. Further studies are required in order to discern the role of this protein.

In addition to the macromolecules that are thought to play a role in lysosomal fusion prevention, a 67-kDa GroEL-like heat-shock protein (HSP) of X-bacteria, named GroELx, has been identified and is also suspected of being involved in the survival of bacteria (Choi et al., 1991; Ahn et al., 1994). The *groELx* gene shares a high degree of identity in nucleotide sequence with other *groEL* genes of intracellular pathogenic bacteria such as *Legionella* sp. and *Coxiella burnetii* (Ahn et al., 1994). This protein is believed to play the role of a molecular chaperone, such as enhancing export of symbiont-produced proteins and stabilizing proteins imported from the host cell cytoplasm (Jeon, 1995).

Additionally, the presence of two promoters, a consensus heat-shock promoter (P1) and a second strong promoter (P2), and a large amount of GroELx in X-bacteria are thought to be an adaptation by the endosymbiotic bacteria, enabling them to survive within a potentially hostile intracellular environment (Ahn et al., 1994; Jeon, 1995).

Three macromolecules synthesized by the host amoebae are also found in symbiosomes: actin, spectrin, and myosin (Jeon, 1992a). All of them were accumulated in/around the symbiosomes of xD amoebae. Spectrin was suggested to be involved in the stabilization of symbiosomes (Choi and Jeon, 1989). The role of the other two proteins, which are components of cytoskeleton, is not known. It is noteworthy that actin is accumulated inside symbiosomes as well as around symbiosome membranes and seemed to be associated to X-bacteria (Kim and Jeon, 1987). However, it is unknown how actin is accumulated inside symbiosomes.

B. Lipopolysaccharides

The so-called cell envelope of Gram-negative bacteria is a multilayer structure composed of an outer membrane lying external to a thin peptidoglycan layer. In comparison, a Gram-positive bacterium lacks an outer membrane, the cell wall consisting mainly of a thick peptidoglycan layer (Hammond et al., 1984). Chemically, the unique outer membrane of Gram-negative bacteria is chiefly composed of phospholipids, proteins, and LPS. The outer leaflet is covalently attached to the peptidoglycan by lipoprotein (Rietschel et al., 1988). LPS are located in the external layer of the outer membrane and are involved in a number of membrane functions.

The LPS from different Gram-negative bacteria share a common architecture: they all consist of a hydrophilic polysaccharide component covalently linked to a hydrophobic lipid moiety, lipid A. The polysaccharide component of enterobacterial LPS, and also of LPS from species of some non-enterobacterial families, comprises two distinct structural regions, the O-specific chain and the core oligosaccharide (Hitchcock et al. 1986). In general, O-specific chains are made up of repeating oligosaccharide units that contain anything from one to seven sugar residues forming chains of different lengths. Monosaccharides of neutral, amino, and acidic nature have been found to be constituents of the oligosaccharide units (Kenne and Lindberg, 1983). The number of sugars present varies, even within a genus or species, as do their sequences and types of linkages (Wilkinson, 1977). Even when isolated from a single culture, all LPS show considerable heterogeneity regarding the lengths of their individual O-chains. LPS with O-specific chains are termed smooth(S)-LPS, owing to the colonial morphology of the strain on a nutrient-rich solid medium. Mutants and several non-enterobacterial species may lack the O-specific chain: such LPS are known as rough(R)-LPS (Sonneson, 1992).

The core structures of LPS are more uniform. In enterobacterial LPS, the core region consists of a heterooligosaccharide which formally can be subdivided into an outer core and an inner core. The outer core usually contains D-glucose, D-galactose and N-acetyl-D-glucosamine, whereas the inner core is usually composed of characteristic and LPS-specific components, L-glycero-D-manno-heptose and 2-keto-3-deoxyoctonic acid (KDO). These residues are substituted by charged groups such as phosphate, pyrophosphate and phosphorylethanolamine. The terminal KDO residue is always alpha-ketosidically bound to the lipid A disaccharide backbone (Rietschel et al., 1988).

KDO appears to be indispensable and is probably present as a hinge in all cores.

The ketosidic linkage between the terminal KDO unit of the core and lipid A is acid labile. Hence, lipid A can be easily cleaved off under mild conditions. The lipid A moiety is by far the most conservative part of the LPS molecule. The primary structure of enterobacterial and some non-enterobacterial lipid A consists of a beta-1,6-linked D-GlcN disaccharide carrying two phosphoryl groups, one in position 4' and one in position 1 (Mayer et al., 1989). The hydrophilic lipid A backbone carries generally 4 moles of 3-hydroxy fatty acids, two amide-linked and two ester-linked. These hydroxy fatty acids may carry additional ester-linked non-hydroxylated and 2-hydroxylated fatty acids forming acyloxyacyl residues (Rietschel et al., 1984). In free lipid A the positions 4 and 6' are non-substituted, the latter hydroxyl group constituting the attachment site for the ketosidic linkage of the KDO terminal group of the polysaccharide (Rietschel et al., 1988). Lipid A structures deviating from those in enterobacterial LPS have also been characterized. The following structural variants have been observed: (1) substitution by additional sugar constituents glycosidically linked to the backbone disaccharide at position 4 or 4'; (2) the amide-linked fatty acids are of 3-oxo fatty acid type; (3) the GlcN disaccharide backbone is non-phosphorylated; and (4) 2,3-diamino-2,3-dideoxy-D-Glc substitute the GlcN disaccharide in the lipid A backbone (Weckesser and Mayer, 1988).

C. Phylogenetic Study of The Endosymbionts, X-Bacteria

The classification of organisms has been traditionally based on similarities in their morphological, developmental, and nutritional characteri-

stics. It is, however, now clear that, with microorganisms, classification based on these criteria does not necessarily correlate well with natural relationships (Lane et al., 1985). Thus, a much broader application of molecular phylogenetic analysis in the description of microbes, both eukaryotic and prokaryotic, has been used (Lane et al., 1985).

Of the macromolecules used for phylogenetic analysis, the comparison of ribosomal RNA sequences, particularly 16S rRNA, has proven to be most useful for deducing phylogenetic and evolutionary relationships among bacteria, archaeobacteria, and eukaryotic organisms because of their high information content, conservative nature, and universal distribution (Woese, 1987). The accuracy of phylogenetic inferences from rRNA sequences depends upon the number of bases compared, and at least 1,000 bases should be considered for each organism to be fully effective (Murray et al., 1990).

The sequences have been derived by methods including sequencing of DNA clones, direct sequencing of RNA by using reverse transcriptase (Lane et al., 1985), and sequencing of material amplified by polymerase chain reaction (PCR) (Embley, 1991).

D. Objectives

In a previous study on the symbiotic relationship between *Amoeba proteus* and bacterial endosymbionts, X-bacteria, symbiosomes containing X-bacteria were found not to fuse with host lysosomes. Although several macromolecules synthesized by X-bacteria or derived from the host cells have been identified and thought to be involved in the host-symbiont interaction, the mechanism for the inhibition of lysosomal fusion and the components involved in the prevention of fusion are not known. Among those macromolecules, LPS

are the most probable candidate for the survival of endosymbionts in amoebae in terms of the inhibition of lysosome-symbiosome fusion due to the biological activities and their location both on the surface of X-bacteria and on the cytoplasmic side of symbiosome membranes. Thus whether the LPS from X-bacteria function as a fusion-blocking factor or not was determined in this study. Additionally the components of X-bacterial LPS were determined since the anti-LPS mAb did not show cross-reactivity with other Gram-negative bacteria in a previous study (Choi and Jeon, 1991; 1992). To determine the chemical composition, sugars in the polysaccharide part and fatty acids and sugars in the lipid A part of X-bacterial LPS were determined by gas chromatography and gas chromatography-mass spectroscopy (GC-MS), and the results were compared with those of LPS from other bacteria.

To determine whether LPS on the symbiosome membranes play a role in the prevention of lysosomal fusion, two kinds of monoclonal antibodies (mAbs) specific for X-bacterial LPS and for lysosome membrane proteins (LMP) were produced, and used for double-staining experiment. xD Amoebae were microinjected with an anti-LPS mAb, and then the injected amoebae were stained with an anti-LMP mAb to detect if symbiosomes in the injected amoebae fused with lysosomes.

It has been known that X-bacteria begin to grow from 3 days after infection and reach the number of X-bacteria in established xD amoebae within 3 weeks after infection. If LPS play a role in the survival of X-bacteria, the appearance time of LPS on symbiosome membrane should be consistent with the growth rate of X-bacteria after infection. Hence, it was also investigated to determine the time LPS appeared on symbiosome membranes after infection. In addition, since xD amoebae allow the fusion between symbiosomes and

lysosomes at high temperature, the relationship between the fate of X-bacteria and LPS during high temperature incubation was investigated.

So far, the identity of X-bacteria is unknown even though some bacteria such as *Legionella* sp. and *Coxiella* sp. were suggested by the comparison of nucleotide sequences of GroE homologue. Since the sequence of the 16S ribosomal DNA and the chemical composition of LPS have been used in taxonomic studies of microorganisms, the 16S ribosomal DNA of X-bacteria was amplified and sequenced to find closely related bacteria with X-bacteria. Besides the 16S rDNA sequence, the chemical composition of LPS was also used to obtain information about the identity of X-bacteria.

Besides LPS on symbiosome membranes thought to block lysosomal fusion, a protein component(s) derived from X-bacteria, such as a 25-kDa protein from *Mycobacterium* sp., may be involved in the prevention of lysosomal fusion. To find a new bacterial protein, which may play a role in the prevention of lysosomal fusion, whole symbiosomes were used as an immunogen for the production of mAbs. Using the mAbs as probes, the protein in the inclusions of symbiosomes was identified, and its location was confirmed by confocal microscopy and a double-staining experiment with an anti-LPS mAb. The possible function of this protein in symbiosis was therefore studied.

CHAPTER II

MATERIALS AND METHODS

A. Materials

For gel electrophoresis, acrylamide, bis-acrylamide, and SDS were purchased from United States Biochemical Co (USB, Cleveland, OH). Ammonium persulfate and NNN'N'-tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Laboratories (Richmond, VA).

For the production of mAbs, iron-supplemented calf sera were obtained from Hyclone (Logan, UT), polyethylene glycol 1500 from Sigma Chemical Co. (St. Louis, MO). Dubellco's Modified Eagle's medium, hypoxanthine-aminopterin-thymidine (HAT) medium, and hypoxanthine-thymidine (HT) medium were bought from Flow Laboratories (McLean, VA). Freund's adjuvant and penicillin-streptomycin (10,000 units/ml) were purchased from Gibco-BRL (Grand Island, NY). Protein A-agarose beads for purifying antibodies from hybridoma culture fluids and NHS-Biotin for labeling isolated antibodies were bought from Pierce (Rockford, IL)

For indirect immunofluorescence microscopy, anti-mouse IgG secondary antibodies conjugated with HRP, FITC or Texas Red were obtained from Jackson Immuno Research (West Grove, PA). Paraformaldehyde was purchased from Aldrich Chemical (Milwaukee, WI). For electron microscopy, glutaraldehyde (50% solution) and sodium cacodylate were purchased from EM Science (Fort Washington, PA). Goat anti-mouse IgG antibodies conjugated with 10-nm gold particles were bought from Sigma Chemical Co (St. Louis, MO). Lowicryl K4M was purchased from Polysciences (Warrington, PA).

For the PCR amplification of 16S ribosomal DNA, Taq polymerase and dNTPs were obtained from Promega (Madison, WI). Agarose for DNA gel electrophoresis was purchased from FMC (Rockland, MD) and Centricon-10 and -30 microconcentrators from Amicon (Beverly, MA). For DNA sequencing, TAQuence sequencing kit and urea were purchased from USB and [$\gamma^{32}\text{P}$]ATP from ICN (Costa Mesa, CA). For radioautography, XAR5 film, developer and fixer for the X-ray film were bought from Kodak Co. (Rochester, NY).

Buffer components and other inorganic chemicals were bought from Fisher Chemical Co. (Pittsburgh, PA) or Mallinckrodt Chemical Co. (St. Louis, MO).

B. Organisms

1. *Amoeba proteus*

Two strains of *Amoeba proteus* were used in this study, symbiont-free D strain and symbiont-bearing xD strain of amoebae. For mass culture of amoebae, cells were cultured in Pyrex baking dishes (34 x 22 x 4 cm) in a modified Chalkley's medium (Jeon and Jeon, 1975). Amoebae were fed *Tetrahymena pyriformis* for 2 hr every other day and then washed with fresh culture medium to prevent overfeeding of amoebae. The culture dishes were changed before feeding to remove molds and other contaminants which were harmful to amoebae.

For stock cultures of amoebae, *Colpidium* sp. were used as food organisms (Lorch and Danielli, 1953). A glass pie dish (15.2 cm in diameter) was half-filled with Chalkley's solution and was added five boiled wheat grains, *Colpidia* filtered through absorbent cotton layers, and about 300 amoebae. The cultures were renewed once a month.

2. *Tetrahymena pyriformis*

Tetrahymena were axenically cultured according to the method of Goldstein and Ko (1976) with modification. The culture medium contained 2% (w/v) proteose peptone, 0.2% liver extract, and 4% salts and vitamin mixture. *Tetrahymena* were harvested by centrifugation for 1 min at 320 g and washed twice with Chalkley's solution. After washing, the packed cells were resuspended and diluted 150 times with Chalkley's solution.

C. Subcellular Fractionation

1. X-Bacteria

X-Bacteria were isolated from xD amoebae by the method of Choi and Jeon (1992). Healthy xD amoebae were collected by centrifugation for 1 min at 170 g and washed twice with Chalkley's solution. xD Amoebae were then homogenized in 20-mM Tris-HCl (pH 7.4) containing 1 mM phenyl methyl-sulfonyl fluoride (PMSF), and centrifuged for 5 min at 170 g. The pellet fraction was suspended in the same buffer, homogenized again, and centrifuged for 5 min at 170 g. The first and second supernatants were combined and centrifuged for 5 min at 7,000 g. The pellet was resuspended in the same buffer, frozen and thawed once to break down any intact symbiosomes. The suspension was applied on top of a 30/50% Percoll step gradient. After centrifugation for 10 min at 10,000 g, pure X-bacteria were collected from the bottom of the gradient and washed twice with the homogenization buffer to remove Percoll. Its purity was checked under a phase-contrast microscope and isolated X-bacteria were stored at -70° C until used.

2. Symbiosomes

xD Amoebae were starved for 2 days and harvested by centrifugation for 1 min at 170 g. They were washed twice with Chalkley's solution by centrifugation. The packed cells in a graduated conical tube were added an equal amount of cold homogenizing solution containing 20-mM Tris-HCl (pH 7.4), 10 % sucrose, 2 µg/ml leupeptin, 1 mM PMSF, and 1 µg/ml pepstatin and broken by homogenization until 90% of cells were lysed as checked under a phase-contrast microscope. The cell homogenate was transferred into a 50-ml conical tube and centrifuged for 30 sec at 170 g to remove unbroken cells and large pieces of plasma membranes. The upper milky supernatant was loaded on top of a 10/20/30/40/50% (v/v) Percoll step gradient and centrifuged for 15 min at 7,000 g in a Beckman J-21C centrifuge using a JA-13 rotor. Pure symbiosomes were collected from the 30/40% interface and washed 3 times by centrifugation for 10 min at 15,000 g after being diluted 5 times with the homogenizing solution. The isolated symbiosomes were stored at -70° C until used.

3. Lysosomes

Three day-starved D amoebae were harvested by centrifugation for 1 min at 170 g and homogenized in the same solution used for the purification of symbiosomes. The lysate was filtered through a 45-µm pore-size nylon screen to remove unbroken cells and large pieces of plasma membranes. The filtrate was placed on top of 10-50% linear gradient of Percoll in a 50-ml tube and centrifuged for 20 min at 10,000 g in a Beckman J-21C centrifuge using a JA-13 rotor. From the top of the tube, 1-ml fractions were collected and used for a lysosomal marker enzyme assay. The fractions showing higher enzyme activities were combined together and washed three times by centrifugation for

10 min at 15,000 g after being diluted 5 times with the homogenizing solution.

4. X-bacterial lipopolysaccharides

The LPS were extracted from formaldehyde-inactivated X-bacteria with hot phenol-water by the method of Westphal et al. (1983) with a slight modification. Purified X-bacteria from 10 ml of packed xD amoebae were suspended in 0.5 ml of 10 mM Tris-HCl (pH 7.4), and 0.5 ml of 90% (v/v) phenol and water mixture was added at 68° C. The suspension was vigorously shaken for 20 min in a water bath at 68° C. After cooling and centrifugation for 5 min at 10,000 g, the aqueous phase was collected. The interphase and phenol phase were extracted twice with an equal volume of Tris-HCl buffer, and aqueous phases were pooled and dialyzed against distilled water for 3 days with daily change of distilled water. The crude phenol-water extract was centrifuged for 20 min at 10,000 g at 4° C. This low-speed supernatant was centrifuged again for 15 hr at 100,000 g. The precipitated LPS were suspended in distilled water and lyophilized. The lipid A fraction and the polysaccharides fraction from LPS were prepared by hydrolysis of LPS with 1% acetic acid for 1.5 hr at 100° C.

D. Marker Enzyme Assay

1. β -Hexosaminidase assay

The lysosomal enzyme marker, β -hexosaminidase, was assayed according to Lippincott-Schwartz and Fambrough (1986). After performing Percoll gradient centrifugation and collecting fractions, 100 μ l of each fraction was added to 0.3 ml of substrate solution containing 0.1 ml of 0.4-M sodium acetate (pH 4.4), 0.1 ml of 0.4% Triton X-100, and 0.1 ml of 5-mM 4-methyl-umbelliferyl- β -D-galactoside. After incubation for 30 min at 37° C, a solution

containing 2 ml of 0.5-M glycine and 0.5-M sodium carbonate (pH 10) was added to stop the reaction and the liberated 4-methyl-umbelliferon measured with a fluorometer at 365 nm for excitation and 450 nm for emission. Results were expressed in arbitrary units of fluorescence.

2. Succinate dehydrogenase assay

Succinate dehydrogenase, a mitochondrial marker enzyme, was assayed according to Choi et al. (1992). Each fraction from the Percoll gradient after centrifugation was mixed with 0.5 ml of a solution containing 50-mM potassium phosphate buffer (pH 7.4), 25-mM sucrose, and 0.1% 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride, and incubated for 15 min at 37° C. Then 0.5 ml of 10% trichloroacetic acid was added and the formazan was extracted with 2 ml of ethyl acetate. After 5 min, 1 ml of the extract from the top of each reaction was used to measure the absorbance at 490 nm.

E. Production of Monoclonal Antibodies

There are numerous steps involved in mAb production, and each procedure is discussed under separate headings. All media were stored at 4° C and pre-warmed to 37° C before using unless described otherwise.

1. Media preparation

a. Incomplete Dulbecco's modified Eagles medium

To make 1 L of incomplete DMEM, one package of powdered medium (Flow Laboratory) was dissolved in 900 ml of distilled water. 3.7 g of sodium bicarbonate was added to the medium and then adjusted to pH 6.5 with 1 N

HCl. After being made up to 1L, the medium was sterilized by passage through a 0.45 μm -size filter-sterilizing unit (Corning). The pH of the medium was adjusted to 7.2 after filtration.

b. Complete Dulbecco's modified Eagles medium

To make 500 ml of complete DMEM, 100 ml of iron-supplemented newborn calf serum (Hyclone Laboratory) and 5 ml of penicillin-streptomycin solution (10,000 unit, Gibco-BRL) were added to 400 ml of incomplete DMEM.

c. Hypoxanthine-thymidine (HT) and hypoxanthine-aminopterin-thymidine (HAT) media

To make 500 ml of HT and HAT media, 100x concentrated HT and HAT stock solutions (Flow Laboratory) were added to 500 ml of complete DMEM to make HT and HAT media, respectively.

d. Cell freezing medium

Dimethyl sulfoxide (DMSO) was used to prevent crystal formation inside cells while freezing. To make 100 ml of cell freezing medium, 10 ml of DMSO was mixed with 90 ml of iron-supplemented calf serum.

2. Antigen preparation and injection

Purified X-bacteria or symbiosomes were fixed with 2 % glutaldehyde and 10 % sucrose in 20-mM Tris-HCl (pH 7.4) for 30 min at 4^o C . After being washed three times with PBS by centrifugation for 5 min at 5,000 g, fixed X-bacteria or symbiosomes were resuspended in PBS. For the first injection, 0.5 ml of the suspension was mixed with a same volume of Freund's complete

adjuvant by sonication and injected into each BALB/c mouse (6-8 weeks old, female) intraperitoneally. Three booster injections with Freund's incomplete adjuvant were followed at 3- to 4-week intervals. The final injection was given 3 or 4 days before the cell fusion.

Purified lysosomes were fixed with 2 % glutaldehyde in PBS for 15 min and injected as described for X-bacteria and symbiosomes.

3. Preparation of cells for fusion

a. Feeder layer cells

Since lymphoid cells do not grow well at a low density, mouse peritoneal cells were used as feeder cells. One day before the cell fusion, a mouse was killed by cervical dislocation and its abdominal skin was removed under surgically clean conditions. 5 ml of ice-cold, sterilized sucrose solution (11.6 %) was injected into the peritoneal cavity and retrieved by a syringe and a needle. The collected sucrose solution was centrifuged for 5 min at 650 g, and the pellet containing peritoneal cells was suspended in 60 ml of HAT medium. 0.5 ml of the suspension was placed into each well of five 24-well culture plates using a 10-ml pipette and kept in a CO₂ incubator at 37° C.

b. Spleen cells

A mouse injected with antigens was killed by cervical dislocation, and the spleen was removed under sterile conditions. The removed spleen was transferred into a Petri dish containing 10-ml of incomplete DMEM, and spleen cells were gently released into the medium by using fine forceps and collected in a sterilized 15-ml centrifuge tube. The tube was allowed to stand for 2 min, and 5 ml of the cell suspension from the top was transferred to another tube. After centrifugation for 3 min at 650 g, the pellet was resuspended in 50 ml of

incomplete DMEM. The cells were counted, centrifuged again, and then suspended in 20 ml of incomplete DMEM. About 3×10^7 of spleen cells were used for each fusion.

c. Myeloma cells

The SP 2/0-Ag-14 (abbreviated SP2) was used in this study. Five days before cell fusion, frozen SP2 cells were removed from a liquid nitrogen tank, thawed in a water bath at 37° C quickly and transferred to a 15-ml tube. Then 10 ml of complete DMEM were added over a period of 5 min with constant stirring. The tube was centrifuged for 3 min at 650 g, and the pellet was suspended in 10 ml of complete DMEM. The entire suspension was transferred into a culture flask (75 cm^2) and incubated at 37° C. The cells were renewed every other day. About 5×10^7 cells were used for each cell fusion.

4. Cell fusion

Among the many steps, this is the most important one for the production of monoclonal antibodies. In a 50-ml tube, 5×10^7 myeloma cells were mixed with 3×10^7 spleen cells and centrifuged for 3 min at 650 g. The pellet was washed once with incomplete DMEM by centrifugation. The supernatant was removed thoroughly since the final concentration of polyethylene glycol (PEG) was critical. The pellet was mixed by tapping the tube with fingers, and 1 ml of 50% PEG-1500 in incomplete DMEM was added slowly to the tube over a period of 1 min at 37° C with constant swirling. Then the tube was kept for another 90 sec at 37° C to complete the fusion reaction. To stop the fusion reaction, incomplete DMEM was added very carefully to the tube. For a period of 10 min, a total of 20 ml of incomplete DMEM was added with constant swirling. PEG was removed by centrifugation for 1 min at 650 g

and cells were carefully suspended in 20 ml of HAT medium. After another centrifugation, cells were resuspended in 120 ml of HAT medium and 1 ml of suspension was placed in each well of five 24-well culture plates containing the feeder layer cells. The plates were kept at 37° C in a CO₂ incubator (5 % CO₂ and 95 % air). Three days later, 1 ml of HAT medium was aspirated and 1 ml of HT medium was added to each well. Three days later, most of culture fluid was removed and replaced with new HT medium. Hybridoma colonies usually appeared 4 days after fusion and the cells were screened for antibody production 10-12 days after fusion. To screen the cells, several analytical methods such as indirect immunofluorescence microscopy and immunodot blotting were performed. Positive clones were transferred to 6-well culture plates, then to culture flasks (75 cm²) and frozen in liquid nitrogen for cloning.

For freezing, cells grown in a culture flask were centrifuged for 3 min at 650 g. After the supernatant was decanted, the pellet was suspended in 1 ml of freezing medium and transferred into a freezing vial (Wheaton). Then the vial was wrapped with 5 layers of tissue paper and put in a disposable styrofoam cup. The cup was covered with a small piece of styrofoam, kept at -70° C in a deep freezer for 2 hr, and then placed in a liquid nitrogen tank.

5. Cloning of hybridoma cells

As soon as positive wells were identified, hybridoma cells were cloned by limiting dilution. Frozen hybridoma cells were thawed and incubated with complete DMEM at 37° C. When they were in a log phase of growth, hybridoma cells were diluted with PBS and their number was determined by using a Neubauer cell counter. The numbers of cells were adjusted to 15 cells per ml by serial dilutions with complete DMEM and one drop of cell suspension was dispensed into each well of 96-well culture plates containing feeder cells

prepared 1 day before. Two more drops of complete DMEM were added to each well. The media were renewed every three days. After 4 days, the plates were examined with an inverted microscope and wells containing a single colony were selected. About one week later, cells in the selected wells were transferred to 24-well culture plates. Another 2 more days later, supernatants of 24-well culture plates were screened by immunofluorescence microscopy or immunodot blotting analysis. Positive clones were transferred again to 6-well culture plates and then to culture flasks (75 cm²).

6. Production of monoclonal antibodies

A large amount of mAbs can be produced either by culturing hybridoma cells in complete DMEM or by growing them as tumors. The concentration of antibody in the ascites fluid of a tumor-bearing mouse is much higher than that of an antibody in the supernatant of cell culture, and their concentrations are 10-30 mg/ml and 5-50 ng/ml, respectively.

To produce ascites fluids, BALB/c mice were injected with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane) to induce tumors. After one week, 1×10^7 cells were suspended in 0.5 ml of sterilized PBS and injected into a mouse intraperitoneally. One week later, when accumulation of ascites fluid resulted in the mouse's swollen abdomen, ascites fluid was removed from the mouse; the mouse was anesthetized with metofane for 3 min, a 18-gauge needle was inserted into the peritoneal cavity, and the dripping ascites fluid was collected in a 15-ml glass tube. The tube was centrifuged for 10 min at 7,000 g and the supernatant was dispensed into 0.5-ml Eppendorf tubes and stored at -70° C. Three or four more tapplings were carried out every 2-3 days.

F. Electrophoresis and Related Techniques

1. SDS-polyacrylamide gel electrophoresis

Laemmli's SDS polyacrylamide gel and buffer system (Laemmli, 1970) was used. The separating gel (10% acrylamide) in 0.375 M Tris-HCl buffer (pH 8.8) containing 0.1% SDS was polymerized chemically by the addition of 0.025% TEMED, and 1% ammonium persulfate between two plates. The stacking gel was 4% acrylamide gel in 0.125 M Tris-HCl buffer (pH 6.8) containing 0.1% SDS and was polymerized chemically in the same way as the separating gel. The sample was boiled for 2 min in a solution containing 0.125 M Tris-HCl (pH 6.8), 2% SDS, 0.5% sucrose, 5% β -mercaptoethanol and 0.0025% bromophenol blue to dissociate proteins. Samples containing 20-30 μ g proteins in 5 μ l were applied to each lane. The upper and lower tanks were filled with 0.025 M Tris-HCl buffer (pH 8.3) containing 0.1% SDS. Two gels were run for 2 hrs at constant 100 volts using Ortec 4100 pulsed constant power supply (Oak Ridge, TN) until the tracking dye was about 0.5 cm from the bottom of each gel.

For LPS, the same SDS polyacrylamide gel and buffer system were used except a 15% acrylamide gel was used for the separating gel. The gels were silver-stained to detect LPS.

2. Coomassie blue staining

In order to stain the protein gel, the CB staining method was used (Fairbanks et al., 1971). A 1% CB stock solution was prepared in distilled water and filtered through Whatman No. 1 filter paper. The gel was stained in a solution containing 0.125% CB, 50% methanol, and 10% acetic acid for 30 min. The gel was destained in solution I (50% methanol and 10% acetic acid) for

30 min and then in solution II (10% methanol and 5 % acetic acid) with several changes of the solution.

3. Silver staining

a. Silver staining for protein

A silver-staining method was used to stain proteins in a gel according to Merrill et al. (1981) with modification. The gel was fixed for 1 hr in 200 ml of destaining solution I described in CB staining and further incubated for 2 hrs in 200 ml of destaining solution II to remove excess SDS. The gel was placed in a solution containing 0.0034 M potassium dichromate and 0.0032 N nitric acid for 5 min, and washed 4 times for 30 sec each in 200 ml of distilled water. Then the gel was incubated in 200 ml of 0.012 M silver nitrate solution for 30 min and rinsed twice with distilled water. The development was begun by adding 200 ml of the image-developer solution containing 0.28 M sodium carbonate and 0.15 ml of formaline. The gel was agitated gently until the desired colors appeared. Finally, the developer was removed and the gel was stored in 200 ml of 1% acetic acid.

b. Silver staining for LPS

A silver-staining method was used for LPS staining (Tsai and Frisch, 1982; Hitchcock and Brown, 1983). The gel was fixed in 200 ml of a solution containing 25% isopropanol and 7% acetic acid for 2 hrs. The fixed gel was oxidized for 5 min in 200 ml of a solution containing 0.75% periodic acid in distilled water and 4 ml of 25% isopropanol and 7% acetic acid. After washing for 2 hr with distilled water, the gel was incubated in the silver nitrate solution containing 28 ml of 0.1 N sodium hydroxide, 1 ml of concentrated ammonium hydroxide, 5 ml of 20% silver nitrate, and 115 ml of distilled water. To make the

solution, the ingredients were added sequentially while being stirred vigorously not to make precipitates. The gel was washed again with distilled water for 30 min and to it were added 250 ml of a color development solution containing 50 mg of citric acid, and 0.5 ml of formaldehyde. A 100 ml of 7% acetic acid was added to stop the reaction

3. Immunoblotting

After finishing electrophoresis, proteins in the gel were transferred electrophoretically onto nitrocellulose membrane (Towbin et al., 1979). The nitrocellulose membrane was then washed with distilled water and incubated in 3% non-fat dry milk in phosphate-buffered saline (PBS) for 1 hr to block non-specific binding of antibodies. The membrane was rinsed with PBS three times and treated for 1 hr with either culture supernatants of hybridoma cells or 500x-diluted ascites fluids in PBS. After being washed three times with PBS containing 1% Tween 20, the membrane was incubated with HRP-conjugated goat antibody against mouse IgG for 1 hr and washed three times with PBS. The washed NC membrane was treated with a solution containing 30 mg of 4-chloro-1-naphtol, 30 µl of hydrogen peroxide (30%), and 10 ml of methanol in 50 ml of PBS. Also used was another developing solution containing 30 mg of diaminobenzidine (DAB) and 30 µl of hydrogen peroxide (30%) in 50 ml of PBS.

G. Indirect Immunofluorescence Microscopy

Two methods were used to prepare amoebae for indirect immunofluorescence microscopy. To test a few different antibodies, amoebae were collected in Syracuse watch glasses, and the medium was removed by aspiration. Cells were fixed and permeabilized with cold methanol (-20° C) for 5

min. After cells were washed three times with PBS, they were incubated in the primary antibody solution diluted with PBS for 30 min at room temperature. Supernatant of hybridoma cell culture was diluted with PBS at the ratio of 1:1 and ascites fluids 1:50. Cells were washed three times and further incubated in the secondary antibody diluted 1,000 times in PBS for 30 min at room temperature. The goat anti-mouse IgG antibody conjugated with FITC was used as the secondary antibody. The labeled cells were washed 3 times with PBS and mounted with a mounting solution containing 90% glycerol, 1 mg/ml p-phenylenediamine, and 10% PBS on a glass slide. A Leitz epifluorescence microscope was used to observe labeled cells.

To test many antibodies at the same time, hundreds of amoebae were placed on a glass coverslips (40 x 22 x 0.15 mm) using a fine-tipped mouth pipette under a dissecting microscope. The standard printed microscope slides with varying number of wells (Cell-line) were also used for processing a large number of samples at a time. After removing as much medium as possible, a drop of 45 % acetic acid was added directly on the amoebae. A siliconized glass coverslip was placed diagonally over the coverslip or slide with attached amoebae and quickly placed all together on a flat surface of dry ice. After a few minutes, the coverslip was flipped off. Amoebae on the coverslip or slide were fixed and permeabilized in cold methanol (-20° C) for 5 min each, and dried in air. For antibody staining, amoebae on the coverslip or slide were hydrated in PBS for 5 min and processed as described above.

H. Electron Microscopy

1. Immunoperoxidase method

Healthy amoebae were centrifuged for 1 min at 170 g and fixed with cold

methanol (-20° C) for 5 min. The cells were washed three times with PBS and treated with mAbs for 1 hr at room temperature. Culture fluids were used directly and ascetic fluids were diluted 50 times with PBS. After the cells were washed three times with PBS, they were further incubated with a secondary antibody conjugated with peroxidase and diluted 50 times with PBS for 1 hr at room temperature. After the cells were washed three times with PBS, they were postfixed with 1% glutaldehyde in 0.1-M sodium phosphate buffer (pH 7.2) for 30 min at 4° C to minimize further deterioration of cellular structures. Amoebae were rinsed with 0.1-M sodium phosphate buffer (pH 7.2) and incubated in 10 ml of the same phosphate buffer containing 2 mg of DAB and 0.001% hydrogen peroxide. The reaction was allowed to proceed until cells became dark brown, as monitored with a light microscope. The following steps were the same as those for the preparation of cells for conventional electron microscopy.

Amoebae were postfixed with 1% osmium tetroxide in 0.1-M sodium phosphate buffer (pH 7.2) for 30 min, dehydrated in a series of ethanol and embedded in Epon. Thin sections were cut on an ultramicrotome and collected on copper grids. The sections were stained with uranyl acetate for 30 min and counterstained with lead citrate for 30-45 sec. A Hitachi H-600 electron microscope was used to observe sections on the grids.

2. Immunogold method

Isolated X-bacteria or symbiosomes were incubated in a fixative containing 3% paraformaldehyde, 0.3% glutaldehyde, and 10% sucrose in 0.1-M sodium phosphate buffer (pH 7.2) for 1 hr. The specimen was washed twice with the same buffer and dehydrated in a series of dimethyl formamide (DMF) solutions (Sigma). The incubation times used were 10 min in 50% solution, 15

min in 70%, and 15 min in 90%. The dehydrated specimen was infiltrated and embedded in Lowicryl K4M as follows (Altman et al., 1984): 15 min in 2:1 (DMF:Lowicryl), 15 min in 1:1 (DMF:Lowicryl), 30 min in Lowicryl, and another 30 min in Lowicryl K4M. The polymerization was done at 40° C with exposure to a longer UV light (360 nm) overnight. Hardened blocks were stored at room temperature until used. Gold-colored thin sections were collected on nickel grids and treated with 1% bovine serum albumin for 1 hr to block non-specific binding. Grids were jet-washed with PBS and incubated with mAbs for 1 hr. Goat anti-mouse IgG antibody conjugated with colloidal gold (10 nm in diameter, Sigma) was used as the secondary antibody. The sections were stained with lead citrate for 45 sec and observed with a Hitachi H-600 electron microscope.

3. Negative staining method

The hot phenol-treated X-bacteria were placed on carbon-coated grids and allowed to settle for 1 min. After most of the liquid was removed by touching the top of grid with filter paper using a pair of forceps, the KPTA stain solution containing 2% phosphotungstic acid (pH 7.0 with KOH) was added and a drop of stain was quickly removed. The grid was allowed to air dry and was observed under the electron microscope.

I. Purification of Antibodies

Hundred milliliters of culture fluid from hybridoma cells were centrifuged for 5 min at 650 g and applied to the top of a protein A-coated agarose column (1 ml packed volume, Pierce). The column was then washed extensively until the protein concentration in PBS, which was used to rinse out the unbound

antibodies, came down to the background level. Bound antibodies were eluted with 0.1-M glycine-HCl (pH 2.5), neutralized with the addition of 1-M Tris, and dialyzed against distilled water for 24 h. Then the dialyzed solution containing antibodies was concentrated by using Centricon-10 Microconcentrators (Amicon). All procedures were performed at 4° C. The protein concentration was determined by measuring optical density at 280 nm and the purity of a preparation was checked by SDS-PAGE. Purified antibodies were filtered through a 0.2-µm pore-size nylon screen just before biotinylation or microinjection to eliminate contaminants.

J. Biotinylation of Antibodies

The biotinylation of purified antibodies was performed according to the protocol from Pierce. Purified and concentrated antibodies were adjusted to a final concentration of 10 mg/ml in 50-mM bicarbonate buffer (pH 8.5) in a clean test tube. 0.4 mg of NHS-LC-Biotin (Pierce) were added directly to the tube and the tube was placed in ice for 2 hr. To remove the unreacted biotin, the product was centrifuged three times for 30 min at 1,000 g using Centricon-30 Microconcentrators. After centrifugation, the sample was adjusted to a concentration of 10 mg/ml in 0.1-M phosphate buffer (pH 7.0), and stored at 4° C in 0.1% NaN₃ until ready for use. The protein concentration was determined by the measuring optical density at 280 nm.

K. Microinjection of Antibodies

To prepare agar-coated coverslips (22 x 40 mm), 0.3 g of agar was melted in 50 ml of Chalkley's solution, the solution was filtered and was poured

onto clean glass coverslips. Twenty amoebae were transferred to the top of an agar-coated coverslip with a fine-tipped mouth pipette. Amoebae were usually placed in 4 groups. After removing the excess medium, about 5×10^{-5} μ l of antibody solution (1/50 of amoeba cell volume) were injected into each amoeba using a micropipette mounted on a de Fonbrune micro-manipulator. The glass micropipettes were made with a de Fonbrune microforge. The antibody concentration was adjusted to 2 mg/ml with Chalkley's solution just before injection. For each microinjection experiment, more than 50 amoebae were microinjected.

L. Experimental Infection

Bacteria-free D amoebae were infected with X-bacteria isolated from xD amoebae. Purified X-bacteria were harvested by centrifugation for 5 min at 8,000 g, suspended, and incubated in a solution containing 0.05% poly-L-lysine (MW 350,000, Sigma) for 20 min at 4° C. After being washed by centrifugation, X-bacteria were resuspended in Chalkley's solution to a concentration of 10^9 X-bacteria per ml. Two ml of X-bacterial suspension were added to 1×10^4 D amoebae in a Petri dish (3 cm in diameter). After 2 hr, the supernatant containing unphagocytosed X-bacteria was removed and amoebae were washed several times with Chalkley's solution. The next day amoebae were fed with *Tetrahymena*.

M. Chemical Analyses of Lipopolysaccharides

1. Cleavage of lipopolysaccharides

A sample of 5.6 mg (dried weight) of lyophilized LPS material was

hydrolyzed in 2 ml of 1% (v/v) acetic acid at 100° C for 2 hr (Wilkinson et al. 1973). The lipidic pellet was collected after centrifugation at 1000 g for 30 min at 20° C, and washed twice with 2 ml of distilled water. The pellet was then partitioned in water/methanol/chloroform (1:2:4, v/v/v) to remove uncleaved LPS and 0.7 mg of lipid A was recovered from the organic phase.

The supernatant of the acetic acid hydrolysate and the subsequent aqueous washing of the lipidic material contained the polysaccharide fraction. These fractions were pooled and lyophilized. The water-soluble material obtained was purified from undegraded LPS by solid-phase extraction using disposable C₁₈-columns (Bond-Elut, Varian). The lyophilized polysaccharide was dissolved in water, passed through a C₁₈-column, and 3.8 mg of PS part were obtained by lyophilization of the water eluant.

2. Analytical methods

For neutral sugars, the polysaccharide fraction was hydrolyzed in 0.1-M HCl at 100° C for 48 hr and for the amino sugars, hydrolysis in 4-M HCl at 100° C for 18 hr was used. Additionally, the polysaccharide fraction was also hydrolyzed in 2 N TFA for 30 min at 80° C for the determination of sugars located at the outermost. After hydrolysis, samples were extracted with chloroform and aqueous fractions were dried. The residues were analyzed both as free sugars by high pH anion-exchange chromatography (HPAEC) with pulsed electro-chemical detection (PED) and as alditol acetate derivatives by gas chromatography-mass spectroscopy. The alditol acetate derivatives of sugars were prepared by the reduction of samples with NaBH₄ and peracetylation with acetic anhydride. All the constituents reported here were identified by their mass spectra. Acidic sugar, 2-keto-3-deoxyoctonic acid (KDO), was quantified colorimetrically by the thiobarbituric acid reaction after

hydrolysis with a 0.1-M sodium acetate buffer (pH 4.4) for 1 hr at 100° C (Brade and Brade, 1985).

The fatty acids in the lipid A part of X-bacteria were determined by GC after being released by hydrolysis in 4-M HCl in methanol at 100° C for 18 hr and then extracted with hexane. The organic extract was analyzed for fatty acids and the water fraction for sugars. The ester-linked fatty acids were determined after liberation by 2-M HCl in methanol for 2 hr at 60° C. The amide-linked fatty acids were calculated by the difference (Sonesson et al., 1989). The hydroxy groups of fatty acids were acetylated prior to analysis.

The HPAEC-PED system (Dionex 4500, Sunnyville, CA) and analytical conditions that were used in this study followed the methods of Sonesson and Jantzen (1992). The analyses by GC were performed using fused-silica columns (stationary phase DB-1) and flame-ionization detection. In addition to comparison of retention times, the identification of sugars and fatty acids was verified by GC-MS. The analytical conditions for GC and GC-MS analyses were the same as those described in Sonesson et al. (1994).

N. Purification of the Chromosomal DNA from X-Bacteria

Isolated X-bacteria were suspended in a lysis buffer containing 50-mM Tris-HCl (pH 8.0), 150-mM glucose, and 10-mM EDTA and incubated for 10 min at 37° C. Then, cells were treated with Proteinase K (final concentration, 1 mg/ml) for 30 min at 37° C and further incubated for 10 min at 37° C with the addition of lysozyme (final concentration, 1 mg/ml). After the addition of SDS (final 2%), X-bacteria were incubated for 30 min at 37° C and for another 60 min at 60° C. The supernatant containing DNA was collected by centrifugation for 10 min at 10,000 g, extracted through a mixture of phenol :

chloroform : isoamyl alcohol (25:24:1), and precipitated in cold ethanol (-20° C) in the presence of sodium acetate, pH 7.5 (final concentration, 0.3 M). After being washed with 70% ethanol, the precipitate was dissolved in 30 µl of TE buffer containing 20-mM Tris-HCl (pH 8.0) and 10-mM EDTA. The RNA was removed with a treatment of RNase (final concentration, 20 µg/ml) for 30 min at 37° C. The purity of chromosomal DNA from X-bacteria was determined by agarose gel electrophoresis.

O. PCR Amplification of 16S Ribosomal DNA

The master mix was made as follows; each amplification tube contained ca. 10-100 ng of template DNA, 0.5 mM each of the two primers, 200 mM each of dNTP mixture (dATP, dCTP, dGTP, dTTP), 2-mM MgCl₂, 10 ml of buffer (X10), and sterile distilled water to 99.5 µl. The buffer (X10) contained 100-mM Tris-HCl (pH 9.0 at 25° C), 500-mM KCl, and 1% Triton X-100. Each tube was overlaid with 100 µl of mineral oil to prevent the evaporation of the reaction mixture. Following 5 min of incubation at 94° C, 0.5 µl of Taq polymerase (2.5 units) were added to each reaction tube. The thermal program consisted of 35 cycles of denaturation for 1 min at 95° C, annealing for 1 min at 55° C, and extension for 3 min at 72° C. An additional cycle containing a 5 min extension at 72° C was included as the final step. The two primers (5' to 3' orientation) used for amplification were **forward 16S7f** AGAGTTTGATCMTGGCTCAG (position 7 to 26, *E. coli* numbering) and **reverse 16S1510r** GGTTACCTTGTTACGACTT (1510 to 1492). Besides the purified X bacterial chromosomal DNA, the lambda DNA containing the genomic DNA library of X-bacteria and whole X-bacteria were also used as a template DNA. After amplification, mineral oil was aspirated and 1 volume of the mixture containing chloroform

and isoamylalcohol (24:1) was added to each reaction tube to remove proteins and oil. The aqueous phase was collected after microcentrifugation for 5 min at 10,000 g, and analyzed by using the 1% agarose gel in Tris-acetate (0.04 M) EDTA (1 mM) buffer.

P. Direct Cycling Sequencing and Computer Analysis

For sequencing, the PCR products were filtered through a Centricon-30 to remove remaining primers. Two picomoles of the sequencing primer were end-labeled using T4 polynucleotide kinase (USB) and 1 ml of [$\gamma^{32}\text{P}$] ATP (7000 Ci/mmol, ICN) in a reaction volume of 10 μl according to the manufacture's instructions. Six different sequencing primers were used to sequence the X-bacterial 16S rDNA (Table 1). Labeled primers were used directly in sequencing reactions without purification. The TAQuence DNA sequencing kit (USB) was used to sequence the amplified DNA. Each sequencing reaction was applied in the following order, 3 μl of template (ca. 50 to 200 ng), 9.5 μl of water, 1 μl of labeled primer (0.2 pmol), 1 μl of reaction buffer (x10), and 2 μl of diluted TAQ polymerase (2 units). This mixture was spun down and 4 μl were added to each of four tubes containing 4 μl of termination mixture (ddATP, ddCTP, ddGTP, ddTTP). Each tube was overlaid with 10 μl of mineral oil. The thermal program for sequencing consisted of 30 cycles of denaturation for 40 sec at 95 $^{\circ}$ C, annealing for 30 sec at 55 $^{\circ}$ C, and extension for 90 sec at 72 $^{\circ}$ C. The sequencing reactions were terminated by adding 4 μl of TAQuence stop mix to each tube. The products of sequencing reactions were analyzed on standard 5% acrylamide sequencing gels. The gels were fixed using aqueous 10% methanol and 5% acetic acid solution, dried at 80 $^{\circ}$ C, and radioautographed on Kodak XAR5 film after exposure for 15 to 24 h at room temperature.

The nucleotide sequence of X-bacterial 16S rDNA was compared with those of other bacteria in GenBank using the GCG package. The secondary structure of X-bacterial 16S rDNA was also constructed with the same package.

Table 1. List of Primers Used for the Amplification and Sequencing of X-Bacterial 16S Ribosomal DNA

Primers	Sequences	Length
16S7f	5' AGAGTTTGATC <u>M</u> TGGCTCAG 3'	20-mer*
16S308f	GGAGGCAGCAGTGGGGAA	19-mer
16S472r	G <u>W</u> ATTACCGCGGCK <u>G</u> CTG	18-mer+
16S705f	GCGAAAGCGTGGGGAGC	17-mer
16S821r	CCGTACTCCCCAGGCGG	17-mer
16S1510r	GGTTACCTTGTTACGACTT	19-mer*

Glossary: K={GT}, M={AC}, W={AT}

* Amplification conserved primer (Embley, 1991)

+ Conserved 16S rRNA sequence (Lane et al., 1985)

CHAPTER III

RESULTS

A. Chemical Composition of X-Bacterial LPS

1. General properties of LPS isolated from X-bacteria

The LPS of X-bacteria were recovered from the aqueous phase using a hot phenol-water extraction described in Materials and Methods. Phenol-treated X-bacteria were negatively stained and observed under an electron microscope (Fig. 1). Only the outer cell membrane, where LPS are present, was released from the bacterium. The released outer cell membranes were further processed to isolate LPS. The silver-stained SDS-PAGE of isolated X-bacterial LPS showed a distinct ladder-like band pattern, indicating a repeated chain of polysaccharides (Fig. 2). Therefore, X-bacteria have a characteristic smooth-type LPS containing a polysaccharide and a lipid A part.

The cleavage of LPS by mild hydrolysis in 1% acetic acid yielded 12.5 % of lipid A and 67.8 % of polysaccharides (of LPS dry weight) for X-bacteria.

2. Chemical analysis of X-bacterial LPS

a. The polysaccharide (PS) part

The constituents of the PS part of X-bacterial LPS is presented in Table 2. The hydrolyzed PS part was divided and treated separately to determine the three kinds of sugars; neutral, amino, and acidic sugars (Fig. 3). The conditions for each sugar are described in Materials and Methods. The seven different kinds of neutral sugars identified were rhamnose, fucose, glucose, mannose, galactose, yersiniose A and 3-O-methyl hexose (Fig. 3B). Rhamnose was the

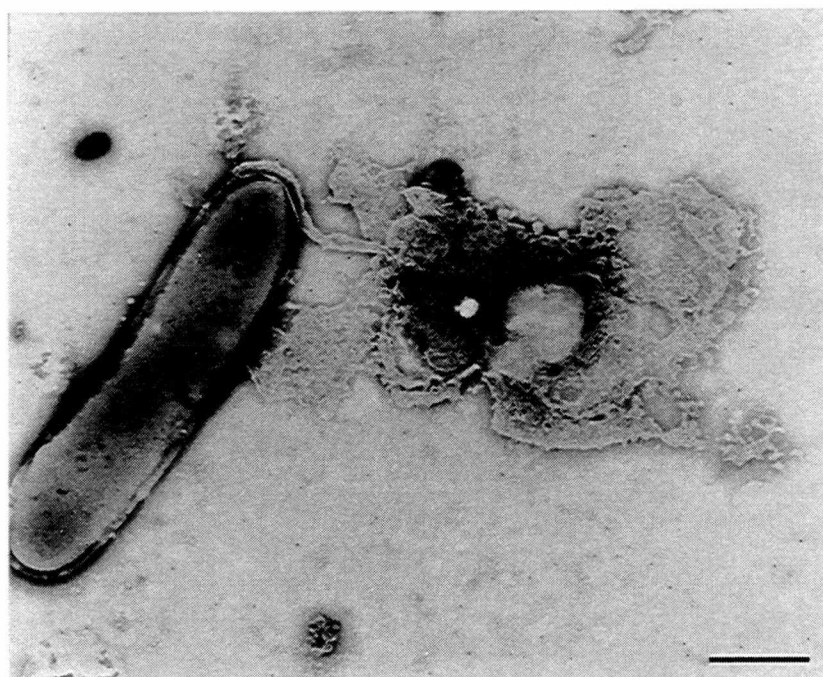


Fig. 1. Negative staining of an X-bacterium treated with 45% hot phenol/ water reagent to isolate the outer membrane containing LPS
Bar = 0.2 μm .

Arrow indicates the outer membrane of X-bacteria.

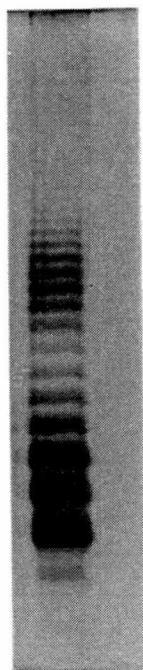


Fig. 2. SDS-PAGE of LPS purified from X-bacteria

Fig. 3. Gas chromatograms of sugar components in the polysaccharide part of X-bacterial LPS

- A: Standard
- B: X-Bacterial LPS hydrolyzed with 4 N HCl for 18 hr at 100° C
- C: X-Bacterial LPS hydrolyzed with 0.01 N HCl for 48 hr at 100° C
- D: X-Bacterial LPS hydrolyzed with 2 N TFA for 30 min at 80° C

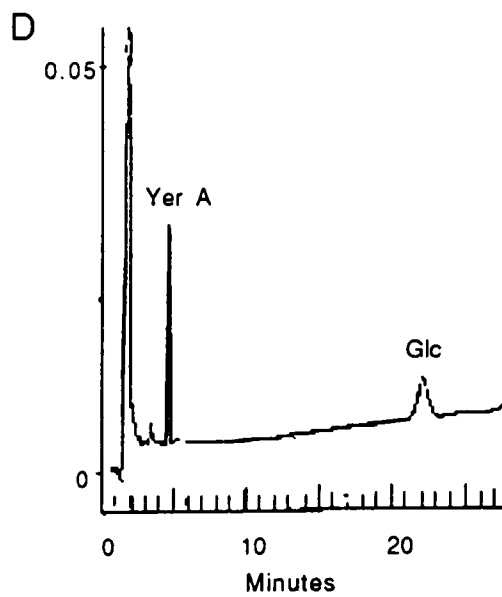
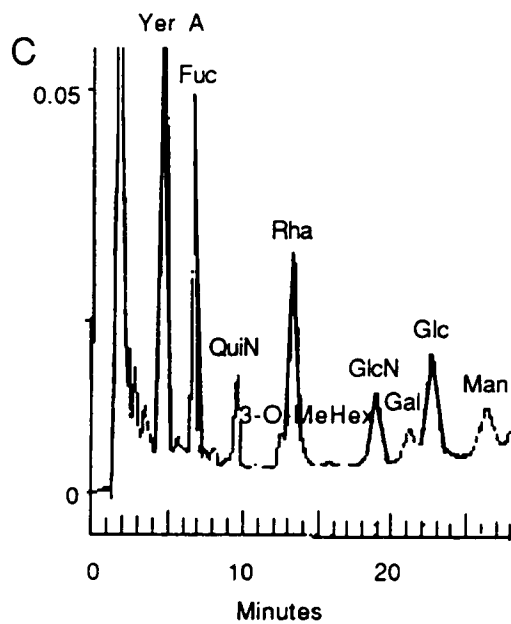
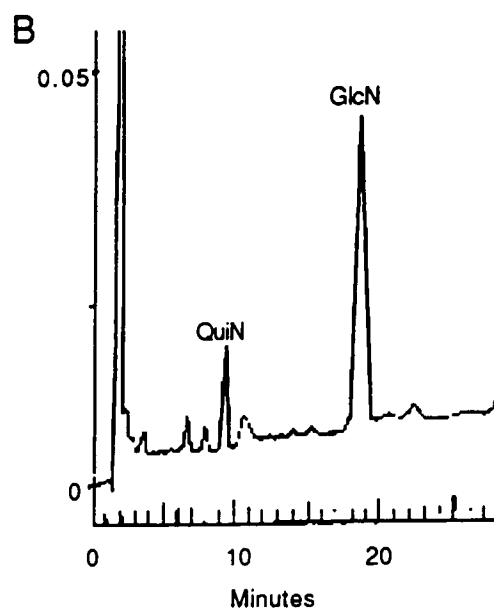
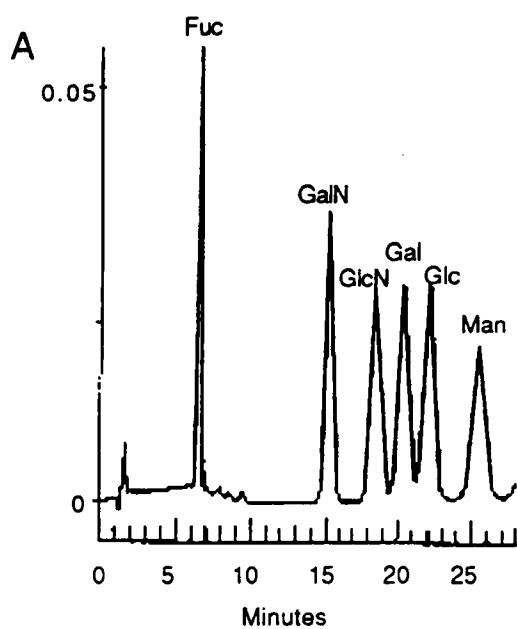


Table 2. Constituents of the Polysaccharide Part of X-Bacterial LPS

Sugar	Concentration (nmol/mg)
Rhamnose (Rha)	330
Fucose (Fuc)	270
Glucose (Glc)	200
Mannose (Man)	160
Galactose (Gal)	68
Yersiniose A (Yer A)	311
3-O-Methyl hexose	96
Quinovosamine (QuiN)	78
Glucosamine (GlcN)	480
Inositol (Ino)	310
2-Keto-3-deoxyoctonic acid (KDO) *	20

* KDO was quantified colorimetrically by thiobarbituric acid method.

Note: Results are expressed as nmol/mg of LPS dry weight.

most abundant among them and followed by the rare branched-chain cotose, yersiniose A. The amino sugars such as glucosamine and quinovosamine were found in the PS part, and glucosamine was the most abundant sugar among those identified in the PS of X-bacterial LPS (Fig. 3C, Table 2). KDO, an acidic sugar and ubiquitous LPS component, was also found with a thiobarbituric acid method. Therefore, LPS of X-bacteria contained sugars which are found in LPS of other Gram-negative bacteria (Keene and Lindberg, 1983). Further analysis of the PS part under a mild hydrolysis condition in 2 N trifluoroacetic acid (TFA) for 30 min at 80° C revealed that yersiniose A and glucose were the only sugars released from LPS (Fig. 3D). This result suggested that yersiniose A may be linked at the terminus of the X-bacterial LPS.

b. Lipid A part

Lipid A of LPS is composed of a sugar backbone and attached fatty acids. The fatty acid profile of X-bacterial LPS showed 13 kinds of 3-hydroxylated fatty acids and 4 kinds of non-hydroxylated fatty acids, in the range of C₁₂ - C₂₁ and C₁₆ -C₁₈, respectively (Fig. 4, Table 3). Their molar distribution in X-bacterial LPS was 72 % of 3-hydroxy fatty acids and 28 % non-hydroxy fatty acids. Both fatty acids include linear (normal) and methyl branched-chain (*anteiso* and *iso*) acids. Most of the 3-hydroxylated fatty acids were amide-linked, but all non-hydroxylated fatty acids were found to be exclusively ester-linked. The most prominent hydroxylated fatty acid was 3-hydroxymethyltridecanoic acid (3-OH-i14:0), and 3-hydroxycosanoic acid (3-OH-n20:0) was the second most. However, the long-chain fatty acids containing longer than C₂₇ such as 27-oxooctacosanoic acids, found in members of the genus *Legionella*, were not detected.

Sugars identified in the lipid A part were glucosamine and inositol, and

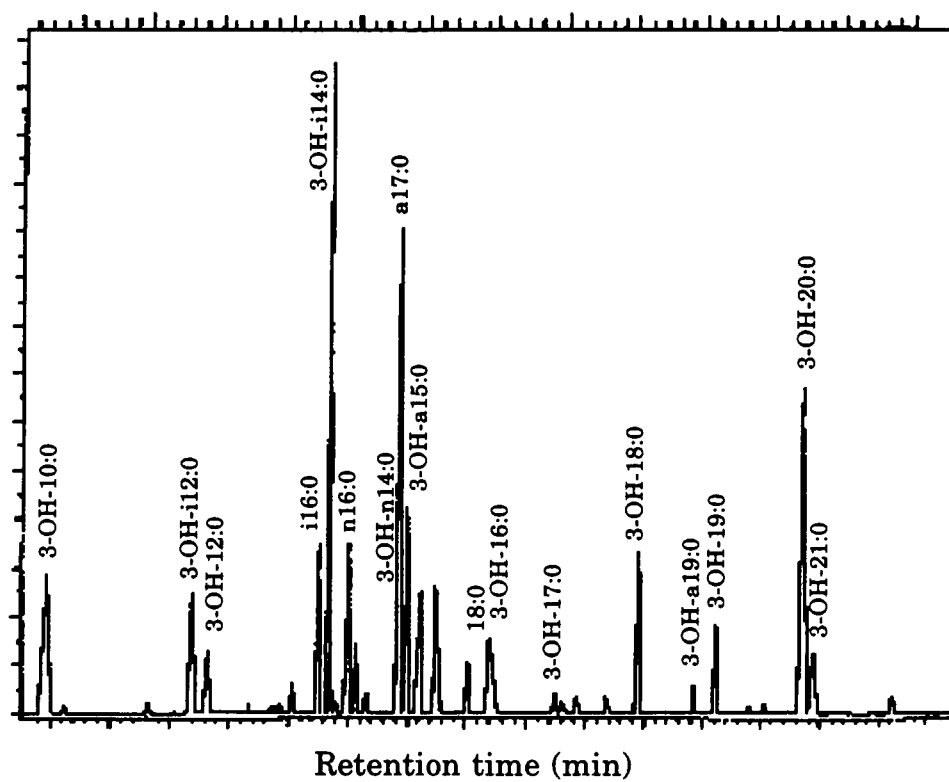


Fig. 4. Gas chromatogram of fatty acids in the lipid A part of X-bacterial LPS

Table 3. Constituents of the Lipid A Part of X-Bacterial LPS**1. Amide-linked fatty acids**

3-OH-n10:0	2.6	(nmol/mg)
3-OH- i12:0	1.6	
3-OH-n12:0	0.8	
3-OH- i14:0 *	6.8	
3-OH-n14:0	0.5	
3-OH-a15:0	2.2	
3-OH-n16:0	0.6	
3-OH-n17:0	0.2	
3-OH-n18:0	1.6	
3-OH-a19:0	0.3	
3-OH-n19:0	0.8	
3-OH-n20:0	4.4	
3-OH-n21:0	0.5	

(* 3-OH-n14:0 was found both ester- and amide-linkages)

sum 3-OH-Fatty acids 22.9 nmol/mg

2. Ester-linked fatty acids

i16:0	1.5	(nmol/mg)
n16:0	1.5	
a17:0	4.9	
n18:0	1.1	

sum Fatty acids 9.0 nmol/mg

3. Sugars

Glucosamine	34	(nmol/mg)
Inositol	21	

glucosamine seems to be the lipid A backbone sugar since it was present both in the PS part and in the lipid A part of X-bacterial LPS and was the most common backbone sugar identified in the LPS (Rietschel et al., 1988).

B. Studies on the Role of LPS in the Prevention of Fusion Between Symbiosomes and Lysosomes

1. Localization of X-bacterial LPS

Two mAbs against X-bacterial LPS (KJL18 and KJL36) were produced and their specificity was analyzed by indirect immunofluorescence staining and immunoelectron microscopy. Both mAbs stained X-bacteria and surrounding symbiosome membranes in xD amoebae, but none in D amoebae, by indirect immunofluorescence staining (Fig. 5). Immunoelectron microscopy with colloidal-gold particles showed the specific localization of LPS on bacterial cell surfaces as expected (Fig. 6). Since newly produced mAbs and the mAb previously reported by Choi and Jeon (1991) showed the exactly same pattern of immunoblot and immunofluorescence staining, one anti-LPS mAb (CJL20) produced by Choi and Jeon was used for further studies due to its strong reactivity.

2. Appearance of LPS on symbiosome membranes

Since both anti-LPS mAbs stained symbiosome membranes in xD amoebae, they were used to determine the time that LPS first appeared on the symbiosome membrane in newly infected D amoebae by indirect immunofluorescence staining (Fig. 7). Before performing the experimental infection, X-bacteria were pre-treated with poly-L-lysine to make various sizes of bacterial lumps, that resulted in an increase in the number of bacteria phagocytosed

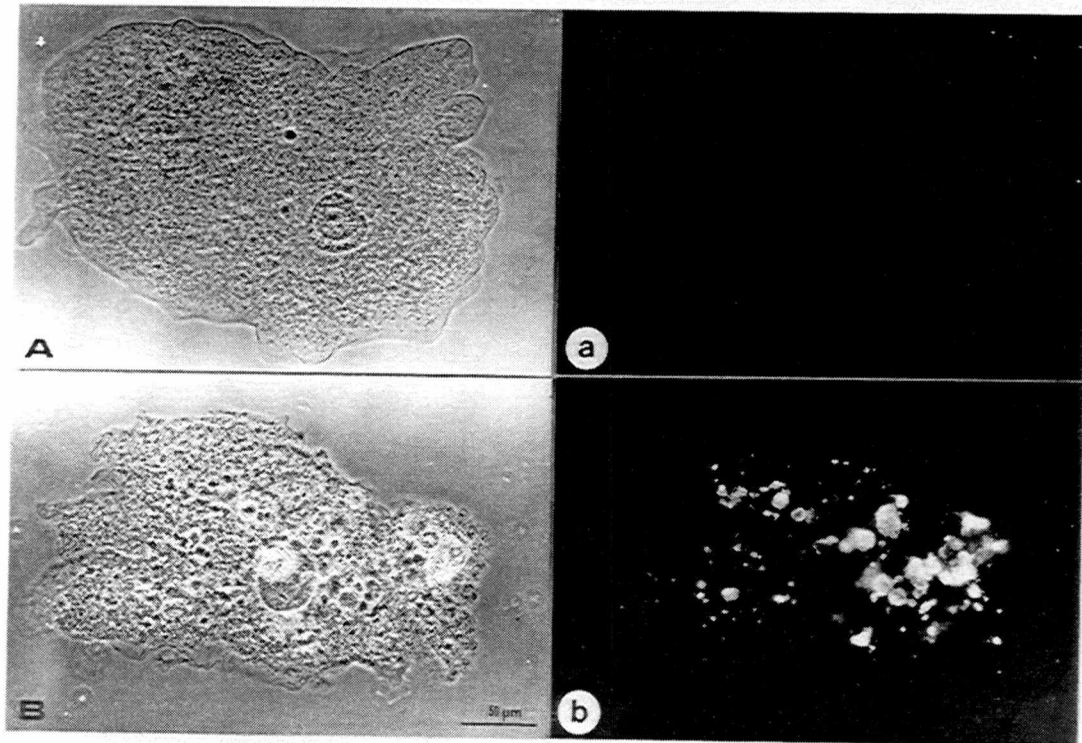


Fig. 5. Phase-contrast (A and B) and immunofluorescence (a and b) micrographs of D and xD amoebae treated with a mAb against X-bacterial LPS (KJL36) Bar = 50 μ m.

- A, a: A symbiont-free D amoeba
- B, b: A symbiont-bearing xD amoeba

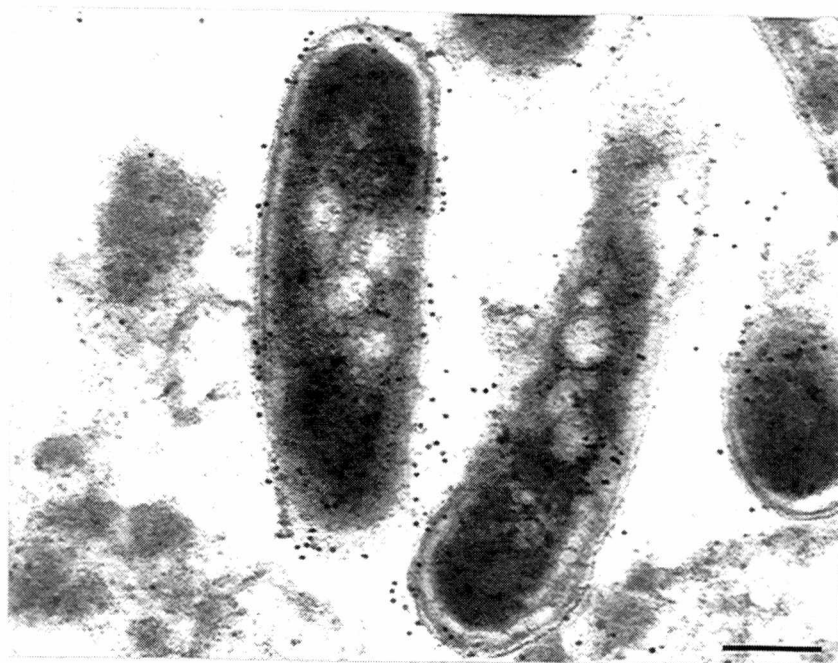
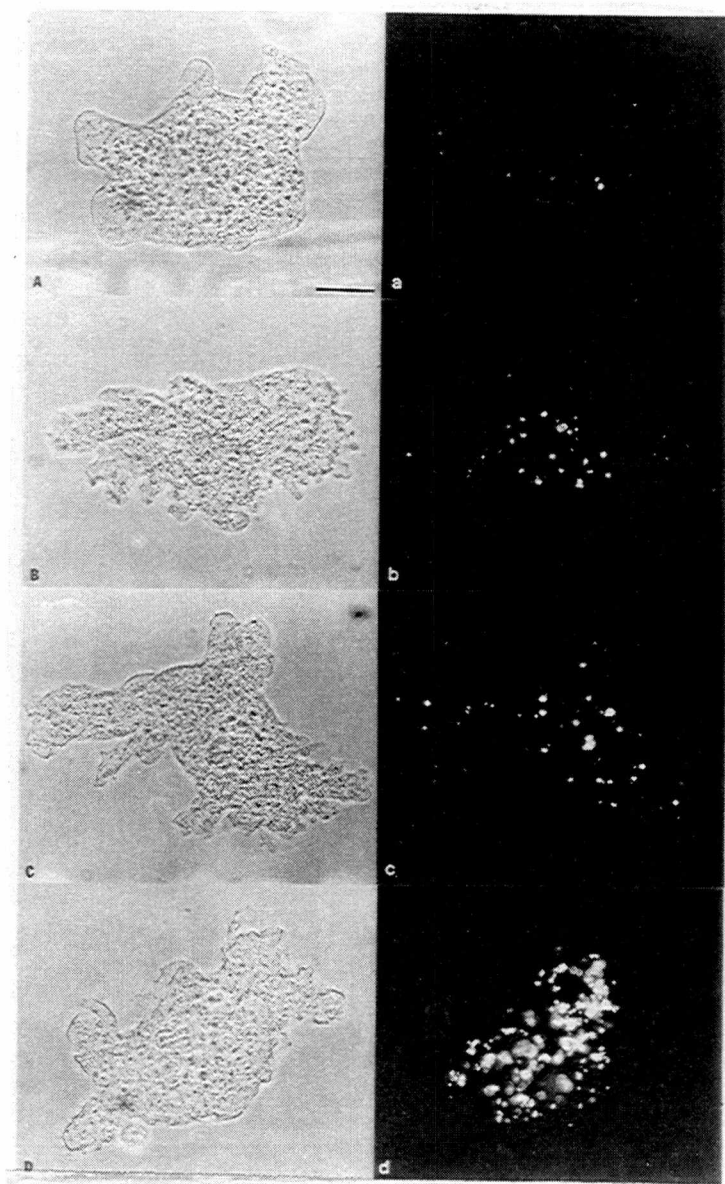


Fig. 6. An immunoelectron micrograph of X-bacteria treated with a mAb against LPS (KJL36) and a colloidal gold-conjugated secondary antibody Bar = 0.2 μ m.

Fig. 7. Phase-contrast (A, B, C, and D) and immunofluorescence with CjL20 (a, b, c, and d) micrographs of newly infected D amoebae to show the appearance of LPS on the newly forming symbiosome membranes Bar = 50 μ m.

- A, a: A D amoeba 3 days after infection
- B, b: A D amoeba 7 days after infection
- C, c: A D amoeba 14 days after infection
- D, d: A D amoeba 21 days after infection



into amoebae. After infection of D amoebae for 2 hr, free bacteria were washed away three times with Chalkley's solution and observed under the phase-contrast microscope. Newly infected D amoebae contained bacteria surrounded by membranes in the form of bacterial lumps as well as single bacteria.

Three days after infection, a few bacteria were visible in the cytoplasm of newly infected D amoebae under the phase-contrast microscope. When processed for indirect immunofluorescence staining with an anti-LPS mAb (CJL20), X-bacteria showed fluorescence, but vesicle membranes containing them did not (Fig. 7A, a). After 7 days, there were more X-bacteria seen, and all of them were enclosed in symbiosomes. Some of the symbiosome membranes were stained by CJL20 (Fig. 7B, b). Thus, it appeared that LPS were first added to symbiosome membranes at a detectable level between 3 and 7 days after infection.

At 14 days after infection, all the symbiosome membranes were stained by CJL20, although the numbers of X-bacteria and symbiosomes were lower than those of established xD amoebae (Fig. 7C, c). At 21 days after infection, newly infected D amoebae could not be distinguished from established xD amoebae either by the number of X-bacteria or by the degree of immunofluorescence staining of symbiosomes (Fig. 7D, d).

3. mAbs against lysosomal membrane proteins (LMPs) as a marker of lysosomes

In order to monitor lysosomal fusion with symbiosomes, a marker for amoeba lysosomes was required, and three different mAbs against amoeba lysosomes (KJL3, KJL8, and KJL9) were produced by immunizing mice with whole lysosomes fractionated by Percoll gradient centrifugation. Before each

injection, the marker enzyme assay for lysosome was performed, and fractions showing high β -hexosaminidase activity were pooled and used for both mAb production and SDS-PAGE analysis (Fig. 8).

Indirect immunofluorescence staining of amoebae with all three mAbs showed small vesicles in the cytoplasm of host amoeba, which had been proven to be lysosomes in the previous report of Choi et al. (1992) (Fig. 9). An electron microscopic observation showed that the mAb against lysosome (KJL9) stained lysosomal membranes of the amoeba (Fig. 10). Immunoblot results, however, revealed that each of three anti-lysosome mAb had recognized different lysosomal proteins (Fig. 11). Two mAbs (KJL3 and KJL8) stained a protein having a molecular mass of 56 kDa and a mAb (KJL9) stained a 50-kDa protein. It is not known whether the two mAbs, KJL3 and KJL8, recognize the same 56-kDa protein, but each mAb showed different fusion pattern with phagosomes containing ciliates, *Tetrahymena pyriformis*. To determine the time of fusion, 3-day-starved amoebae were fed with freshly harvested ciliates for 5 min, washed with Chalkley's solution 4-5 times, and then processed for immunofluorescence with three mAbs against LMP (Fig. 12, 13, and 14).

The protein (56 kDa) recognized by KJL3 appeared on the phagosomal membranes within 30 min, and fluorescence reached a maximum intensity in 90 min after phagocytosis. This protein disappeared from phagolysosomes 6 hr after phagocytosis (Fig. 12A, a; 13A, a). The protein (56 kDa) recognized by KJL8 appeared on the phagolysosomal membranes within 30 min, and fluorescence reached a maximum intensity in 90 min after phagocytosis like that recognized by KJL3 (Fig. 12B, b; 13B, b; 14A). However, they stayed on phagolysosomes up to 27 hr after phagocytosis. The other 50-kDa protein recognized by KJL9 showed a totally different pattern. This protein appeared

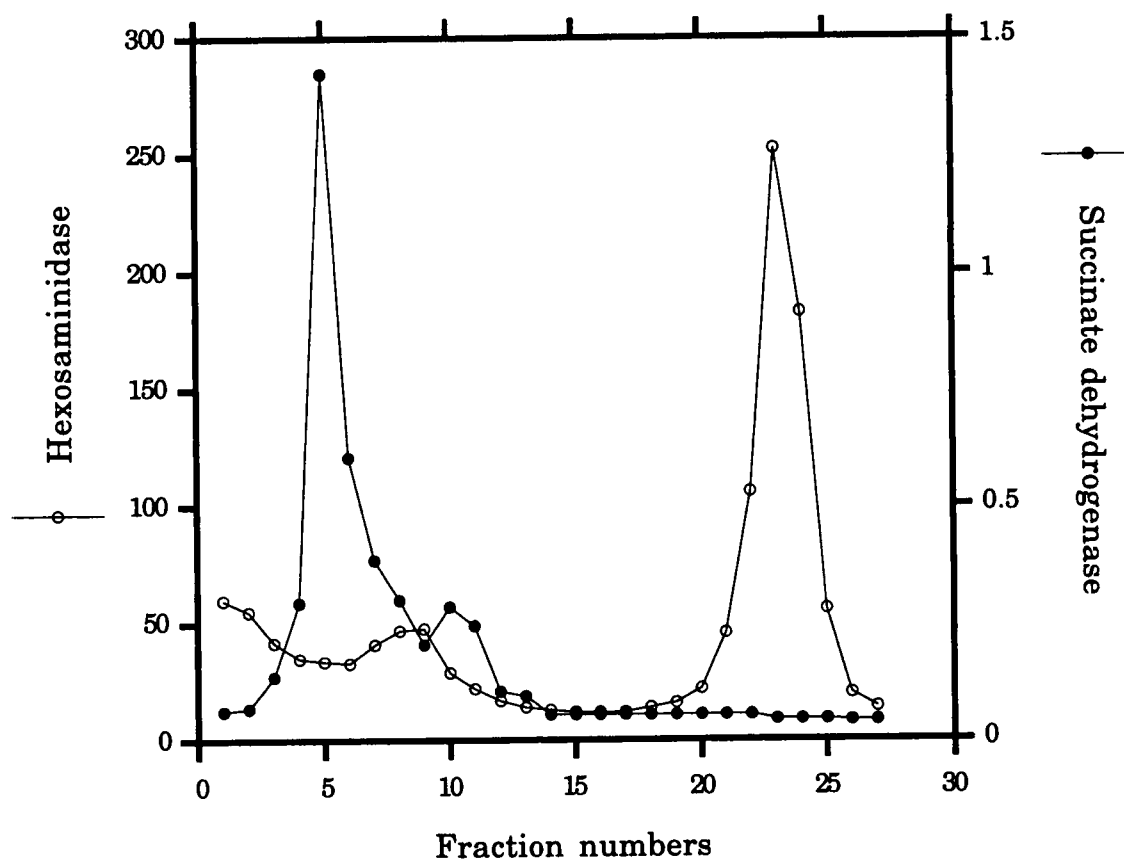


Fig. 8. A graph showing activities of β -hexosaminidase and succinate dehydrogenase in fractions collected from the top of a Percoll gradient

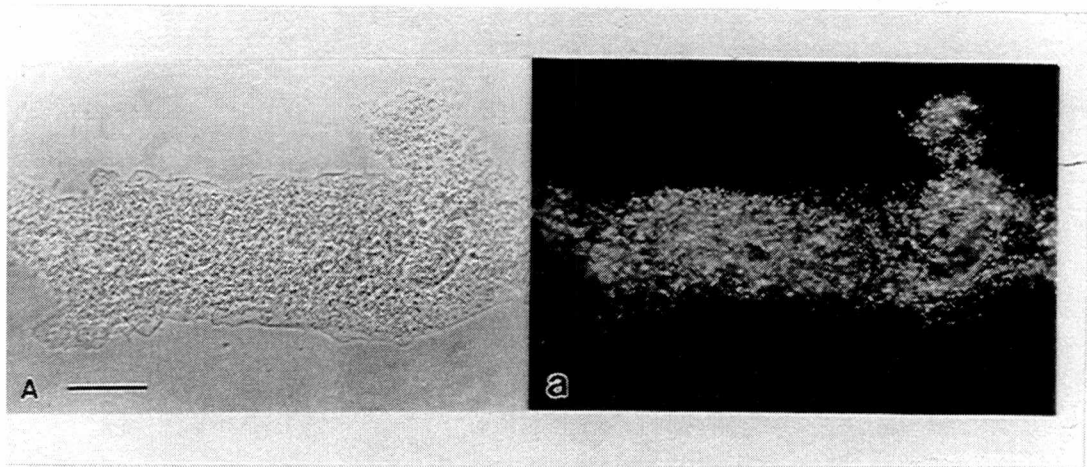


Fig. 9. Phase-contrast (A) and immunofluorescence (B) micrographs of a 3-days starved *D. amoeba* treated with a mAb against LMP (KJL9) and FITC-conjugated secondary antibody Bar = 50 μ m.

The mAbs (KJL3 and KJL8) showed the same patterns.

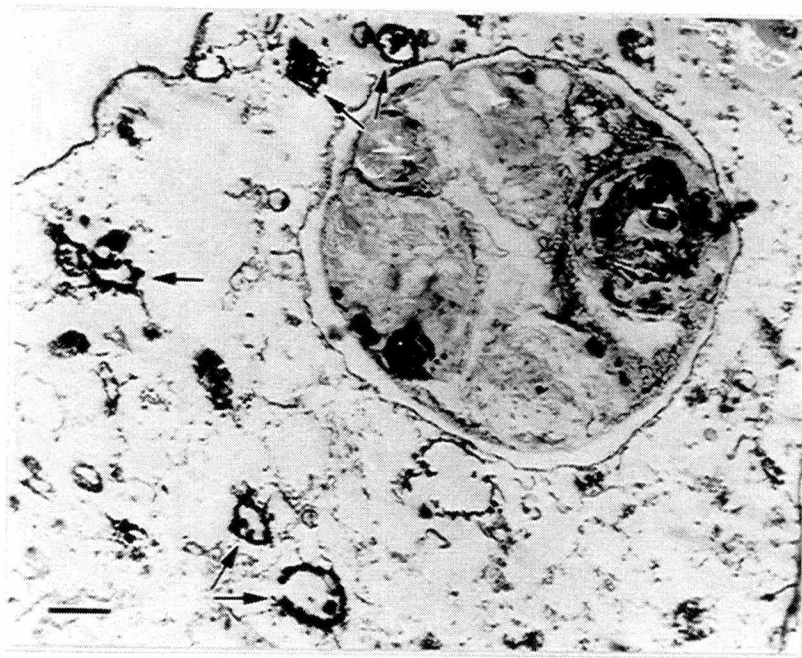


Fig. 10. Immunocytochemistry micrograph of a *D* amoeba treated with a mAb against a lysosomal membrane protein (KJL9) Bar = 0.1 μ m.

Arrows indicate lysosomal membranes stained with antibody

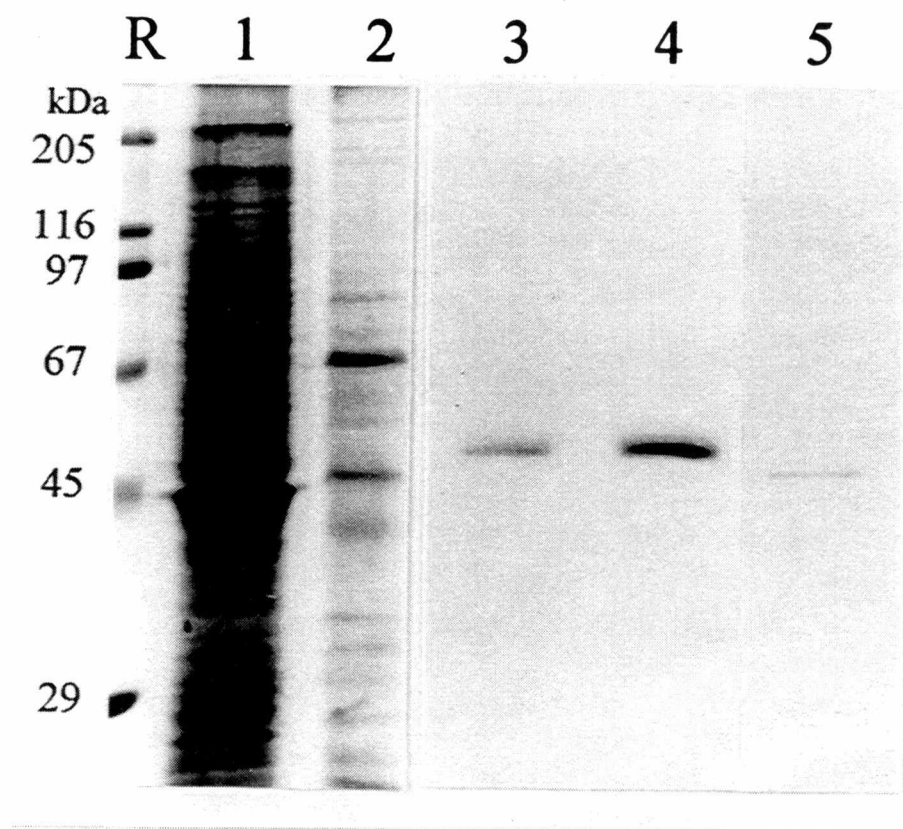


Fig. 11. SDS PAGE (1, and 2) and corresponding immunoblots (3, 4, and 5)

- R: Molecular standard
- 1: Whole homogenates of *D. amoebae*
- 2: Purified lysosomes
- 3: Purified lysosomes probed with a KJL3 mAb
- 4: Purified lysosomes probed with a KJL8 mAb
- 5: Purified lysosomes probed with a KJL9 mAb

Fig. 12. Phase-contrast (A, B, and C) and immunofluorescence (a, b, and c) micrographs of a D amoeba 30 min after phagocytosis Bar = 50 μ m.

Arrows indicate phagosomes with lysosomal proteins recognized by antibody and arrowheads indicate phagosomes without lysosomal proteins.

- A, a: A KJL3-treated D amoeba
- B, b: A KJL8-treated D amoeba
- C, c: A KJL9-treated D amoeba

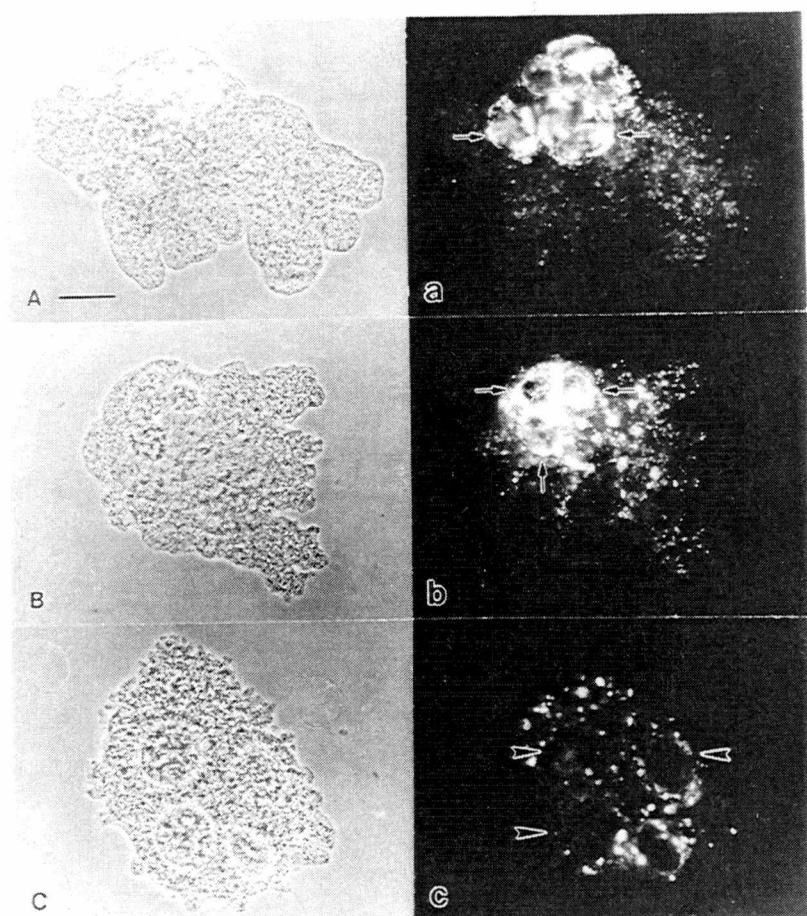


Fig. 13. Phase-contrast (A, B, and C) and immunofluorescence (a, b, and c) micrographs of a D amoeba 6 hrs after phagocytosis Bar = 50 μ m.

Arrows indicate phagosomes with lysosomal proteins recognized antibody and arrowheads indicate phagosomes without lysosomal proteins.

- A, a: A KJL3-treated D amoeba
- B, b: A KJL8-treated D amoeba
- C, c: A KJL9-treated D amoeba

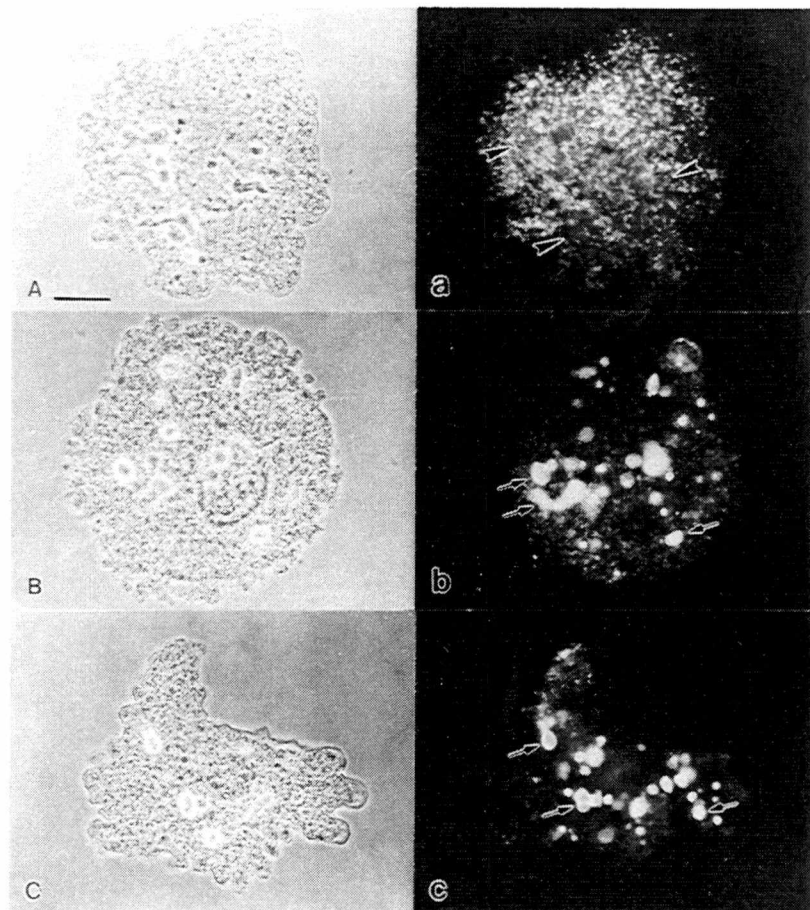
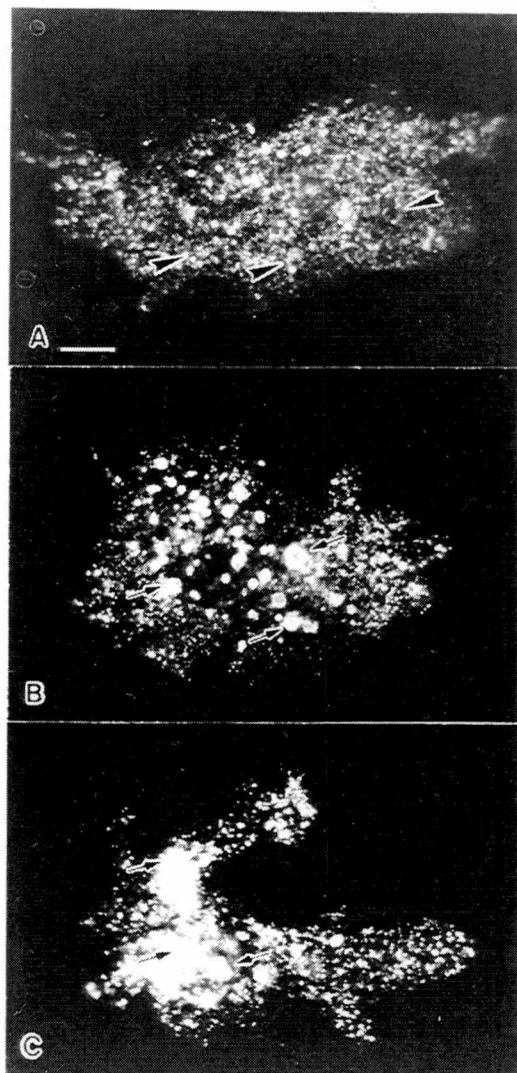


Fig. 14. Immunofluorescence (A, B, and C) micrographs of a D amoeba 27 hrs (A, and B) and 3 days (C) after phagocytosis Bar = 50 μ m.

Arrows indicate phagosomes with lysosomal proteins recognized by antibody and arrowheads indicate phagosomes without lysosomal proteins.

- A: A KJL8-treated D amoeba
- B: A KJL9-treated D amoeba
- C: A KJL9-treated D amoeba



on phagosomal membranes 90 min or longer after phagocytosis, and the fluorescence intensity increased for up to 6 hr. This protein persisted there even 3 days after phagocytosis (Fig. 12C, c; 13C, c; 14B, C). Whether the proteins recognized by three anti-LMP mAbs are present on the same vesicles is not known but preliminary double staining results showed that two 56-kDa LMPs, which fuse early with lysosomes, seemed to locate on the same vesicles, but the 50-kDa protein did not.

It is interesting to note that two LMPs, 100- and 150-kDa, previously reported by Choi et al. (1992), showed different fusion pattern from any of those described above. The proteins recognized by CJL53 and CJL57 appeared on the phagosome membrane within 30 min or earlier after phagocytosis like the 56-kDa LMPs and stayed there even after 3 days like a 50-kDa LMP. Therefore, LMPs of amoebae could be classified into 4 groups (Table 4) and an anti-LMP mAb (KJL8) was used for further study.

4. Microinjection and double-staining experiments

If LPS on the symbiosome membranes were involved in prevention of lysosomal fusion, microinjection of an anti-LPS antibody would remove their ability to block lysosomal fusion with symbiosomes and allow symbiosome-lysosome fusion to occur, which normally does not occur in xD amoebae. To test this hypothesis, two mAbs, anti-LPS (CJL20) and anti-LMP (KJL8) mAbs, were purified separately by using protein A-conjugated agarose columns. Since both mAbs were produced from mice, the secondary antibodies against mouse IgG can recognize both so that the anti-LMP mAb (KJL8) was further labeled with biotin as described in Materials and Methods. Texas Red-conjugated streptavidin was used for the detection of anti-LMP mAb (CJL20), and a FITC-conjugated goat antibody against mouse IgG for anti-LPS mAb

Table 4. List of Lysosomal Membrane Proteins Found in Amoebae and Their Fusion Pattern

MAbs	Protein recognized by a mAb (kDa)	Appearance on phagosomes	Maximum intensity of fluorescence	Dis- appearance from phagosomes
KJL 3	56	> 30 min	> 90 min	> 6 hr
KJL 8	56	> 30 min	> 90 min	> 27 hr
KJL 9	50	2 hr	6 hr	3 days
*CJL 53 / 57	100/150	15 min	> 90 min	3 days

* These mAbs were reported previously (Choi et al., 1992)

(KJL8). The biotin-labeled anti-LMP mAb was checked to determine if it still maintained the specificity for LMP of amoebae (Fig. 15A, B). Without prior injection of a mAb (CJL20), none of the symbiosomes were stained with the anti-LMP mAb (KJL8) (Fig. 15B).

A purified anti-LPS mAb (CJL20) was first microinjected into xD amoebae individually, and injected amoebae were washed three times and then processed for double immunostaining with an anti-LMP mAb (KJL8) to localize symbiosome-specific LPS and lysosome-specific LMP. In an xD amoeba injected with an anti-LPS mAb (CJL20), some, not all, symbiosomes were stained with two antibodies, an anti-LPS mAb and an anti-LMP mAb. This result indicated that some symbiosomes had fused with lysosomes after LPS on symbiosome membranes were masked by the microinjected anti-LPS mAb (CJL20) (Fig. 15C, D). Therefore the result supports the hypothesis that X-bacteria-derived LPS on symbiosome membranes actively play a role in the lysosome-symbiosome fusion prevention.

5. High temperature treatment and LPS

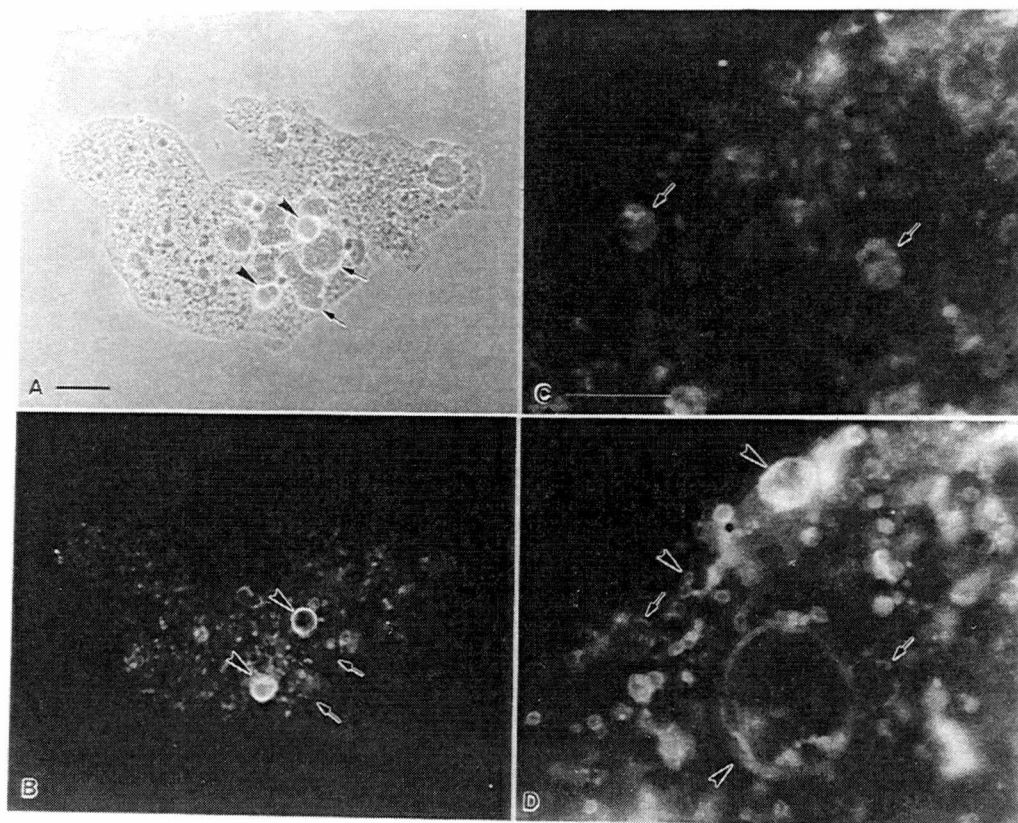
As described in Introduction, high temperature (27° C) treatment induced fusion between lysosomes and symbiosomes in xD amoebae, and X-bacteria were completely eliminated within 14 days of incubation (Ahn and Jeon, 1979). In order to obtain more information about the function of LPS, xD amoebae were incubated at 27° C and the LPS pattern and morphology of bacterial surface were investigated with indirect immunofluorescence and electron microscopy, respectively

On the 3rd day, symbiosomes of a xD amoeba stained with a mAb against LPS (CJL20) declined in number compared with that in a control xD

Fig. 15. Phase-contrast (A) and immunofluorescence (B, C, and D) micrographs of xD amoeba treated with an anti- LMP mAb (KJL8) (B and D) and with an anti-LPS mAb (CJL20) (C) Bar = 50 μ m (A and B), 25 μ m (C and D).

- A: An xD amoeba containing numerous symbiosomes (small arrows), phagolysosomes (arrowheads), and newly formed phagosomes containing *Tetrahymena* cells (large arrows)
- B: The same amoeba as in panel A stained with a mAb against LMP
- C: An xD amoeba stained with an mAb against LPS and examined with a FITC filter. Arrows indicate symbiosomes.
- D: The same amoeba examined with a rhodamine filter. Arrows indicate symbiosomes fused with lysosomes and arrowheads indicate lysosomes.

* C and D: The amoeba was first injected with a mAb against LPS and stained with FITC-conjugated anti-mouse IgG. Then, the same amoebae were processed for double-staining with a biotinylated anti-LMP mAb as the primary antibody and stained with Texas-Red conjugated streptavidin.



amoeba (Fig. 16A, B). On the 4th day of treatment, the number of symbiosomes decreased further (Fig. 16C). After 5 days of treatment, some symbiosomes became much bigger than those in a control xD amoeba, and LPS seemed to be depleted from symbiosome membranes and dispersed inside symbiosomes (arrow) (Fig. 16D). After 7 days of treatment, only a few small symbiosomes were observed (Fig. 16 E).

Electron micrographs of high temperature-treated xD amoebae, together with immunofluorescence staining results, revealed interesting changes in symbiosome and X-bacteria (Fig. 17). The outer membrane of X-bacteria came off from the inner membrane after 3 days of incubation (Fig. 17A). After 5 days of incubation it was completely detached from X-bacteria and seemed to be released into the lumen of symbiosomes (Fig. 17B, C). X-Bacteria were confined to the center of the symbiosome and were being digested whereas active vesicular fusions were seen on the symbiosome membrane (Fig. 17B). Additionally the outside of X-bacteria in symbiosome was filled with unknown materials and is thought to contain LPS since there was no membrane structure observed (Fig. 17C) and the lumen of symbiosome was stained with anti-LPS mAb (Fig. 16D).

Based on the results, high temperature treatment might force X-bacteria appear to lose their outer membranes containing LPS and shed LPS from symbiosome membranes, resulting in the lysosomal fusion.

C. Phylogenetic Studies of X-Bacteria by the Comparison of 16S Ribosomal DNA Sequences

1. Amplification of the 16S rDNA of X-bacteria with PCR

The X-Bacterial 16S ribosomal DNA was amplified with the asymmetric

Fig. 16. Immunofluorescence micrographs of xD amoebae incubated at high temperature (27° C) Bar = 50 μ m.

Amoebae were treated with an anti-LPS mAb (CJL20) and FITC-conjugated secondary antibody.

- A: A control xD amoeba
- B: An xD amoeba incubated for 3 days
- C: An xD amoeba incubated for 4 days
- D: An xD amoeba incubated for 5 days
- E: An xD amoeba incubated for 7 days

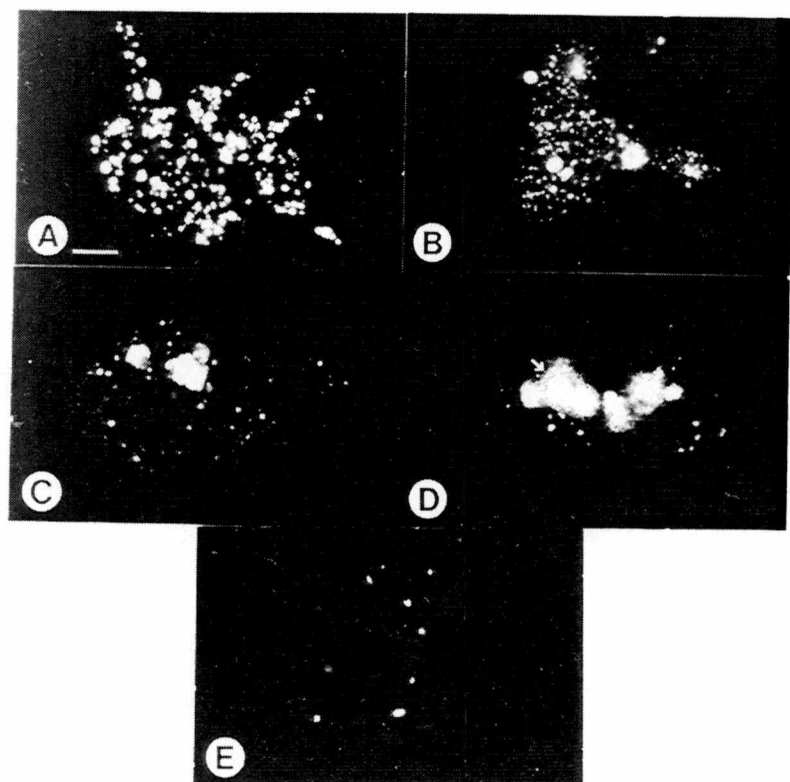


Fig. 17. Electron micrographs of symbiosomes in xD amoebae incubated at high temperature Bar = 1 μ m.

- A: X-Bacteria in an xD amoeba incubated for 3 days at 27° C
- B: A symbiosome in an xD amoeba incubated for 5 days at 27° C
- C: A highly magnified symbiosome in an xD amoeba incubated for 5 days at 27° C



PCR method by using two primers known to be conserved among eubacteria (Table 1). The products of PCR reaction were examined by agarose gel electrophoresis, and a single DNA fragment of about 1,500 nucleotides was generated from three different sources of template DNAs; purified chromosomal DNA of X-bacteria, a genomic expression library of X-bacteria, and isolated X-bacteria (Fig. 18). The amplified DNA fragments were used directly for sequencing after filtration to remove unincorporated primers.

2. Direct sequencing and comparison of 16S rDNA of X-bacteria

The amplified double-stranded DNA fragment was sequenced by a linear PCR procedure (Embley, 1991). The primers used for sequencing are listed in Table 1, and a total of 1,407 base pairs of the amplified 1.5 kb fragment was sequenced (Fig. 19). The partial nucleotide sequences (about 100 nucleotides) of PCR products from three different sources were the same so that the possibility of contamination during PCR amplification could be eliminated. On the basis of the primary nucleotide sequence, the secondary structure of X-bacterial 16S rDNA was depicted (Fig. 20)

A comparison with known 16S rDNA sequences showed that X-bacteria belong to the gamma subclass of *proteobacteria* and have the highest nucleotide-sequence identity (97.1%) with *Flavobacterium lutescens*, which is a member of *Pseudomonas* superfamily. The 16S rDNA sequences of other members in the pathogenic *Pseudomonas* genus, including *P. mendocina*, *P. aruginosa*, *P. flavescens*, also have a high identity (94-96%) whereas those of *Legionella pneumophila* and *Coxiella burnetii* have less (85% and 79%, respectively).

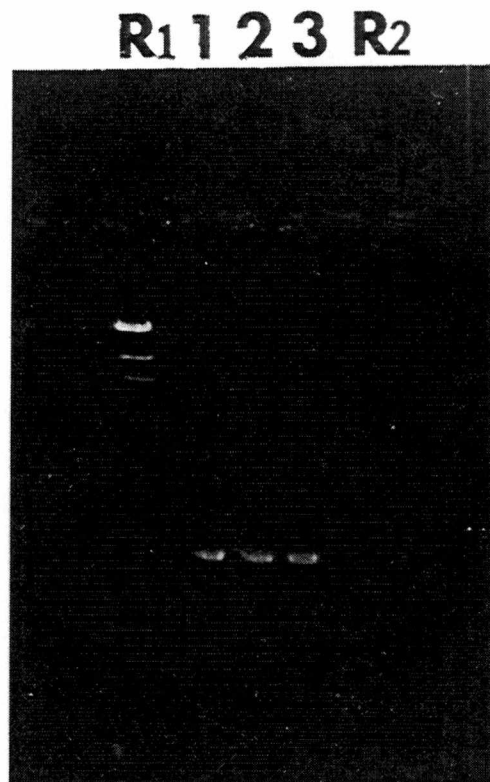


Fig. 18. Agarose gel electrophoresis of amplified X-bacterial 16S rDNA with PCR

R1: *Hind* III-treated lambda DNA

1: Intact X-bacteria as a template

2: Chromosomal DNA of X-bacteria as a template

3: Genomic expression library of X-bacteria as a template

R2: Molecular weight markers

TGGCGGCAGG CCTAACACAT GCAAGTCGAA CGGATGAGTG GAGCTTGCTC CATGATTCAG 60
 CGGCGGACGG GTGAGTAATG CCTAGGAATC TGCCTGGTAG TGGGGGACAA CGTTTCGAAA 120
 GGAACGCTAA TACCGCATAC GTCTACGGAG AAAGTGGGGG ATCTTCGGAC CTCACGCTAT 180
 CAGATGAGCC TAGGTCCGGAT TAGCTAGTTG GTGAGGTAAA GGCTCACCAA GGCACGATC 240
 CGTAACTGGT CTGAGAGGAT GATCAGTCAC ACTGGAAGTG AGACACGGTC CAGACTCCTA 300
 CGGGAGGCAG CAGTGGGGAA TATTGGACAA TGGCGAAAGC CTGATCCAGC CATGCCGCGT 360
 GTGTGAAGAA GGTCTTCGGA TTGTAAAGCA CTTTAAGTTG GGAGGAAGGG CAGTAAGTTA 420
 ATACCTTGCT GTTTTGACGT TACCAACAGA ATAAGCACCG GCTAACTTCG TGCCAGCAGC 480
 CGCGGTAATA CGAAGGGTGC AAGCGTTAAT CGGAATTACT GGGCGTAAAG CGCGCGTAGG 540
 TGGTTCGTTA AGTTGGATGT GAAAGCCCCG GGCTCAACCT GGGAACTGCA TCCAAAAGTG 600
 GCGAGCTAGA GTATGGCAGA GGGTGGTGGG ATTTCTGTG TAGCGGTGAA ATGCGTAGAT 660
 ATAGGAAGGA ACACCAAGTG CGAAGGCGAC CACCTGGGCT AATACTGACA CTGAGGTGCG 720
 AAAGCGTGGG GAGCAAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA ACGATGTCGA 780
 CTAGCCGTTG GGATCCTTGA GATCTTAGTG GCGCAGTAAC GCATTAAGTC GACCGCCTGG 840
 GGAGTACGGC CGCAAGTTAA AACTCAAATG AATTGACGGG GGCCCGCACA AGCGGTGGAG 900
 CATGTGGTTT AATTCGAAGC AACGCGAAGA ACCTTACCAG GCCTTGACAT GCAGAGAACT 960
 TTCCAGAGAT GGATTGGTGC CTTCGGGAAC TCTGACACAG GTGCTGCATG GCTGTGCTCA 1020
 GCTCGTGTCG TGAGATGTTG GGTAAAGTCC CGTAACGAGC GCAACCCTTG TCCTTAGTTA 1080
 CCAGCACGTT AAGGTGGGCA CTCTAAGGAG ACTGCGGTGA CAAACCGGCG GAAGGTGGGG 1140
 ATGACGTCAA GTCATCATGG CCCTTACGGC CTGGGCTACA CACGTGCTAC AATGGTCGGT 1200
 ACAAAGGGTT GCTAAGCCGC GAGGTGGAGC TAATCCCATA AAACCGATCG TAGTCCGGAT 1260
 CGCAGTCTGC AACTCGACTG CGTGAAGTCG GAATCGCTAG TAATCGTGAA TCAGAATGTC 1320
 ACGGTGAATA CGTTCCCGGG CCTTGTACAC ACCGCCCGTC ACACCATGGG AGTGAATTGT 1380
 ACCAGAAGCG GGTAGGCTAA CCTTCGG 1407

Fig. 19. Partial sequence of the X-bacterial 16S ribosomal DNA

SQUIGGLES of: 16s.connect December 7, 1994 12:43

FOLDRNA of: 16s.;2 Check: 7193 from: 1 to: 1407 December 6, 1994 15:09

Length: 1407 Energy: -430.9

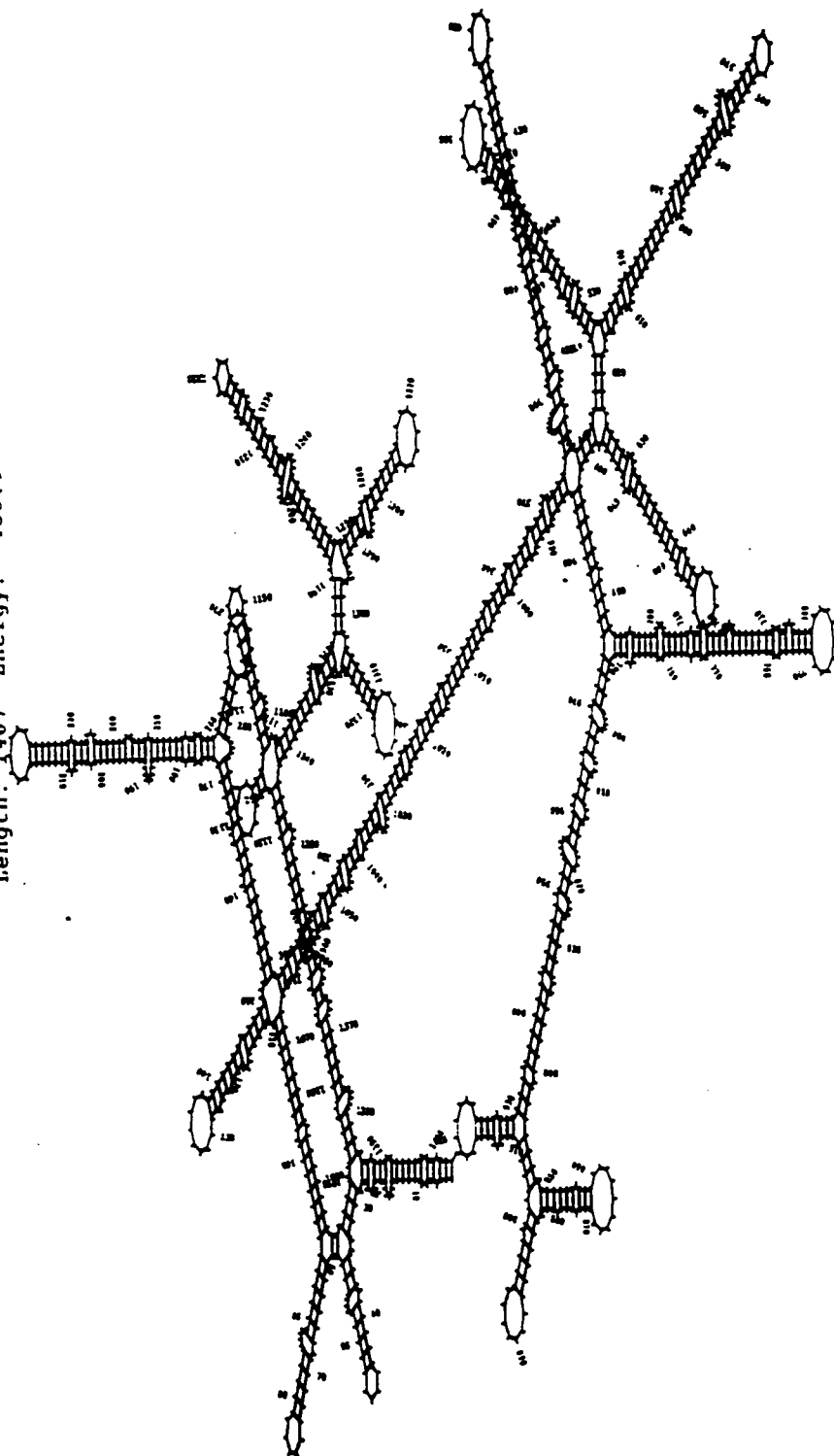


Fig. 20. The proposed secondary structure of X-bacterial 16S ribosomal DNA

D. A New Bacterial Protein in the Symbiosome Inclusions:

85-kDa Protein

1. A mAb against an 85-kDa protein in the inclusions

Symbiosomes in established xD amoebae contain fibrous inclusions, besides X-bacteria, when observed under the electron microscope (Fig. 21). Their presence was reported previously, but little was known about their composition and function. A mAb against the inclusions inside symbiosomes was produced by immunization of mice with the entire fixed symbiosome fraction and screened by immunoblotting and indirect immunofluorescence staining for its specificity. The mAb against inclusions appeared to stain an 85-kDa protein present both in X-bacteria and symbiosome fractions that include X-bacteria, inclusions and symbiosome membrane (Fig. 22). Immunofluorescence staining showed that an 85-kDa protein was mainly present in the inclusions rather than in X-bacteria because the fluorescence intensity of X-bacteria was weak and not comparable with that of inclusions (Fig. 23). A confocal micrograph of an xD amoeba treated with the same mAb clearly showed that this mAb recognizes inclusions in symbiosomes (Fig. 24). A double staining experiment with two mAbs, anti-LPS and anti-85 kDa mAb, showed that an 85-kDa protein was present only in the inclusions of symbiosomes (Fig. 25). On the basis of these results, the 85-kDa protein seemed to be synthesized by X-bacteria and to localize mainly in the inclusions of symbiosomes.

2. Appearance of an 85-kDa protein in symbiosomes

The time that it takes for the 85-kDa protein to appear in symbiosomes after experimental infection was investigated (Fig. 26). The protein first

emerged in a newly infected D amoeba 3 days after infection and reached the maximum amount in 21 days after infection. This result was the same as the appearance time of LPS on symbiosome membranes. The role of this protein was not known but the location and appearance time suggested that it was involved with the amoeba-bacteria symbiosis.

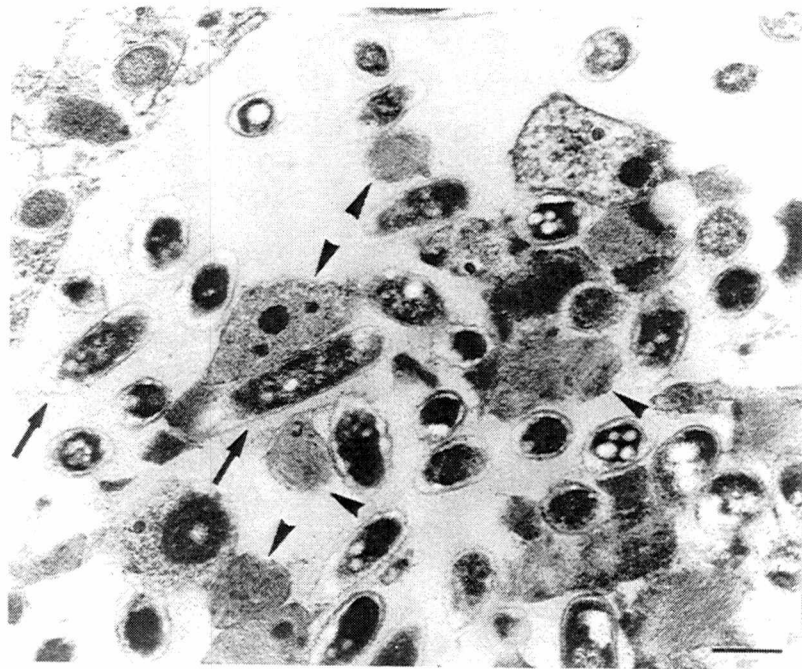


Fig. 21. An electron micrograph of a symbiosome showing inclusions (arrowheads) and X-bacteria (arrows) Bar = 0.5 μ m.

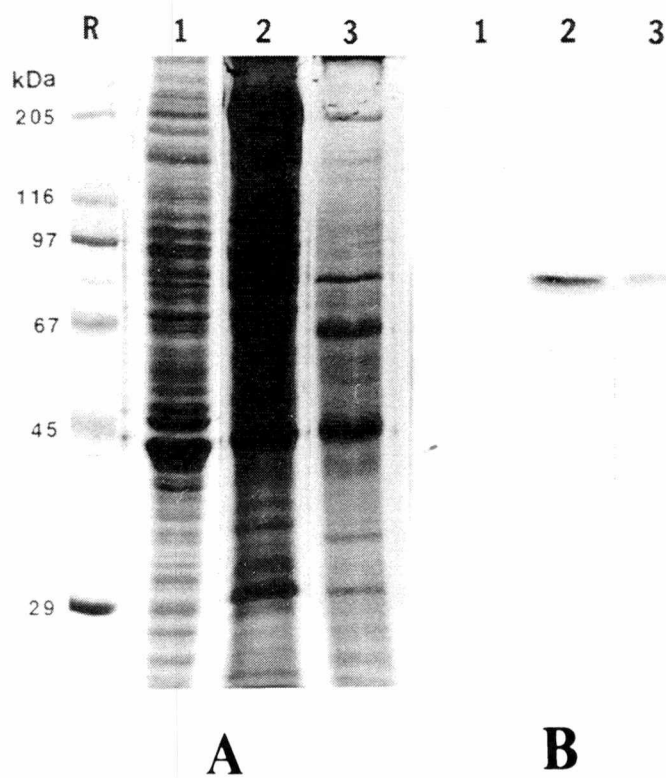


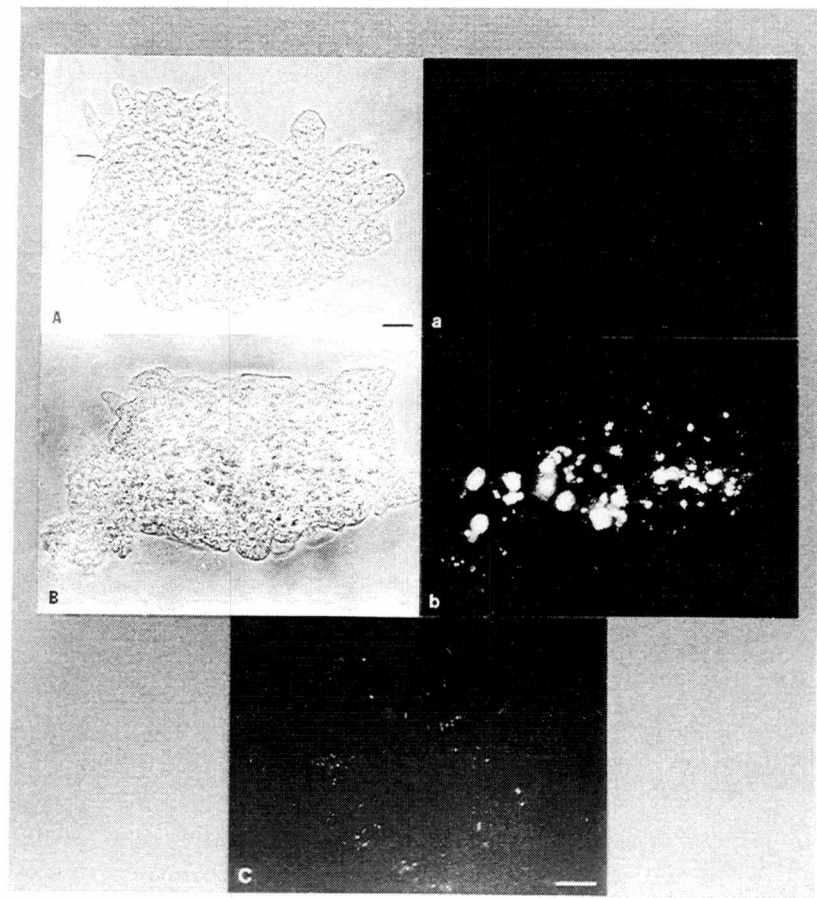
Fig. 22. SDS-PAGE (A) and corresponding immunoblot (B)

A1, B1:	Whole xD amoebae
A2, B2:	Isolated symbiosomes
A3, B3:	Isolated X-bacteria

Fig. 23. Phase-contrast (A and B) and immunofluorescence (a, b, and C) micrographs of an xD amoeba (A, a, B, and b) and X-bacteria (C) treated with a mAb against 85-kDa protein

Bar = 25 μm for A, B, a, b and 5 μm for C.

- A, a: A symbiont-free D amoeba
- B, b: A symbiont-bearing xD amoeba
- C: X-Bacteria



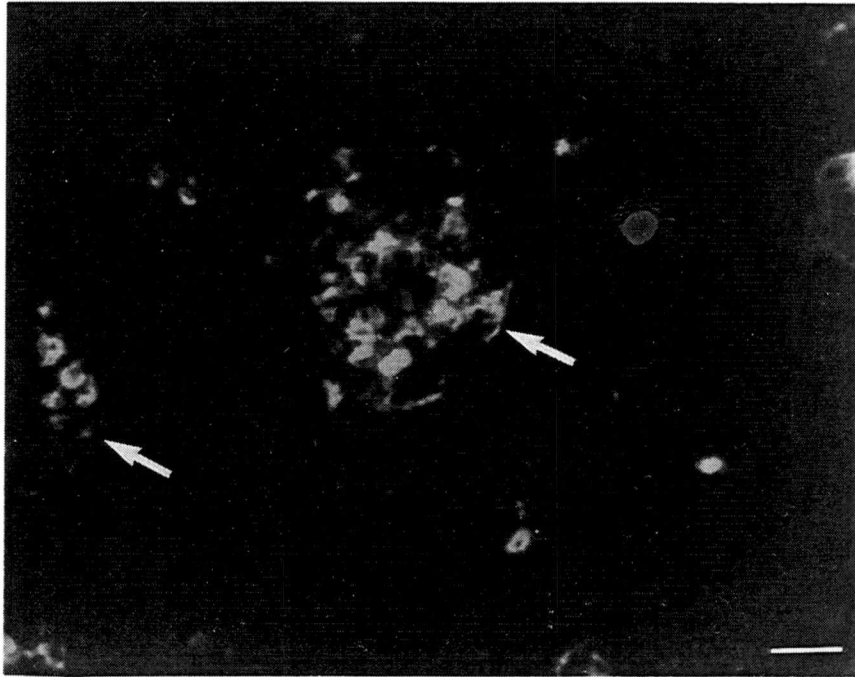


Fig. 24. A confocal micrograph of symbiosomes in an xD amoeba stained with a mAb against 85-kDa protein Bar = 5 μ m.

Arrows indicate the inclusions of the symbiosome.

Fig. 25. Phase-contrast (A) and immunofluorescence (B, and C) micrographs of an xD amoeba double stained with mAbs against LPS (CJL20) (B) and 85-kDa protein (C) Bar = 25 μ m.

Arrows indicate symbiosomes stained with both mAbs and arrowheads symbiosomes stained with only mAb against LPS.

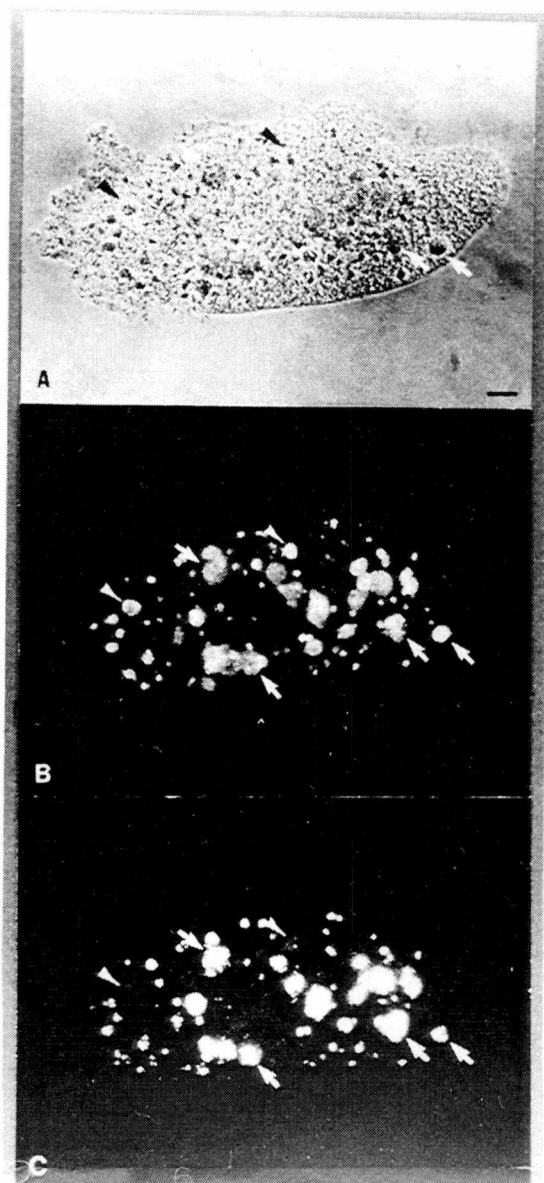
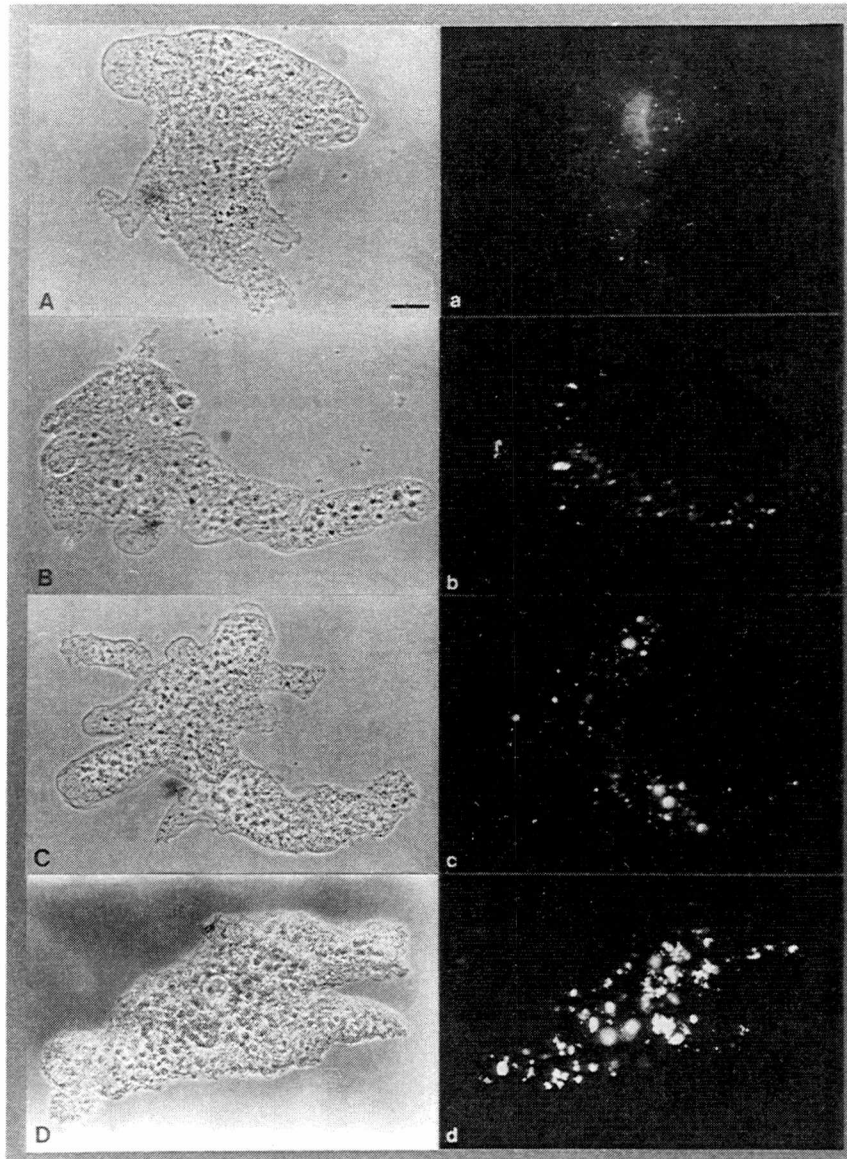


Fig. 26. Phase-contrast (A, B, C, and D) and immunofluorescence (a, b, c, and d) micrographs of newly infected xD amoebae stained with a mAb against 85-kDa protein. Bar = 25 μ m.

- A, a: An xD amoeba 5 days after infection
- B, b: An xD amoeba 7 days after infection
- C, c: An xD amoeba 14 days after infection
- D, d: An xD amoeba 21 days after infection



CHAPTER IV

DISCUSSION

A. Composition of X-Bacterial Lipopolysaccharides

The LPS of X-bacteria were isolated and analyzed by chemical methods. The SDS-PAGE profile of LPS showed that X-bacteria have a smooth-type of LPS structure containing a repeated chain of polysaccharides. The chemical analyses to determine their composition proved that X-bacteria have a complete LPS structure including the polysaccharide and lipid A parts (Tables 2 and 3).

The presence of KDO among sugars identified in X-bacterial LPS is the evidence that X-bacteria have the true LPS since KDO has been known as the marker sugar of LPS among Gram-negative bacteria of known LPS compositions (Rietschel et al., 1988). The sugars identified are common constituents of LPS, except yersiniose A (Table 2), a sugar originally identified in the O-antigens of *Yersinia pseudotuberculosis* serogroup VI and *Y. frederiksenii* O:16,29 (Gorshkova et al., 1984, 1989). This was also found recently in LPS of *Legionella maceachernii* and *L. micdadei* (Sonesson et al., 1994). The role of yersiniose A is not known, however. Since yersiniose A was found only in pathogenic bacteria and is linked at the terminus of the X-bacterial LPS, this sugar may be involved in symbiosis.

X-Bacterial LPS have a remarkably complex and distinct pattern of hydroxylated fatty acids (Table 3), which is less common in Gram-negative bacteria (Keene and Lindberg, 1983). The same complexity was found in *Legionella* sp. (Sonesson et al., 1994).

Lipid A of X-bacteria was found to contain glucosamine and inositol in the molar proportion of 3:2. Since glucosamine is present both in the PS part and in the lipid A part of the X-bacterial LPS, it seems to be the lipid A backbone sugar, as in most Gram-negative bacteria (Mayer et al., 1989). Inositol was also found in both parts, but it seems to be the contaminant of amoeba membrane since inositol is known to be a component of eukaryotic cell membrane rather than that of prokaryotic cell membrane. However, inositol could make links like glucosamine structurally although there is no such case using inositol as a backbone sugar of lipid A. Further study is therefore needed.

Bacterial systematics deals with diversity and relationships among bacteria and their ordering into taxonomic groups or taxa (Austin and Priest, 1986). Chemotaxonomy or chemosystematics, deals with relatedness, or lack of relatedness, as determined by chemical techniques. Contemporary chemosystematic analysis of bacterial cells includes the study of nucleic acids (e.g., DNA-DNA hybridization, sequence comparison of 16S ribosomal RNA), electrophoretic patterns of whole cell proteins, the amino acid sequence of heat-shock proteins, cell envelope constituents (e.g., peptidoglycans and lipids), and metabolic products (e.g., short-chain fatty acids) (Goodfellow and Minnikin, 1985).

The chemical composition of LPS has been shown to be useful in taxonomic studies of Gram-negative bacteria (Nikaido, 1970). Monomeric units and types of linkages vary considerably between taxa. Almost a hundred different sugars and sugar derivatives, several of them unique, have so far been identified in LPS (Wilkinson, 1977). The number of fatty acids may vary between 1-3 up to 10-15, generally constituting 15-20 mol-% of total cellular fatty acids (Wilkinson, 1988). The predominant 3-hydroxy fatty acids usually constitute 60-65 mol-% of fatty acids in lipid A (Jantzen and Bryn, 1985). For a

great number of species, lipid A compositions have been shown to correlate with 16S rRNA homologues of respective species (Weckesser and Mayer, 1988).

Comparison of sugars and fatty acid profiles led the conclusion that X-bacterial LPS are similar to those of *Legionella* sp., which was considered as the most closely related species (Ahn et al., 1991; 1994). Both X-bacteria and *Legionella* sp. were found to have a complex hydroxylated fatty acid profile and a rare sugar yersiniose A in this study. However, some differences between them were also found. The LPS of all members of *Legionella* sp. are known to have the same backbone sugar, 2,3-diamino-2,3-dideoxyglucose, and contain several long-chain fatty acids such as 27-oxooctacosanoic acids, 29-oxotriacontanoic acids, and 29-hydroxy-triacontanoic acid (Sonneson et al., 1994), whereas the X-bacterial LPS were found to use glucosamine as a backbone sugar and did not have such a long-chain fatty acid.

In fact, the anti-LPS mAb did not crossreact with LPS from Gram-negative bacteria including *Legionella* sp. (Choi and Jeon, 1992). Thus, it is possible that LPS of X-bacteria are changed during the long period of symbiosis with amoebae.

B. The Role of X-Bacterial LPS

The LPS derived from X-bacteria appeared on symbiosome membranes during the first 3 to 7 days after the introduction of X-bacteria into bacteria-free D amoebae and reach the maximum amount 21 days after infection. These results are consistent with the previous finding of Ahn and Jeon (1979), who counted the number of X-bacteria during high temperature incubation.

However, it is not yet known how the X-bacterial LPS are transported and added to symbiosome membranes in xD amoebae.

There are known examples in which phagocytosed organisms actively block phagosome acidification and hence prevent phagosome-lysosome fusion, although the precise mechanism is not yet known (Berger and Isberg, 1993; Goren and Mor, 1990; Weidner and Sibley, 1985). Whereas polyanions and a 25-kDa glycolipoprotein of *Mycobacterium* sp. have been known to interfere with phagosome-lysosome fusion by acting on the lysosomal membranes in other cells, symbiosome membranes seem to be altered for X-bacteria to avoid fusion with lysosomes by the addition of a symbiont-derived substance such as LPS or the 96-kDa protein (Choi and Jeon, 1992). Present results from a double-staining experiment in conjunction with microinjection of an anti-LPS antibody showed that LPS are actively involved in the prevention of lysosome-symbiosome fusion.

As surface structures, LPS represent one of the main antigens (O-antigens) of Gram-negative bacteria and play an important role for the pathogenesis and manifestations of bacterial infections. They have various important biological activities (Friedman et al., 1990), some of which include their role as a permeability barrier, especially to hydrophobic compounds, for the outer membrane (Nikaido, 1979), their involvement in the bacterial resistance to phagocytosis (Rest et al., 1977; Tan et al., 1986), and their role in the establishment of effective symbiosis of *Rhizobium* bacteroids (de Maagd et al., 1989).

Although the mechanism by which the LPS on symbiosome membranes block lysosomal fusion is not known, there are some lines of evidence which suggest that LPS may affect the microtubule network of the host cell. It has recently been reported that LPS bind to β -tubulin and microtubule-associated

protein-2 (MAP-2) specifically *in vitro* (Ding et al., 1992). LPS-tubulin interaction has also been detected by gel filtration and electron microscopy, and LPS-MAP2 interaction by ligand-blot assays (Risco et al., 1993). MAP-2, known to promote microtubule assembly, is excluded from microtubules when particular concentrations of LPS and microtubular proteins are added to the polymerization mixture, a result suggesting a specific interaction between LPS and MAP-2 (Risco et al., 1993). The displacement of the associated protein from polymerized microtubules could be the result of the binding of LPS to MAP-2, although the binding of LPS to tubulin in its site for MAP-2 cannot be eliminated (Risco et al., 1993). In addition, LPS preferentially interact with β -tubulin but not α -tubulin in polymerized microtubules, a preference shared by several MAPs, including MAP-2, tau protein, and the *mos* protooncogene product (Littauer et al., 1986; Zhou et al., 1991). These findings support the hypothesis that microtubules may be an intracellular target for LPS (Ding et al., 1990).

There is also a report showing that LPS can inhibit the polymerization of microtubules *in vitro* (Risco et al., 1993). This effect could be a consequence of the polyanionic nature of LPS (Olins and Warner, 1967). In fact, it has been shown that some polyanions such as nucleic acids also inhibit microtubule assembly *in vitro* (Corces et al., 1980). The interaction between LPS and microtubules could be a consequence of the capacity of tubulin to interact with LPS in membranes and liposomes (Stephens, 1986).

Microtubules are known to serve as intracellular highways for movement of a variety of membrane-bounded organelles such as mitochondria, lysosomes, endoplasmic reticulum, and assorted vesicles that participate in both secretory and endocytic pathways (Bloom, 1992). To generate the forces for most, if not all, of these movements, the cell relies upon mechanochemical

enzymes known as microtubule motor proteins, e.g., kinesin (Hollenbeck and Swanson, 1990), dynein (Witman, 1992), and dynamine (Shpetner and Vallee, 1989). In fact, Swanson et al. (1992) showed that an anti-kinesin antibody inhibits radial movement of lysosomes along microtubules in permeabilized macrophages, a result implying that kinesin is the mechanochemical motor of lysosome extension along microtubules.

Early electron microscopic studies on the cytoplasm of *A. proteus* showed that amoebae, unlike tissue cells, seemed to possess a less complex cytoskeleton mainly composed of microfilaments (Taylor, 1977) and, possibly, intermediate filaments (Paulin-Levasseur and Gicquaud, 1984). Microtubules in *A. proteus* were first detected in the mitotic apparatus (Roth et al., 1960), and cytoplasmic microtubules were found later during interphase (Christiani et al., 1986). Whereas microfilaments are known to be responsible for protoplasmic streaming and other phenomena, the function of cytoplasmic microtubules in amoebae is still unknown. However, since microtubules in amoebae are also connected to other cytoskeletal components such as microfilaments and intermediate filaments, they may play the same role as in the mammalian cells, that is, transporting cell organelles such as lysosomes. If that is the case, LPS on symbiosome membranes may block the transport of lysosomes to the symbiosomes by affecting the stability of microtubules or by inhibiting the polymerization of microtubules near symbiosomes.

Another possible explanation is that LPS may prevent vesicle fusion rather than transporting of lysosomes. According to the SNARE hypothesis, the targeting and fusion of transport vesicles are mediated mainly by protein-protein interaction, assembly of a SNARE complex composed of v-SNARE on the transporting vesicle and t-SNARE on the targeting organelle (Bennett, 1995). Proteins known to be involved in targeting and fusion of vesicles have

not been identified in amoeba, but since LPS are present on the cytoplasmic side of symbiosome membranes (Choi and Jeon, 1992), a long polysaccharide chain of LPS may interfere with the protein-protein interaction required for the symbiosome-lysosome fusion.

How are LPS derived from X-bacteria incorporated into symbiosome membranes? The results from studies of LPS binding to murine lymphocytes led to the conclusion that LPS associates with cell membranes in a two-step process (Price and Jacobs, 1986). The first step is the association of the LPS aggregate with the cell surface (adherence); the second is the incorporation of individual LPS molecules into the cell membrane by a hydrophobic interaction between the lipid A part of LPS and the hydrophobic region of the membrane lipid bilayer (coalescence). The intrusion of such a large segment of lipid covalently linked would be expected to alter the fluidity of the membrane lipid bilayer. Indeed, LPS were found to decrease the stability of phospholipid bilayers at a concentration of 50 µg/ml, as measured by survival time and dielectric breakdown studies (Benedetto et al., 1973). LPS were also able to penetrate the phospholipid monolayer. Their penetration was dependent on the temperature and was increased by unsaturation and by the presence of cyclopropane in the fatty acyl chains of phospholipids, resulting in a tendency to increase the size of intermolecular cavities (Benedetto et al., 1973). Psychrophilic bacteria are known to contain a cyclopropane among their fatty acids, and its concentration is increased when the bacteria are cultured at low temperature (Kate and Hagen, 1964).

X-Bacteria are considered to be psychrophilic since they cannot live above the temperature of 27° C (Ahn and Jeon, 1979) and fail to infect amoebae after a high temperature treatment (Han and Jeon, 1980). The results of high temperature incubation showed that the outer membranes of X-

bacteria containing LPS were detached from the inner membranes after a 3-day incubation and were completely released to the lumen of symbiosomes after 5 days of incubation, resulting in inflated symbiosomes. At the same time, active vesicle fusions were observed all over the symbiosome membranes (Fig. 17).

Therefore, it is possible to suggest that LPS of X-bacteria are secreted and penetrate symbiosome membranes at a concentration sufficient to prevent the lysosomal fusion. However, high temperature incubation appeared to release outer membranes from X-bacteria, which would make bigger LPS pools inside symbiosomes. To explain active lysosomal fusion at high temperature, two possible hypotheses are suggested. The first is that X-bacteria may lose the ability to transport LPS to symbiosome membranes at high temperature. Insufficient supply of LPS would lower the concentration of LPS in symbiosome membranes and allow lysosomes to fuse. It could be tested by using the 29-kDa protein (S29x), which is transported continuously into the cytoplasm of host amoebae. Although it is not known whether X-bacteria use the same machinery to transport S29x and LPS or not, the result could be used to test the hypothesis indirectly.

The second is that X-bacteria may have different LPS at high temperature, which cannot prevent lysosomal fusion; symbiosome membranes having nonfunctional LPS would allow lysosomes to fuse. To test this hypothesis, the compositions of LPS isolated from control X-bacteria and high-temperature treated X-bacteria should be compared. Although comparison of LPS on symbiosome membranes in control and in high-temperature treated xD amoebae is better, it is impossible to do so since the amount of LPS on membranes is too small. Either hypothesis could explain the depletion of LPS on symbiosome membranes seen in xD amoebae incubated

for 5 days at 27° C (Fig. 16 D). Active lysosomal fusion would decrease relative concentrations of LPS on symbiosome membranes so that LPS may not be detected with an antibody.

In conclusion, LPS appeared on symbiosome membranes between 3 and 7 days after infection and play a role in the prevention of symbiosome-lysosome fusion. In addition, how LPS can be incorporated into membranes and how they function are discussed.

C. Phylogenetic Studies of Bacterial Endosymbionts, X-Bacteria

In order to obtain information about the identity of X-bacteria, a phylogenetic analysis was performed by using the nucleotide sequence of 16S ribosomal DNA of X bacteria and the LPS composition. A 16S rDNA fragment was amplified from X-bacteria by PCR, and was sequenced by using dideoxy cycling sequencing method with PCR. The computer-generated prediction of secondary structure of X-bacterial 16S rDNA showed a typical ribosomal DNA structure.

The nucleotide sequence of X-bacterial 16S rDNA showed that X-bacteria were classified in the gamma subclass of *Proteobacteria*, and *Flavobacterium lutescen* was found to have the highest sequence identity. Pathogenic *Pseudomonas* species such as *P. aeruginosa* and *P. mendosina* also have the high identity. *F. lutescen* is a non-motile species and has a complex fatty acid profile (Wilkinson, 1988).

Protozoa such as amoebae and flagellates have long been known to be associated with both extracellular and intracellular prokaryotic symbionts (Lee et al., 1985). In amoebae, all known bacterial symbionts are intracellular. This is understandable since amoebae do not have a firm cortex and

ectosymbionts cannot permanently attach to them (Jeon, 1992b). Several bacterial pathogens surviving within amoebae have been reported, including *Legionella*, *Listeria*, *Vibrio*, *Mycobacterium*, *Pseudomonas*, *Flavobacterium* species (Barker and Brown, 1994; Harf, 1993/94).

Legionella pneumophila were considered to be related to X-bacteria since they have an almost identical nucleotide sequence of GroEL, and have the similar chemical composition, especially a complex fatty acid profile. They are also known to live inside various kinds of amoebae such as *Hartmannella*, *Acanthamoeba* and *Naegleria* sp. (Rowbotham, 1980; 1993), and ciliated protozoa, *T. pyriformis* (Fields, 1993). But, besides the differences pointed out earlier, the consensus sequence found in all *Legionella* species was not found in the 16S rDNA sequence of X-bacteria, and the 16S rDNA sequence of *L. pneumophila* turned out to have less homology than that of *Pseudomonas* sp. (85% vs. 94-97%, respectively). Additionally, *Legionella* sp. is known to be killed by *A. proteus*, used in this study (Jeon, 1983).

Pathogenic *Pseudomonas* sp. and *F. lutescen* showed high identity in nucleotide sequence of the 16S rDNA, but they have different LPS composition and pathogenicity from those of X-bacteria (Wilkinson et al., 1973).

The present cultures of xD amoebae were derived from clones established and maintained in the laboratory for over 30 years. Many hundreds of generations of xD amoebae have been cultured since the initial infection, and only viable, symbiont-bearing clones have been maintained. Therefore, it is difficult to determine the identity of X-bacteria. In fact, mAbs against X-bacterial LPS did not crossreact with LPS of any other Gram-negative bacteria including *E. coli* and *Legionella* sp. (Choi and Jeon, 1992).

In conclusion, X-bacteria appear to belong to the gamma subclass of Proteobacteria on the basis of the 16S rDNA sequence analyses and LPS composition comparisons,.

D. A Novel 85-kDa Bacterial Protein in the Symbiosome Inclusion

A novel, 85-kDa bacterial protein was identified in the inclusions of symbiosomes, which are non-fusible with lysosomes. This protein was found to be synthesized by X-bacteria and localized mainly in the inclusions of symbiosomes. Experimental infection with isolated X-bacteria showed that the protein appeared inside symbiosomes 5 days after infection and reached the maximum amount after 21 days. This result is consistent with the previous finding that the number of X-bacteria in newly infected amoebae reached that of X-bacteria in established amoebae 21 days after infection (Ahn and Jeon, 1979) and appearance time of LPS determined in this study. Thus, the protein is suggested to be involved in symbiosis, but further characterization is needed to determine the role.

In the pathogenesis of *Toxoplasma gondii*, dense granule proteins and the rhoptry proteins are discharged into parasitophorous vacuoles surrounding *Toxoplasma* after parasitic invasion and increase in amount as the vacuoles mature (Sibley and Krahenbuhl, 1988). Some of the dense granule proteins such as GRA1, 2, and 4 are confined to the vacuolar space and are associated with the network of parasitophorous vacuoles whereas others are associated with vacuolar membranes and are known to be involved in the modification of vacuole membranes to prevent lysosomal fusion (Joiner et al., 1994). The exact function of the dense granule proteins remaining inside vacuoles is not

yet known, but they ,like an 85-kDa protein found in symbiosomes of xD amoebae, are suggested to be involved in the survival of pathogens

Including an 85-kDa protein reported in this study, several macromolecules were identified in/around symbiosomes of xD amoebae (Fig. 27). Some of them, such as the 96-kDa protein and LPS, are synthesized by X-bacteria and found on symbiosome membranes like the proteins (GRA3, GRA5) found in *Toxoplasma* pathogenesis. LPS are revealed to play a role in the prevention of lysosome-symbiosome fusion in this study. The roles of other macromolecules are not yet known, but they are also thought to be involved in the symbiotic interactions between X-bacteria and amoebae.

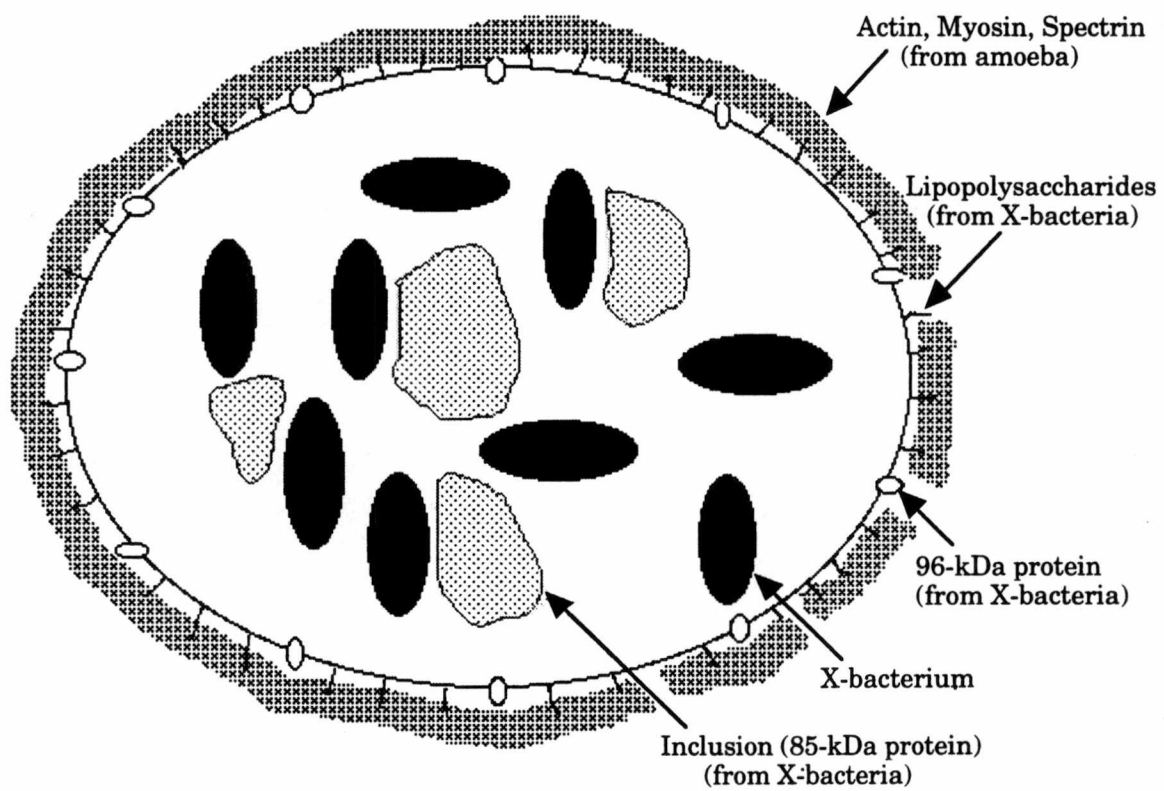


Fig. 27. A diagram showing a symbiosome and macromolecules in xD amoebae

BIBLIOGRAPHY

BIBLIOGRAPHY

- Ahn, T. I., and Ahn, K. S. 1987. Effects of the bacterial genetic components on the maintenance of the nucleus morphology of the symbiotic strain of *Amoeba proteus*. *Kor. J. Genet.* 9: 19-28.
- Ahn, K. S., Choi, E. Y., and Jeon, K. W. 1990. A symbiosome-membrane-specific protein in symbiont-bearing *Amoeba proteus* as studied with a monoclonal antibody. *Endocyt. Cell Res.* 7:45-50.
- Ahn, T. I., and Jeon, K. W. 1979. Growth and electron microscopic studies on an experimentally established bacterial endosymbionts in amoebae. *J. Cell. Physiol.* 98: 49-58.
- Ahn, T. I., and Jeon, K. W. 1982. Structural and biochemical characteristics of the plasmalemma and vacuole membranes in amoebae. *Exp. Cell Res.* 137:253-268.
- Ahn, T. I., Leeu, H. K., Kwak, I. H., and Jeon, K. W. 1991. Nucleotide sequence and temperature-dependent expression of XgroEL gene isolated from symbiotic bacteria of *Amoeba proteus*. *Endocyt. Cell Res.* 8:33-44.
- Ahn, T. I., Lim, S. T., Leeu, H. K., and Jeon, K. W. 1994. A novel strong promoter of the groEx operon of symbiotic bacteria in *Amoeba proteus*. *Gene* 128:43-49.
- Alexander, J., and Vickerman, K. 1975. Fusion of host cell secondary lysosomes with the parasitophorous vacuoles of *Leishmania mexicana*-induced macrophages. *J. Protozool.* 22: 502-508.
- Altman, L. G., Schneider, B. G., and Papermaster, D. S. 1984. Rapid embedding of tissues in Lowicryl K4M for immunoelectron microscopy. *J. Histochem. Cytochem.* 32:1217-1223.
- Andrews, N. W. 1995. Lysosome recruitment during host cell invasion by *Trypanosoma cruzi*. *Trends Cell Biol.* 5:133-137.
- Armstrong, J. A., and Hart, P. D. 1971. Response of cultured macrophages to *Mycobacterium tuberculosis*. *J. Exp. Med.* 134:713-740.
- Austin, E., and Priest, F. 1986. *Modern Bacterial Taxonomy*. Van Nostrand Reinhold, Workinham. United Kingdom.
- Barker, J., and Brown, M. R. W. 1994. Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. *Microbiol.* 140:1253-1259.

- Benedetto, D. A., Shands, J. W., and Shah, D. O. 1973. The interaction of bacterial lipopolysaccharide with phospholipid bilayers and monolayers. *Biochim. Biophys. Acta* 298:145-157.
- Bennett, M. K. 1995. SNAREs and the specificity of transport vesicle targeting. *Curr. Opin. Cell Biol.* 7:581-586.
- Berger, K. H., and Isberg, R. R. 1993. Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. *Mol. Microbiol.* 7:7-19.
- Bielecki, I., Youngman, P., Connelly, P., and Portnoy, D. A. 1990. *Bacillus subtilis* expressing a haemolysin gene from *Listeria monocytogenes* can grow in mammalian cells. *Nature* 345:175-176.
- Brade, L., and Brade, H. 1985. Characterization of two different antibody specificities recognizing distinct antigenic determinants in free lipid A of *Escherichia coli*. *Infect. Immun.* 48:776-781.
- Bloom, G. S. 1992. Motor proteins for cytoplasmic microtubules. *Curr. Opin. Cell Biol.* 4:66-73.
- Bryn, K., and Jantzen, E. 1982. Analysis of lipopolysaccharides by methanolysis, trifluoroacetylation, and gas chromatography on a fused-silica capillary column. *J. Chromatogr.* 240:405-413.
- Chang, K. P., and Dwyer, D. M. 1976. Multiplication of human parasite (*Leishmania donovani*) in phagolysosomes of hamster macrophages in vitro. *Science* 193:679-681.
- Choi, E. Y., Ahn, G. S., and Jeon, K. W. 1991. Elevated levels of stress proteins associated with bacterial symbiosis in *Amoeba proteus* and soybean root nodule cells. *Biosystems* 25:205-212.
- Choi, E. Y., and Jeon, K. W. 1989. The presence of spectrin-like protein on symbiosome membrane of symbiont-bearing *Amoeba proteus* as studied with monoclonal antibodies. *Endocyt. Cell Res.* 6:99-108.
- Choi, E. Y., and Jeon, K. W. 1991. The presence of bacterial-endosymbiont-derived lipopolysaccharides on eukaryotic host vesicle membranes, as studied using monoclonal antibodies. *Endocyt. C. Res.* 6:99-108.
- Choi, E. Y., and Jeon, K. W. 1992. Bacterial-endosymbiote-derived lipopolysaccharides on amoeba symbiosome membranes. *J. Protozool.* 39:205-210.
- Choi, E. Y., Kim, K. J., and Jeon, K. W. 1992. Lysosomal membrane proteins of *Amoeba proteus* as studied with monoclonal antibodies. *J. Protozool.* 39:671-677.

Christiani, A., Hugelmeyer, P., and Stockem, W. 1986. Morphological evidence for the existence of a more complex cytoskeleton in *Amoeba proteus*. *Cell Tiss. Res.* 246:163-168.

Corces, V. G., Manso, R., de la Torre, J., and Avila, J. 1980. Effects of DNA on microtubule assembly. *Eur. J. Biochem.* 105:7-16.

de Maagd, R. A., Rao, A. S., Mulders, I. H. M., Goosen-de Roo, L., van Loosdrecht, M. C. M., Wijffelman, C. A., and Lugtenberg, B. J. J. 1989. Isolation and characterization of mutants of *Rhizobium leguminosarum* bv. viciae 248 with altered lipopolysaccharides: possible role of surface charge or hydrophobicity in bacterial release from the infection thread. *J. Bacteriol.* 171:1143-1150.

Ding, A., Porteu, F., Sanchez, E., and Nathan, C. F. 1990. Shared actions of endotoxin and taxol on TNF receptors and TNF release. *Science* 248:370-372.

Ding, A., Sanchez, E., Tandinco, M., and Nathan, C. 1992. Interactions of bacterial lipopolysaccharide with microtubule proteins. *J. Immunol.* 148:2853-2858.

El-On, J., Bradley, D. J., and Freeman, J. C. 1980. *Leishmania donovani*: Action of excreted factor on hydrolytic enzyme activity of macrophages from mice with genetically different resistance to infection. *Exp. Parasitol.* 49:167-174.

Embley, T. M. 1991. The linear PCR reaction: a simple and robust method for sequencing amplified rRNA genes. *Lett. Appl. Microbiol.* 13:171-174.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochem.* 10:2606-2030.

Fields, B. S. 1993. Legionella and Protozoa: interaction of a pathogen and its natural host, p. 129-136. In Barbaree, J. M., Breiman, R. F., and Dutour, A. P. (eds.), *Legionella: Current Status and Emerging Perspectives*, American Society for Microbiology, Washington, DC.

Friedman, H., Klein, T. W., Nakano, M., and Nowotny, A. (eds.) 1990. *Endotoxin*. Plenum Press, New York.

Goldstein, L., and Ko, C. 1976. A method for the mass culturing of large free-living amoebas. *Methods Cell Biol.* 13:239-246.

Goodfellow, M., and Minnikin, D. E. 1985. Introduction to chemosystematics, p. 1-15. In Goodfellow, M., and Minnikin, D. E. (eds.), *Chemical Methods in Bacterial Systematics*. The Society for Applied Bacteriology Technical Series No. 20, Academic press, New York.

- Goren, M. B., and Mor, N. 1990. Influence of phagosomal contents on the apparent inhibition of phagosome-lysosome fusion mediated by polyanionic substances in mouse peritoneal macrophages. *Biochem. Cell Biol.* 68:24-32.
- Gorshkova, R. P., Zubkov, V. A., Isakov, V. V., and Ovodov, Y. S. 1984. Yersiniose, a new branched-chain sugar. *Carbohydr. Res.* 126:308-312.
- Gorshkova, R. P., Zubkov, V. A., Isakov, V. V., and Ovodov, Y. S. 1989. The structure of O-specific polysaccharides of *Yersinia fredericksonii* serotype O:16,29 lipopolysaccharide. *Bioorg. Khim.* 15:1627-1633.
- Hackstard, T., and Williams, J. C. 1981. Biochemical stratagem for obligate parasitism of eukaryotic cells by *Coxiella burnetii*. *Proc. Natl. Acad. Sci. USA* 78:3240-3244.
- Hammond, S. M., Lambert, P.A., and Rycroft, A. N. 1984. *The Bacterial Cell Surface*. Croom Helm, London.
- Han, J. H., and Jeon, K. W. 1980. Isolation and partial characterization of two plasmid DNAs from endosymbiotic bacteria in *Amoeba proteus*. *J. Bacteriol.* 141:1466-1469.
- Harf, C. 1993/94. Free-living amoeba: interactions with environmental pathogenic bacteria. *Endosym. Cell Res.* 10:167-183.
- Hitchcock, P. J., and Brown, T. M. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide. *J. Bacteriol.* 154:269-277.
- Hitchcock, P. L., Levine, L., Makela, P. H., Rieschel, E. T., Strittmatter, W., and Morrison, D. C. 1986. Lipopolysaccharide nomenclature-past, present and future. *J. Bacteriol.* 166:699-705.
- Hollenback, P. J., and Swanson, J. A. 1990. Radial extension of macrophage tubular lysosomes supported by kinesin. *Nature* 346:864-866.
- Horwitz, M. A. 1983. The legionnaire's disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. *J. Exp. Med.* 15:2108-2126.
- Jantzen, E., and Bryn, K. 1985. Whole-cell and lipopolysaccharide fatty acids and sugars of gram-negative bacteria, p. 145-171. *In* Goodfellow, M., and Minnikin, D. E. (eds.), *Chemical Methods in Bacterial Systematics*. The Society for Applied Bacteriology Technical Series No. 20, Academic press, New York.
- Jeon, K. W. 1972. Development of cellular dependence on infective organisms; micrurgical studies in amoeba. *Science* 176:1122-1123.

- Jeon, K. W. 1983. Integration of Bacterial Endosymbionts in Amoebae. *Int. Rev. Cytol.* 14(suppl):29-47.
- Jeon, K. W. 1992a. Macromolecules involved in the amoeba-bacteria symbiosis. *J. Protozool.* 39:199-204.
- Jeon, K. W. 1992b. Prokaryotic symbionts of amoebae and flagellates. p. 3855-3864. *In* Balows, A., Truper, H. G., Dworkin, M., Harder, W., and Schleifer, K. (eds.) *The Prokaryotes* (2nd ed.), vol. 4, Springer-Verlag, New York.
- Jeon, K. W. 1994. Survival and adaptation of microbes inside eukaryotic cells, *Mol. Cells* 4:259-265.
- Jeon, K. W. 1995a. The large, freeliving amoebae: wonderful cells for biological studies. *J. Euk. Microbiol.* 42:1-7.
- Jeon, K. W. 1995b. Bacterial endosymbiosis in amoebae. *Trends Cell Biol.* 5:137-140.
- Jeon, K. W., and Ahn, T. I. 1978. Temperature sensitivity: a cell character determined by obligate endosymbionts in amoebas. *Science* 202:635-637.
- Jeon, K. W., and Jeon, M. S. 1975. Cytoplasmic filaments and cellular wound healing in *A. proteus*. *J. Cell Biol.* 67:243-249.
- Jeon, K. W., and Jeon, M. S. 1976. Endosymbiosis in amoebae: Recently established endosymbionts have become required cytoplasmic component. *J. Cell. Physiol.* 89:337-347.
- Jeon, K. W., and Lorch, I. J. 1967. Unusual intracellular bacterial infection in Large, free living amoebae. *Exp. Cell Res.* 46:615-619.
- Joiner, K. A., Fuhrman, S. A., Miettinen, H. M., Kasper, L. H., and Mellman, I. 1990. *Toxoplasma gondii*: Fusion competence of parasitophorous vacuole in Fc receptor-transfected fibroblast. *Science* 249:641-649.
- Jones, T. C., and Hirsch, J. G. 1972. The interaction between *Toxoplasma gondii* and mammalian cells. *J. Exp. Med.* 135:1173-1194.
- Kate, M., and Hagen, P. -O. 1964. Influence of temperature on fatty acid composition of psychrophilic and mesophilic *serratia* species. *Can. J. Biochem.* 42:481-488.
- Kenne, L., and Lindberg, B. 1983. Bacterial polysaccharides, p. 287-363. *In* Aspinall, G. O. (ed.), *The Polysaccharides*, vol.2. Academic Press, New York.
- Kim, H. B., and Jeon, K. W. 1986. Protein synthesis by bacterial endosymbionts in amoebae. *Endocyt. C. Res.* 3:299-309.

- Kim, H. B., and Jeon, K. W. 1987. Actin-like host protein accumulated within symbiont-containing vesicles of amoebae as studied using a monoclonal antibody. *Endocyt. Cell Res.* 3:151-166.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., and Pace, N. R. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proc. Natl. Acad. Sci. USA* 82:6955-6959.
- Lee, J. J., Soldo, A. T., Reisser, W., Lee, M. J., Jeon, K. W., and Gortz, H. 1985. The extent of algal and bacterial endosymbioses in protozoa. *J. Protozool.* 32:391-403.
- Lippincott-Schwartz, J., and Fambrough, D. M. 1986. Lysosomal membrane dynamics: Structure and intraorganellar movement of a major lysosomal membrane glycoprotein. *J. Cell Biol.* 102:1593-1605.
- Littauer, U. Z., Givon, D., Thierauf, M., Ginzberg, I., and Ponstingl, H. 1986. Common and distinct tubulin binding sites for microtubule-associated proteins. *Proc. Natl. Acad. Sci. USA* 83:7162-7166.
- Lorch, I. J., and Danielli, J. F. 1953. Nuclear transplantation in amoebae. I. some characters of *Amoeba proteus* and *Amoeba discoides*. *Quart. J. Microsc. Sci.* 94:445-460.
- Lorch, I. J., and Jeon, K. W. 1980. Resuscitation of amoebae deprived of essential symbionts: micrurgical studies. *J. Protozool.* 27:423-426.
- Lorch, I. J., and Jeon, K. W. 1982. Nuclear lethal effect and nucleocytoplasmic incompatibility induced by endosymbionts in *Amoeba proteus*. *J. Protozool.* 29:468-470.
- Lorch, I. J., and Jeon, K. W. 1985. Differential effect of colcemid on mitotic apparatus in amoebae as studied using anti-tubulin monoclonal antibodies. *Eur. J. Cell Biol.* 39:290-294.
- Luderitz, O., Tanamoto, K., Galanos, C., Westphal, O., Zahringer, U., Rietschel, E. T., Kusumoto, S., and Shiba, T. 1983. p. 3-17. In Anderson, and Unger (eds.) *Bacterial Lipopolysaccharides*, American Chemical Society, Washington, DC.
- Luderitz, O., Westphal, O., Staub, A. M., and Nikaido, H. 1971. Isolation and chemical and immunological characterization of bacterial lipopolysaccharides. p. 145-233. In Weinbaum, Kadis, and Ajl (eds.) *Microbial Toxins*, vol. IV. Academic Press, New York.

Mayer, H., Bhat, U. R., Masoud, H., Radziejewska-Lebrecht, J., Widermann, C., and Krauss, J. H. 1989. Bacterial lipopolysaccharides. *Pure Appl. Chem.* 61:1271-1282.

Mayer, H., Tharanathan, R. N., and Weckesser, J. 1985. Analysis of lipopolysaccharides of Gram-negative bacteria. *Method Microbiol.* 18:157-207.
Merril, C. R., Goldman, S. A., and Ebert, M. H. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid protein. *Science* 211:1473-1438.

Milder, R., and Kloetzel, J. 1980. The development of *Trypanosoma cruzi* in macrophages *in vitro*. Interaction with lysosomes and host cell fate. *Parasitol.* 80:139-145.

Moulder, J. W. 1985. Comparative biology of intracellular parasitism. *Microbiol. Rev.* 49:298-337.

Murray, R. G. E., Brenner, D. J., Colwell, R. R., De Vos, P., Goodfellow, M., Grimont, P. A. D., Pfennig, N., Stacke-Brandt, E., and Zavarzin, G. A. 1990. Report of the ad hoc committee on approaches to taxonomy within the Proteobacteria. *Int. J. Systematic Bacteriol.* 40:213-215.

Nakai, T., Kawahara, K., Danbara, H., and Kume, K. 1991. The epitope structure in lipopolysaccharide recognized by the serotype 2-specific monoclonal antibody of *Actinobacillus pleuropneumoniae*. *FEMS Microbiol. Letters* 82:143-148.

Nikaido, H. 1970. Lipopolysaccharide in the taxonomy of *Enterobacteriaceae*. *Int. J. Syst. Bacteriol.* 20:383-406.

Nikaido, H. 1979. Non-specific transport through the outer membrane. p. 361-405. *In* Inouye, M. (ed.), *Bacterial Outer Membranes: Biogenesis and Functions*. J. Wiley and Sons, New York.

Olins, A. L., and Warner, R. C. 1967. Physicochemical studies on a lipopolysaccharide from the cell wall of *Azotobacter vinelandii*. *J. Biol. Chem.* 242:4994-5001.

Pak, J. W., and Jeon, K. W. 1995. Synthesis and export of a novel protein by bacterial endosymbiont in xD amoebae. *Mol. Biol. Cell* 6 (suppl) :399a.

Paulin-Levasseur, M. P., and Gicquaud. 1984. Observation of 10nm filaments in cytoplasmic preparations of *Amoeba proteus*. *Eur. J. Cell Biol.* 34:60-63.

Pepe, J. C. and Miller, V. L. 1993. *Yersinia enterocolitica* invasin: a primary role in the initiation of infection. *Proc. Natl. Acad. Sci. USA*, 90:6473-6477.

Pluschke, G., and Achman, M. 1984. Degree of antibody-independent activation of the classical complement pathway by K1 *Escherichia coli* differs

with O antigen type and correlates with virulence of meningitis in newborns. *Infect. Immun.* 43:684-692.

Price, R. M., and Jacobs, D. M. 1986. Fluorescent detection of lipopolysaccharide interactions with model membranes. *Biochim. Biophys. Acta* 859:26-32.

Rest, R. F., Coohy, M. H., and Spitznagel, J. K. 1977. Susceptibility of lipopolysaccharide mutants to the bactericidal action of human neutrophil lysosomal fractions. *Infect. Immun.* 16:145-151.

Rietschel, E. T., Brade, L., Schade, U., Seydel, U., Zahringer, U., Kusumoto, S., and Brade, H. 1988. Bacterial endotoxins: properties and structure of biologically active domains, p. 1-41. *In* Schrinner, E., Richmond, M. H., Seibert, G., and Schwarz, U. (eds.), *Surface Structures of Microorganisms and Their Interactions with the Mammalian Host*. VCH Publishers, Weinheim.

Risco, C., Dominguez, J. E., Bosch, M. A., and Carrascosa, J. L. 1993. Biochemical and electron microscopy analysis of endotoxin binding to microtubules in vitro. *Mol. Cell. Biochem.* 121:67-74.

Roth, L. E., Jeon, K. W., and Stacey, G. 1988. Homology in endosymbiotic systems: the term "symbiosome". *In* Palacios, R., and Verma, D. P. S. (eds.), *Molecular Genetics of Plant-Microbe Interactions*, APS Press, St. Paul, MN, p. 220-225.

Roth, L. E., Obetz, S. W., and Daniels, E. W. 1960. Electron microscopic studies of mitosis in amoebae. I. *Amoeba proteus*. *J. Biophys. Biochem. Cytol.* 8:207-220.

Rowbotham, T. J. 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J. Clin. Pathol.* 33:1179-1183.

Rowbotham, T. J. 1993. *Legionella*-like amoebal pathogen, p. 137-140. *In* Barbaree, J. M., Breiman, R. F., and Dutour, A. P. (eds.), *Legionella: Current Status and Emerging Perspectives*, American Society for Microbiology, Washington, DC.

Sansonetti, P. J., Ryter, A., Clerc, P., Aurelli, A. T. and Mounier, J. 1986. Multiplication of *Shigella flexneri* within HeLa cells: lysis of the phagocytic vacuole and plasma-mediated contact hemolysis. *Infect. Immun.* 51:461-469.

Schleifer, K. H., and Kandler, O. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* 36:407-477.

Shpetner, H. S., and Vallee, L. B. 1989. Identification of dynamine: a novel mechanochemical enzyme that mediates interactions between microtubules. *Cell* 59:421-432.

- Sibley, L. D. 1995. Invasion of vertebrate cells by *Toxoplasma gondii*. Trends Cell Biol. 5:129-132.
- Sibley, L. D., and Krahenbuhl, J. L. 1988. Modification of host cell phagosomes by *Toxoplasma gondii* involves redistribution of surface proteins and secretion of a 32 kDa protein. Eur. J. Cell Biol. 47:81-87.
- Sonesson, A., Bryn, K., Jantzen, E., and Larsson, L. 1989. Gas chromatographic determination of (phosphorylated) 2-keto-3-deoxy-octonic acid, heptose, and glucosamine in bacterial lipopolysaccharides after treatment with hydrofluoric acid, methanolysis and trifluoroacetylation. J. Chromatogr. 487:1-7.
- Sonesson, A., Jantzen, E., Bryn, K., Larsson, L., and Eng, J. 1989. Chemical composition of a lipopolysaccharide from *Legionella pneumophila*. Arch. Microbiol. 153:72-78.
- Sonesson, A., and Jantzen, E. 1992. The branched-chain octose yersiniose A is a lipopolysaccharide constituent of *Legionella micdadei* and *Legionella maceachernii*. J. Microbiol. Methods 15:241-248.
- Sonesson, A., Jantzen, E., Bryn, K., Tangen, T., Eng, J., and Zahringer, U. 1994. Composition of 2,3-dihydroxy fatty acid-containing lipopolysaccharides from *Legionella israelensis*, *Legionella maceachernii* and *Legionella micdadei*. Microbiol. 140:1261-1271.
- Stephens, R. E. 1986. Membrane tubulin. Biol. Cell. 57:95-110.
- Sturgill-Koszycki, S., Schlesinger, P. H., Chakraborty, P., Haddix, P., Collins, H. L., Fok, A. K., Allen, R. D., Gluck, S. L., Heuser, J., and Russell, D. G. 1994. Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. Science 263:678-681.
- Swanson, J. A., Locke, A., Ansel, P., and Hollenbeck, P. J. 1992. Radial movement of lysosomes along microtubules in permeabilized macrophages. J. Cell Sci. 103:201-209.
- Tan, E. L., Patel, P. V., Parsons, N. J., Martin, P. M. V., and Smith, H. 1986. Lipopolysaccharide alteration is associated with induced resistance of *Neisseria gonorrhoeae* to killing by human serum. J. Gen. Microbiol. 132:1407-1413.
- Taylor, D. L. 1977. The contractile basis of amoeboid movement. IV. The viscoelasticity and contractility of amoeba cytoplasm in vivo. Exp. Cell Res. 105:413-426.
- Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Nat. Acad. Sci. USA 76:4350-4354.

- Tsai, C. M., and Frisch, C. E. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* 119:115-119.
- Vaara, M., and Nikaido, H. 1984. Molecular organization of bacterial outer membrane, p. 1-45. *In* Rietschel, E. T. (ed.), *Handbook of Endotoxin*, vol. 1: Chemistry of Endotoxin. Elsevier, Amsterdam.
- Viale, A. M., and Arakaki, A. K. 1994. The chaperone connection to the origins of the eukaryotic organelles. *FEBS Lett.* 341:146-151.
- Weckesser, J., and Mayer, H. 1988. Different lipid A types in lipopolysaccharides of phototrophic and related non-phototrophic bacteria. *FEMS Microbiol. Rev.* 54:143-154.
- Weidner, E., and Sibley, L. D. 1985. Phagocytized intracellular microsporidian blocks phagosome acidification and phagosome-lysosome fusion. *J. Protozool.* 32:311-317.
- Westphal, O., Jann, K., and Himmelspack, K. 1983. Chemistry and immunochemistry of bacterial lipopolysaccharides as cell wall antigens and endotoxins. *Prog. Allerg.* 33:9-39.
- Wilkinson, S.G., Galbraith, L., and Lightfoot, G. A. 1973. Cell walls, lipids, and lipopolysaccharides of *Pseudomonas* species. *Eur. J. Biochem.* 33:158-174.
- Wilkinson, S. G. 1977. Composition and structure of bacterial lipopolysaccharides, p. 97-175. *In* Sutherland, I. (ed.), *Surface Carbohydrates of the Prokaryotic Cell*. Academic Press, New York.
- Wilkinson, S. G. 1988. Gram-negative bacteria, p. 299-488. *In* Ratledge, C. and Wilkinson, S. G.(eds.), *Microbial Lipids* vol. 1, Academic Press, New York.
- Witman, G. B. 1992. Axonemal dyneins. *Curr. Opin. Cell Biol.* 4:74-79.
- Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* 51:221-271.
- Zhou, R., Oskarsson, M., Paules, R. S., Schulz, N., Cleveland, D., and Vande Woude, G. F. 1991. Ability of the *c-mos* product to associate with and phosphorylate tubulin. *Science*, 251:671-675.

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